illumina®

Indexed Sequencing

on Illumina Systems

ILLUMINA PROPRIETARY
Document # 15057455 v09
April 2021

Revision History

Document	Date	Description of Change
Document # 15057455 v09	April 2021	Added HTML format.
Document # 15057455 v08	November 2020	Updates made to support the MiniSeq Standard and Rapid reagent kits.
Document # 15057455 v07	July 2020	Updates made to support the NovaSeq 6000 v1.5 reagent kit introduction.
Document # 15057455 v06	March 2020	Modified NextSeq Systems reference to account for all versions. Added Instrument Run Setup to introduction.
Document # 15057455 v05	March 2019	Renamed the two dual-indexed workflows on a paired-end flow cell: • Renamed workflow A to the forward strand workflow. • Renamed workflow B to the reverse complement workflow. Updated cycles per Index Read for all dual-index workflows (except forward strand) to eight or 10. Updated the descriptions of single- and dual-indexed libraries. Corrected the Index 1 primer for the HiSeq 3000/4000 SR Cluster Kit, HiSeq SR Cluster Kit v4, and HiSeq PE Cluster Kit v4 to HP12.
Document # 15057455 v04	February 2018	Added the iSeq 100 and HiSeq X flow cells to workflow B for dual-indexing on a paired-end flow cell. Added the IDT for Illumina TruSeq UD Indexes combinations for dual-indexed libraries.

Document	Date	Description of Change
Document # 15057455 v03	February 2017	Updated for the NovaSeq Series: • Added the NovaSeq 5000/6000 Flow Cell to workflow A for dual-indexing on a paired-end flow cell. • For workflow A, increased the number of cycles in an Index Read to a maximum of 20. Updated how many uniquely tagged libraries can be generated: • Up to 48 single-indexed libraries. • Up to 384 dual-indexed libraries. Clarified that this guide is applicable to all Illumina sequencing systems.
Document # 15057455 v02	March 2016	Added the MiniSeq system, which follows the single-index workflow and Workflow B for dual-indexing on a paired-end flow cell. Renamed this guide to *Indexed Sequencing Overview Guide* to emphasize indexing over systems. Organized dual-indexing workflows on paired-end flow cells as Workflow A and Workflow B. Organized dual-indexing workflows on single-read flow cells by sequencing system.
Document # 15057455 v01	August 2015	Added the dual-indexed workflow for a HiSeq 3000/4000 SR flow cell. Added sequencing primers available in the HiSeq 3000/4000 SR Cluster Kit.
Part # 15057455 Rev. B	February 2015	Added the HiSeq 3000/4000 flow cell to the dual-indexed workflow that performs the Index 2 Read after Read 2 resynthesis. This workflow is performed on NextSeq, HiSeq 4000, and HiSeq 3000. Added sequencing primers available in the HiSeq 3000/4000 PE Cluster Kit.

Document	Date	Description of Change
Part # 15057455 Rev. A	July 2014	Initial release.

Table of Contents

Introduction

This documentation provides an overview of indexed sequencing for Illumina sequencing systems. Indexed sequencing is a method that allows multiple libraries to be pooled and sequenced together.

Indexing libraries requires the addition of a unique identifier, or index sequence, to DNA samples during library preparation. BaseSpace Sequence Hub, Local Run Manager, Instrument Run Setup, or bcl2fastq2 Conversion Software process these tags to identify each uniquely tagged library for downstream analysis.

The number of index sequences added to samples differs for single-indexed and dual-indexed sequencing.

