Overview of Indexed Sequencing on the NextSeq, MiSeq, and HiSeq Platforms

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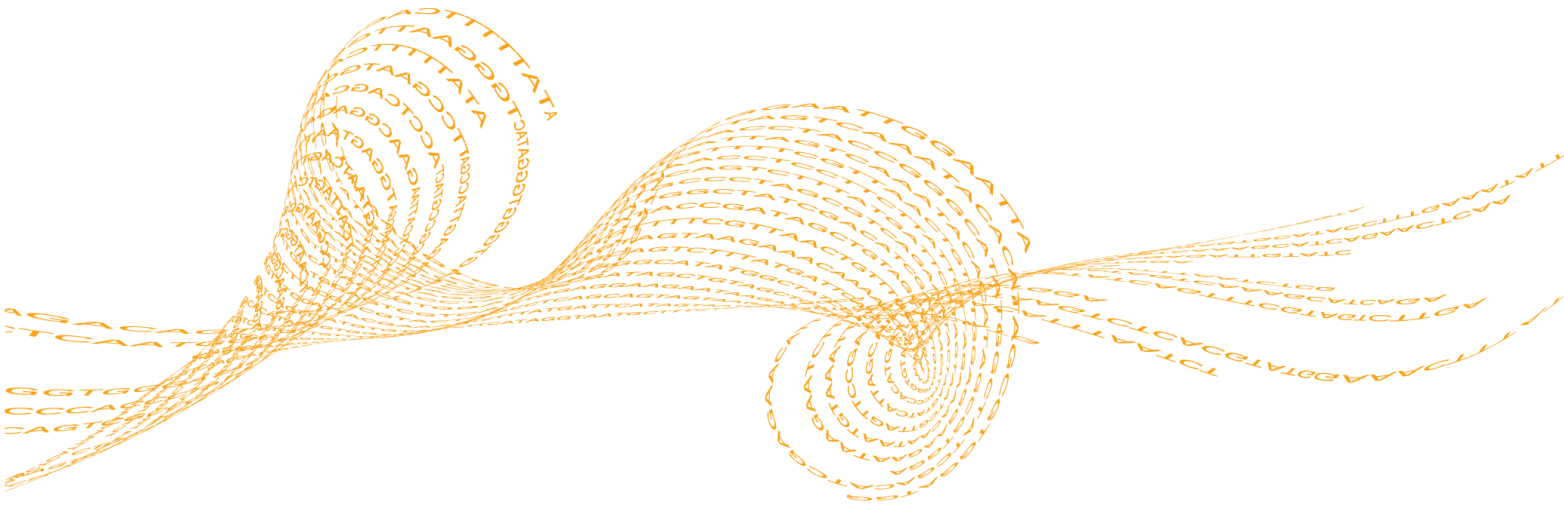

ILLUMINA PROPRIETARY
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Revision History

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

Introduction

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. Sequencing control software on Illumina sequencing platforms processes these tags in an automated sequencing strategy that identifies each uniquely tagged library for downstream analysis.

Single and Dual Indexing

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

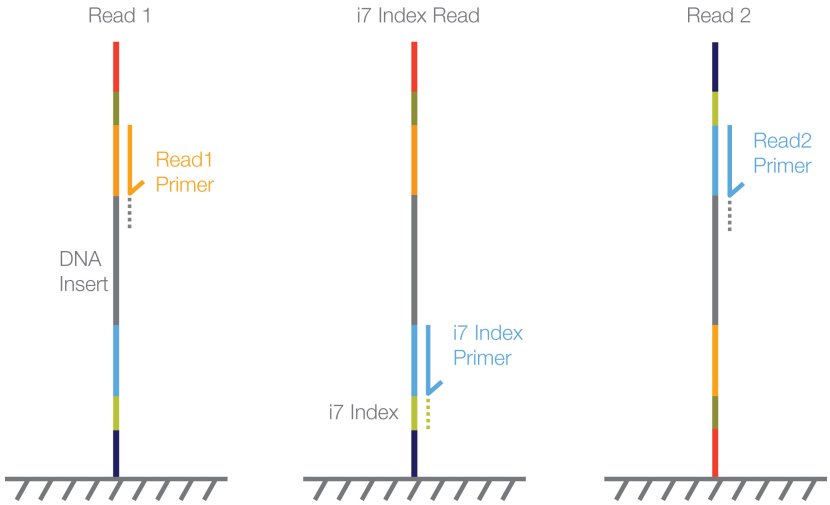
- **Single-indexed libraries**—Adds up to 12 unique 6-base Index 1 sequences for pooling up to 12 uniquely tagged libraries.
- **Dual-indexed libraries**—Adds up to 12 unique 8-base Index 1 (i7) sequences and up to 8 unique 8-base Index 2 (i5) sequences. Index 1 sequences are applied across the columns of 96-well plate and Index 2 sequences are applied down the rows, which creates up to 96 uniquely tagged libraries.

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 2 additional reads, called the Index 1 Read and Index 2 Read.

Single-Indexed Sequencing Overview

Single-indexed sequencing includes 1 Index Read after Read 1. Single-indexed sequencing is performed the same way on all Illumina sequencing platforms.

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 7-cycle Index 1 (i7) Read is performed.

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 2 index reads after Read 1, the Index 1 Read and Index 2 Read. The chemistry applied to the Index 2 Read is different for single-read flow cells than for paired-end flow cells due to the nature of the primers grafted onto the flow cell.

Sequencing kits for the NextSeq, MiSeq, HiSeq 4000, and HiSeq 3000 include a paired-end flow cell only. Sequencing kits for other HiSeq systems are available with either a single-read flow cell or a paired-end flow cell.

Dual-Indexing Workflows

Indexed sequencing is possible on NextSeq, MiSeq, and HiSeq platforms. The sequencing control software performs Read 1, any index reads, and then Read 2 based on the parameters provided for the run in either the sample sheet or during run setup.

On all platforms, the Index 1 Read directly follows Read 1 as it does for single-indexed sequencing. However, the rest of the workflow differs. The Index 2 Read is performed after Read 2 resynthesis on the NextSeq, HiSeq 4000, and HiSeq 3000. On the MiSeq and other HiSeq systems, the Index 2 Read is performed before Read 2 resynthesis. The differences in the workflow require platform-specific chemistry and sequencing primers. For more information, see *Sequencing Primers* on page 10.

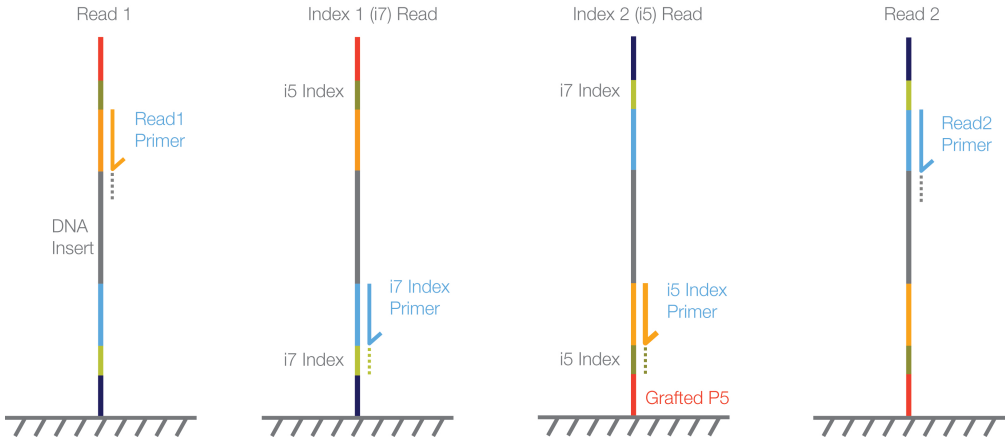
Sequencing Platforms	NextSeq, HiSeq 4000, and HiSeq 3000	MiSeq and HiSeq
Sequencing workflow steps	1. Read 1 2. Index Read preparation 3. Index 1 Read 4. Read 2 resynthesis 5. **Index 2 Read** 6. Read 2 preparation 7. Read 2	1. Read 1 2. Index Read preparation 3. Index 1 Read 4. **Index 2 Read** 5. Read 2 resynthesis 6. Read 2

Dual-Indexed Workflow on a NextSeq or HiSeq 3000/4000 Paired-End Flow Cell

A dual-indexed sequencing run on the NextSeq, HiSeq 4000, or HiSeq 3000 performs the Index 2 Read after the Read 2 resynthesis step. 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 BP13, the dual-indexing primer mix for the NextSeq. For the HiSeq, the Index 2 sequencing primer is part of HP14, an indexing primer mix that contains primers for both index reads.

Figure 2 Dual-Indexed Sequencing on a Paired-End Flow Cell (NextSeq or HiSeq 3000/4000)



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 8 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 8 cycles of sequencing.