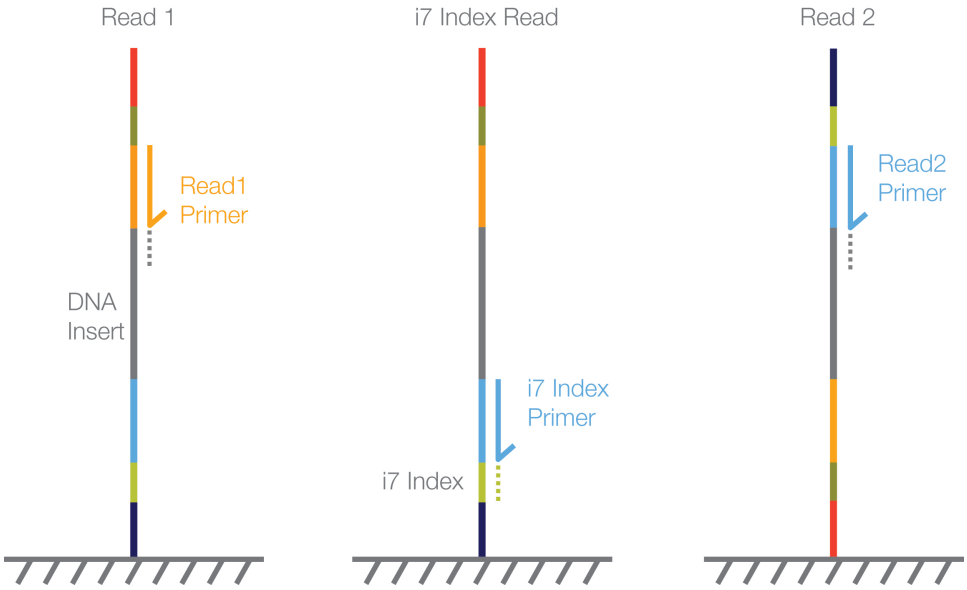
- **Single-indexed libraries**—Adds Index 1 (i7) sequences to generate uniquely tagged libraries.
- **Dual-indexed libraries**—Adds Index 1 (i7) and Index 2 (i5) sequences to generate uniquely tagged libraries.
  - Unique dual (UD) indexes have distinct, unrelated index adapters for both index reads. Index adapter sequences are eight or 10 bases long.
  - Combinatorial dual (CD) indexes have eight unique dual pairs of index adapters, so most libraries share sequences on the i7 or i5 end. Index adapter sequences are eight bases long.

During indexed sequencing, the index is sequenced in a separate read called the Index Read, where a new sequencing primer is annealed. When libraries are dual-indexed, the sequencing run includes two additional reads, called the Index 1 Read and Index 2 Read.

Single-Indexed Sequencing Overview

The single-indexed sequencing workflow applies to all Illumina sequencing platforms, where an Index Read follows Read 1.

Figure 1 Single-Indexed Sequencing



1. **Read 1**—Read 1 follows the standard Read 1 sequencing protocol using SBS reagents. The Read 1 sequencing primer is annealed to the template strand during the cluster generation step.
2. **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.
3. **Index 1 (i7) Read**—Following Index Read preparation, the Index 1 (i7) Read is performed. The read length depends on the system and run parameters.
4. **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.
5. **Read 2**—Read 2 follows the standard paired-end sequencing protocol using SBS reagents.

Dual-Indexed Sequencing Overview

Dual-indexed sequencing includes two index reads after Read 1: the Index 1 Read and the Index 2 Read.

Sequencing kits for HiSeq systems are available with a single-read or paired-end flow cell. For all other systems, sequencing kits include a paired-end flow cell.

The control software performs Read 1, any index reads, and then Read 2 based on the parameters provided for the run in the sample sheet or during run setup.

For all indexing workflows, the Index 1 Read directly follows Read 1. However, for dual-indexing on a paired-end flow cell, the rest of the workflow differs:

- **Forward strand**—The Index 2 Read occurs before Read 2 resynthesis, so the Index 2 (i5) adapter is sequenced on the forward strand.
- **Reverse complement**—The Index 2 Read occurs after Read 2 resynthesis, which creates the reverse complement of the Index 2 (i5) index adapter sequence.

Table 1 Dual-Index Paired-End Sequencing Workflows

Forward Strand	Reverse Complement
Read 1	Read 1
Index Read preparation	Index Read preparation
Index 1 Read	Index 1 Read
Index 2 Read	Read 2 resynthesis
Read 2 resynthesis	**Index 2 Read**
Read 2	Read 2 preparation
	Read 2

Dual-Indexed Workflow on a Paired-End Flow Cell

Dual-index sequencing on a paired-end flow cell follows one of two workflows, depending on the system and software:

- The forward strand workflow is performed on the NovaSeq 6000 with v1.0 reagent kits, MiniSeq with Rapid Reagent kits, MiSeq, HiSeq 2500, and HiSeq 2000.

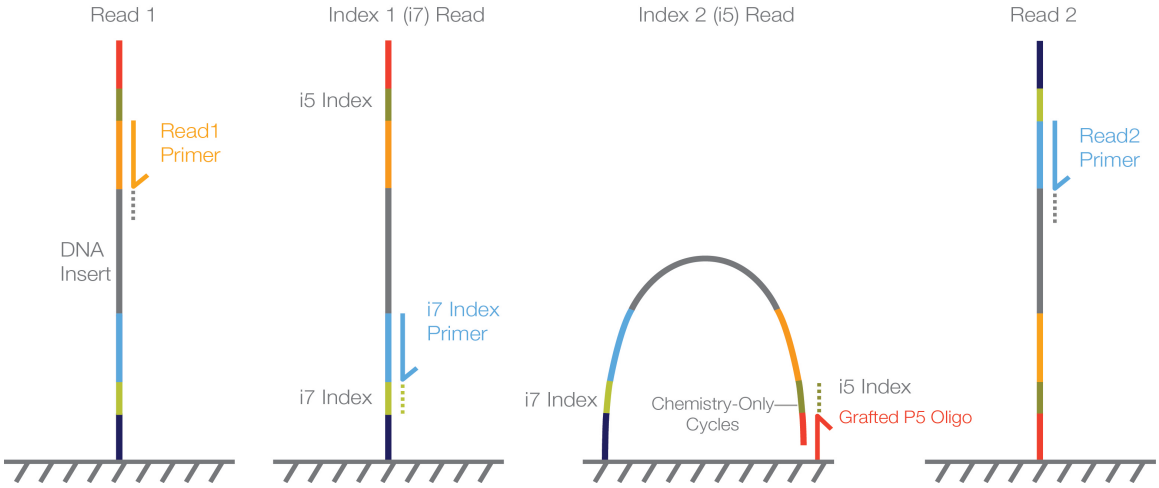
- The reverse complement workflow is performed on the iSeq 100, MiniSeq with Standard reagent kits, NextSeq Systems, NovaSeq 6000 with v1.5 reagent kits, HiSeq X, HiSeq 4000, and HiSeq 3000.

Forward Strand Workflow

The chemistry applied to the Index 2 Read during a paired-end, dual-indexed run on the NovaSeq 6000 with v1.0 reagent kits, MiniSeq with rapid reagent kits, MiSeq, HiSeq 2500, or HiSeq 2000 System is specific to the paired-end flow cell. Reading the i5 index requires seven additional chemistry-only cycles. This step uses the resynthesis mix, a paired-end reagent, during the Index 2 Read process.

> While MiniSeq Rapid reagents allow for dual indexing, Read 2 cannot be performed with the MiniSeq Rapid reagent kit.

Figure 2 Dual-Indexed Sequencing on a Paired-End Flow Cell (Forward Strand)



1. **Read 1**—Read 1 follows the standard Read 1 sequencing protocol using SBS reagents. The Read 1 sequencing primer is annealed to the template strand during the cluster generation step.
2. **Index Read preparation**—The Read 1 product is removed and the Index 1 (i7) sequencing primer is annealed to the same template strand.
3. **Index 1 (i7) Read**—Following Index Read preparation, the Index 1 (i7) Read performs up to 20 cycles of sequencing.

   > The maximum number of cycles in each Index Read depends on the system and run parameters.

4. **Index 2 (i5) Read**—The Index 1 (i7) Read product is removed and the template anneals to the grafted P5 primer on the surface of the flow cell. The run proceeds through an additional seven chemistry-only cycles (no imaging occurs), followed by up to 20 cycles of sequencing.