NOTE
The dual-indexing workflow on the NextSeq, HiSeq 4000, and HiSeq 3000 does not require an additional 7 chemistry-only cycles.

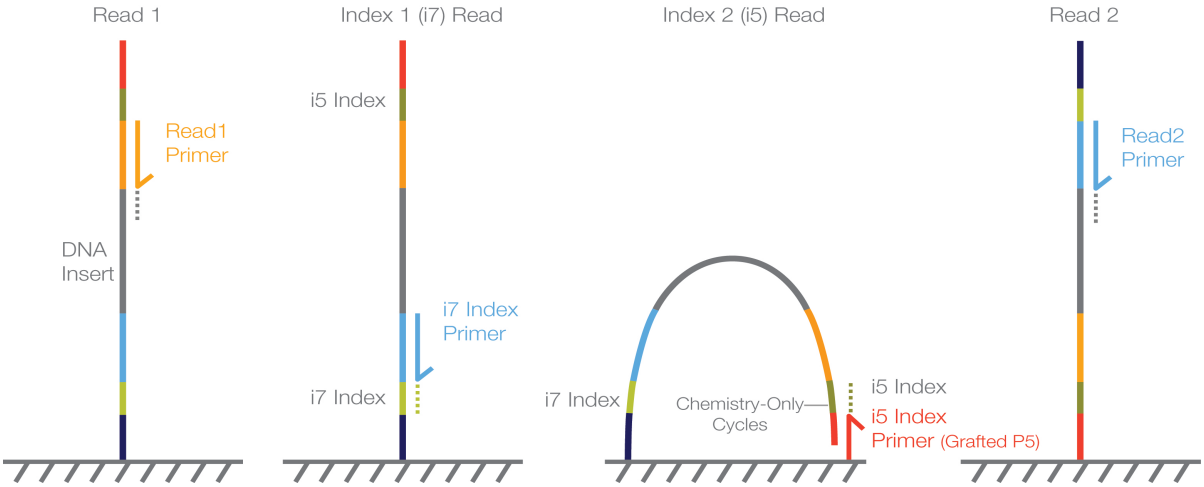
6 **Read 2 preparation**—The Index 2 Read product is removed and the Read 2 sequencing primer is annealed to the same template strand.

7 **Read 2**—Read 2 follows the standard paired-end sequencing protocol using SBS reagents.

Dual-Indexed Workflow on a MiSeq or HiSeq Paired-End Flow Cell

The chemistry applied to the Index 2 Read during a paired-end dual-indexed run on the MiSeq platform, HiSeq 2500, HiSeq 2000, or HiSeq 1500 is specific to the paired-end flow cell. Seven additional chemistry-only cycles are required to attach the i5 index to the complementary primer grafted to the flow cell. This step uses the resynthesis mix, a paired-end reagent, during the Index 2 Read process.

Figure 3 Dual-Indexed Sequencing on a Paired-End Flow Cell (MiSeq or HiSeq)

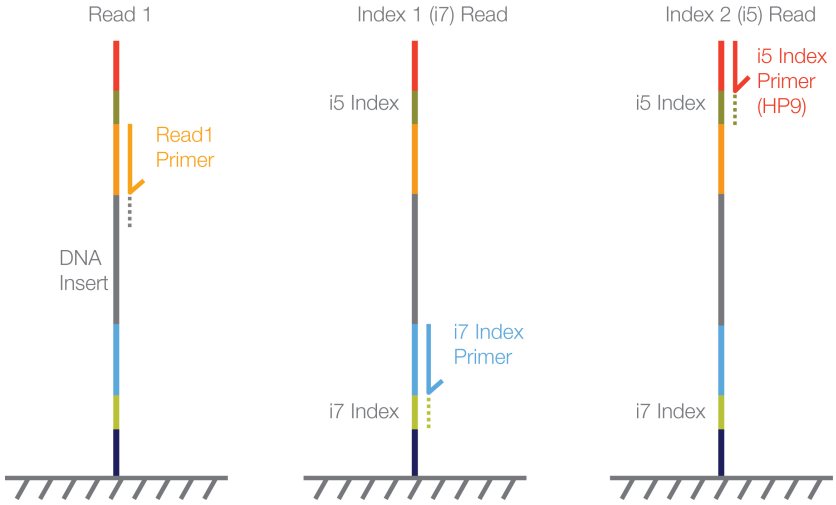


1. **Read 1**—Read 1 follows the standard Read 1 sequencing protocol using SBS reagents. The Read 1 sequencing primer is annealed to the template strand during the cluster generation step.
2. **Index Read preparation**—The Read 1 product is removed and the Index 1 (i7) sequencing primer is annealed to the same template strand.
3. **Index 1 (i7) Read**—Following Index Read preparation, the Index 1 (i7) Read performs 8 cycles of sequencing.
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 7 chemistry-only cycles (no imaging occurs), followed by 8 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. Then, the original template strand is 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.