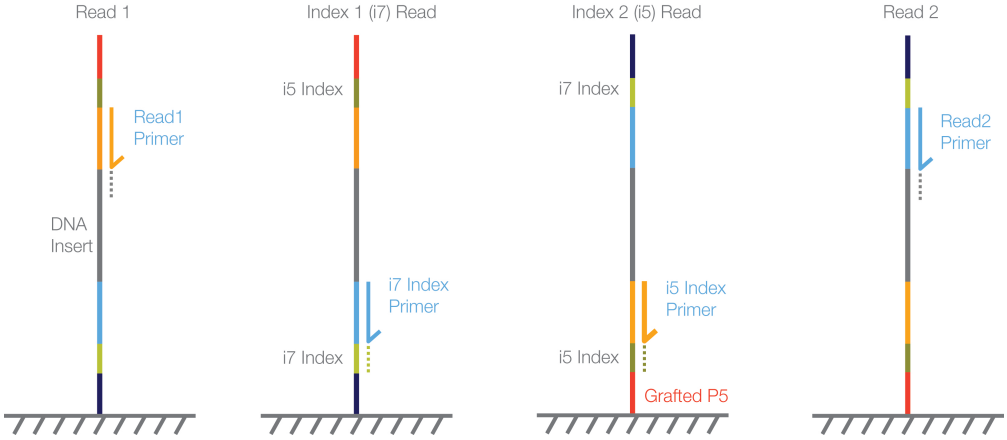
5. **Read 2 resynthesis**—The Index Read product is removed and the original template strand is used to regenerate the complementary strand. The original template strand is then removed to allow hybridization of the Read 2 sequencing primer.
6. **Read 2**—Read 2 follows the standard paired-end sequencing protocol using SBS reagents.

Reverse Complement Workflow

A dual-indexed run on the iSeq 100, MiniSeq with standard reagent kits, NextSeq Systems, NovaSeq 6000 with v1.5 reagent kits, HiSeq X, HiSeq 4000, or HiSeq 3000 System performs the Index 2 Read after Read 2 resynthesis. This workflow requires a reverse complement of the Index 2 (i5) primer sequence compared to the primer sequence used on other Illumina platforms.

The Index 2 sequencing primer is part of the dual-indexing primer mix for the iSeq 100, MiniSeq with standard reagent kits, NextSeq Systems, and NovaSeq 6000 with v1.5 reagent kits. For the HiSeq X, HiSeq 4000, and HiSeq 3000 Systems, the Index 2 sequencing primer is part of HP14. HP14 is an indexing primer mix that contains primers for both index reads.

Figure 3 Dual-Indexed Sequencing on a Paired-End Flow Cell (Reverse Complement)



1. **Read 1**—Read 1 follows the standard Read 1 sequencing protocol using SBS reagents. The Read 1 sequencing primer is annealed to the template strand during the cluster generation step.
2. **Index Read preparation**—The Read 1 product is removed and the Index 1 (i7) sequencing primer is annealed to the same template strand.
3. **Index 1 (i7) Read**—Following Index Read preparation, the Index 1 (i7) Read performs eight or 10 cycles of sequencing.
4. **Read 2 resynthesis**—The Index 1 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 Index 2 (i5) sequencing primer.
5. **Index 2 (i5) Read**—Following Read 2 resynthesis, the Index 2 (i5) Read performs eight or 10 cycles of sequencing.

This workflow does not require seven additional chemistry-only cycles.

6. **Read 2 preparation**—The Index 2 Read product is removed and the Read 2 sequencing primer is annealed to the same template strand.
7. **Read 2**—Read 2 follows the standard paired-end sequencing protocol using SBS reagents.

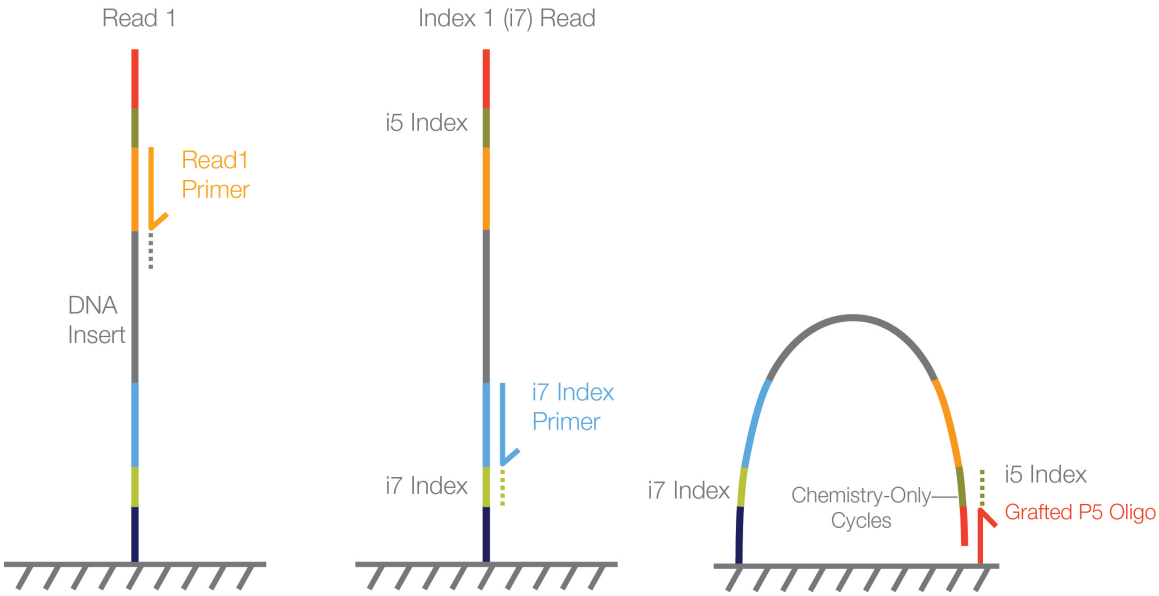
Dual-Indexed Workflow on a Single-Read Flow Cell

Single-read sequencing is possible on all HiSeq systems. Dual-index sequencing on a single-read flow cell follows one of two workflows, depending on the system.

HiSeq 4000 and HiSeq 3000 Systems

The chemistry applied to the Index 2 Read during a single-read dual-indexed run on the HiSeq 4000 or HiSeq 3000 System is specific to the single-read flow cell. Reading the i5 index requires seven additional chemistry-only cycles. This step uses the resynthesis mix during the Index 2 Read.

Figure 4 Dual-Indexed Sequencing on a Single-Read Flow Cell (HiSeq 4000 or HiSeq 3000)

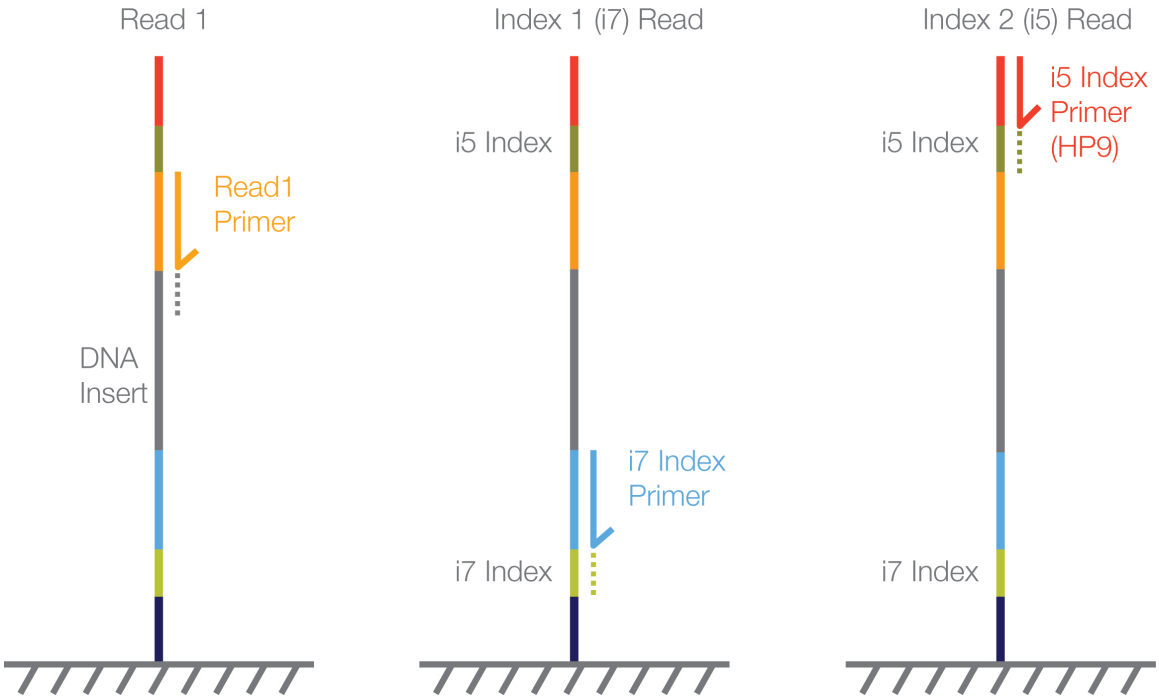


1. **Read 1**—Read 1 follows the standard Read 1 sequencing protocol using SBS reagents. The Read 1 sequencing primer is annealed to the template strand during cluster generation.
2. **Index Read preparation**—The Read 1 product is removed and the Index 1 (i7) sequencing primer is annealed to the same template strand.
3. **Index 1 (i7) Read**—Following Index Read preparation, the Index 1 (i7) Read performs eight or 10 cycles of sequencing.
4. **Index 2 (i5) Read**—The Index 1 (i7) Read product is removed and the template anneals to the grafted P5 oligo on the surface of the flow cell. The run proceeds through seven chemistry-only cycles (no imaging occurs), followed by eight or 10 cycles of sequencing.

HiSeq 2500 and HiSeq 2000 Systems

The chemistry applied to the Index 2 Read during a single-read, dual-indexed run on the HiSeq 2500 or HiSeq 2000 System is specific to the single-read flow cell. Performing the Index 2 Read on a HiSeq single-read flow cell requires HP9, an Index 2 sequencing primer.

Figure 5 Dual-Indexed Sequencing on a Single-Read Flow Cell (HiSeq 2500 or HiSeq 2000)



1. **Read 1**—Read 1 follows the standard Read 1 sequencing protocol using SBS reagents. The Read 1 sequencing primer is annealed to the template strand during the cluster generation step.
2. **Index Read preparation**—The Read 1 product is removed and the Index 1 (i7) sequencing primer is annealed to the same template strand.
3. **Index 1 (i7) Read**—Following Index Read preparation, the Index 1 (i7) Read performs eight or 10 cycles of sequencing.
4. **Index 2 (i5) Read**—The Index 1 (i7) Read product is removed and the Index 2 (i5) sequencing primer is annealed to the same template strand. The run proceeds through eight or 10 cycles of sequencing.