Dual-Indexed Workflow on a HiSeq Single-Read Flow Cell

The chemistry applied to the Index 2 Read during a single-read dual-indexed run on the HiSeq platform is specific to the single-read flow cell. The Index 2 sequencing primer, **HP9**, is required to perform the Index 2 Read on a HiSeq single-read flow cell.

Figure 4 Dual-Indexed Sequencing on a Single-Read Flow Cell



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 8 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 8 cycles of sequencing.

Sequencing Primers

The following tables list available sequencing kits and the associated sequencing primers used for each step in an indexed sequencing run.

Sequencing Primers in NextSeq Kits

Run Type	Read 1	Index 1 (i7)	Index 2 (i5)	Read 2
NextSeq 500 High Output Kit v2	BP10	BP14	BP14	BP11
NextSeq 500 Mid Output Kit v2	BP10	BP14	BP14	BP11
NextSeq 500 High Output Kit	BP10	BP12	BP13	BP11
NextSeq 500 Mid Output Kit	BP10	BP12	BP13	BP11

Sequencing Primers in MiSeq Kits

Run Type	Read 1	Index 1 (i7)	Index 2 (i5)	Read 2
MiSeq Reagent Kit v3	HP10	HP12	--[1]	HP11
MiSeq Reagent Kit v2	HP10	HP12	--[1]	HP11

1 The resynthesis mix, a paired-end reagent provided in the reagent cartridge, is used to perform the Index 2 Read.

Sequencing Primers in HiSeq Kits

Run Type	Read 1	Index 1 (i7)	Index 2 (i5)	Read 2
HiSeq 3000/4000 PE Cluster Kit	HP10	HP14	HP14	HP11
HiSeq PE Cluster Kit v4	HP10	HP12	--[1]	HP11
HiSeq SR Cluster Kit v4	HP10	HP12	HP9	--
TruSeq PE Cluster Kit v3 [2]	HP6	HP8	--[1]	HP7
TruSeq SR Cluster Kit v3 [2]	HP6	HP8	--[3]	--

1 The resynthesis mix, a paired-end reagent provided in the paired-end cluster kit, is used to perform the Index 2 Read.
2 The TruSeq Dual Index Sequencing Primer Box is required in addition to the TruSeq Cluster Kit v3 when sequencing any Nextera libraries, except Nextera mate pair libraries. Sequencing primers provided in TruSeq v3 kits are not compatible with most Nextera libraries. Sequencing primers provided in the TruSeq Dual Index Sequencing Primer Box are compatible with all library types.
3 The TruSeq Dual Index Sequencing Primer Box for single reads is required to perform dual-indexed sequencing on a single-read flow cell, regardless of library type.

Additional Primers for TruSeq Cluster Kit v3

The following kit is required when sequencing Nextera libraries (except Nextera mate pair libraries) using TruSeq Cluster Kit v3, regardless of the type of run to be performed.

The sequencing primers provided in the TruSeq Cluster Kit v3 are not compatible with most Nextera libraries. To confirm primer compatibility, see the documentation for the kit used to prepare libraries.

The single-read kit is required to perform dual-indexed sequencing on a single-read flow cell, regardless of the libraries to be sequenced.

This kit is not required for any workflow if you use the HiSeq Cluster Kit v4.

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, is used to perform the Index 2 Read.

Notes

Technical Assistance

For technical assistance, contact Illumina Technical Support.

Table 1 Illumina General Contact Information

Website	www.illumina.com
Email	techsupport@illumina.com

Table 2 Illumina Customer Support Telephone Numbers

Region	Contact Number	Region	Contact Number
North America	1.800.809.4566	Italy	800.874909
Australia	1.800.775.688	Netherlands	0800.0223859
Austria	0800.296575	New Zealand	0800.451.650
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

Safety Data Sheets

Safety data sheets (SDSs) are available on the Illumina website at support.illumina.com/sds.html.

Illumina
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