> This workflow does not require seven additional chemistry-only cycles.

Sequencing Primers for HiSeq Systems

Indexing workflow differences require system-specific chemistry and sequencing primers. The following tables list available HiSeq reagent kits and the associated sequencing primers, which are used with each step of an indexed run.

Sequencing primers for all other systems are provided in the prefilled reagent cartridge.

Sequencing Primers in Cluster Kits

Run Type	Read 1	Index 1 (i7)	Index 2 (i5)	Read 2
HiSeq 3000/4000 PE Cluster Kit	HP10	HP14	HP14	HP11
HiSeq 3000/4000 SR Cluster Kit	HP10	HP12	--[1]	--
HiSeq PE Cluster Kit v4	HP10	HP12	--[1]	HP11
HiSeq SR Cluster Kit v4	HP10	HP12	HP9	--
TruSeq PE Cluster Kit v3	HP6	HP8	--[1]	HP7
TruSeq SR Cluster Kit v3	HP6	HP8	--[2]	--

[1] The Index 2 Read uses resynthesis mix.
[2] The TruSeq Dual Index Sequencing Primer Box for single reads is required for dual-indexed sequencing on a single-read flow cell, regardless of library type.

Additional Primers for the TruSeq Cluster Kit v3

Using the TruSeq Cluster Kit v3 to sequence any Nextera libraries except Nextera Mate Pair libraries requires the TruSeq Dual Index Sequencing Primer Box. Sequencing primers in TruSeq v3 kits are not compatible with most Nextera libraries, while sequencing primers provided in the TruSeq Dual Index Sequencing Primer Box are compatible with all library types. To confirm primer compatibility, see the documentation for the library prep kit.

Dual-indexed sequencing on a single-read flow cell requires the single-read kit, regardless of the libraries being sequenced.

Run Type	Read 1	Index 1 (i7)	Index 2 (i5)	Read 2
TruSeq PE Dual Index Sequencing Primer Box (For use with paired-end flow cells)	HP10	HP12	--[1]	HP11
TruSeq SR Dual Index Sequencing Primer Box (For use with single-read flow cells)	HP10	HP12	HP9	--

[1] The resynthesis mix, a paired-end reagent provided in the TruSeq PE Cluster Kit v3, is used to perform the Index 2 Read.

Illumina
5200 Illumina Way
San Diego, California 92122 U.S.A.
+1.800.809.ILMN (4566)
+1.858.202.4566 (outside North America)
techsupport@illumina.com
www.illumina.com

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

illumina®