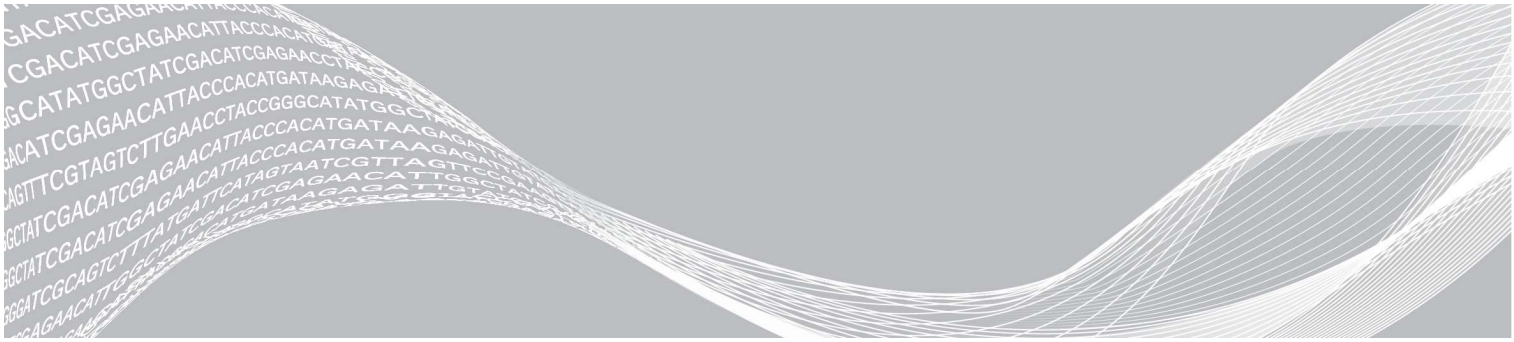
illumina®

Indexed Sequencing

Overview Guide



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Introduction

This guide provides an overview of indexed sequencing for all 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, or standalone bcl2fastq2 process these tags to identify 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.

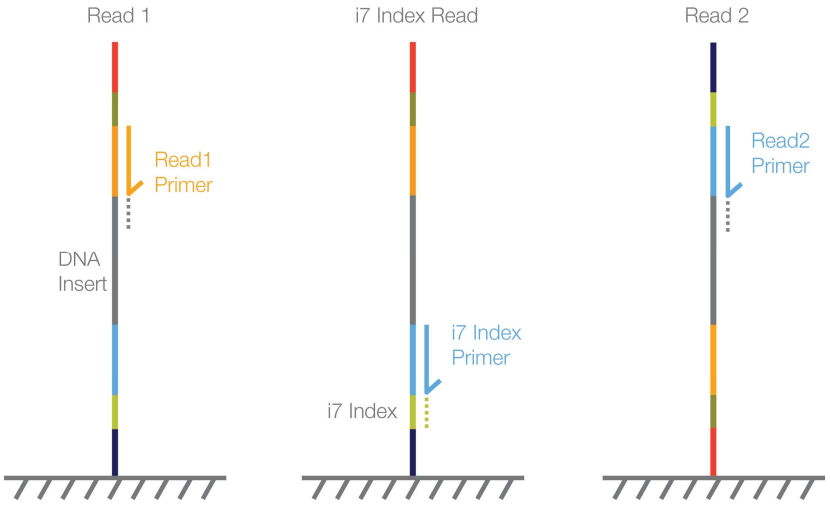
- **Single-indexed libraries**—Adds up to 48 unique six-base Index 1 (i7) sequences to generate up to 48 uniquely tagged libraries.
- **Dual-indexed libraries**—Adds up to 24 unique eight-base Index 1 (i7) sequences and up to 16 unique eight-base Index 2 (i5) sequences, generating up to 384 uniquely tagged libraries. The IDT for Illumina TruSeq UD Indexes are provided as index pairs and can generate up to 96 uniquely tagged libraries. These indexes add up to 96 unique eight-base Index 1 sequences and up to 96 unique eight-base Index 2 indexes.

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.

Dual-Indexing Workflows

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:

- **Workflow A**—The Index 2 Read is performed before Read 2 resynthesis, so the Index 2 (i5) adapter is sequenced on the forward strand.
- **Workflow B**—The Index 2 Read is performed 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

Workflow A	Workflow B
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

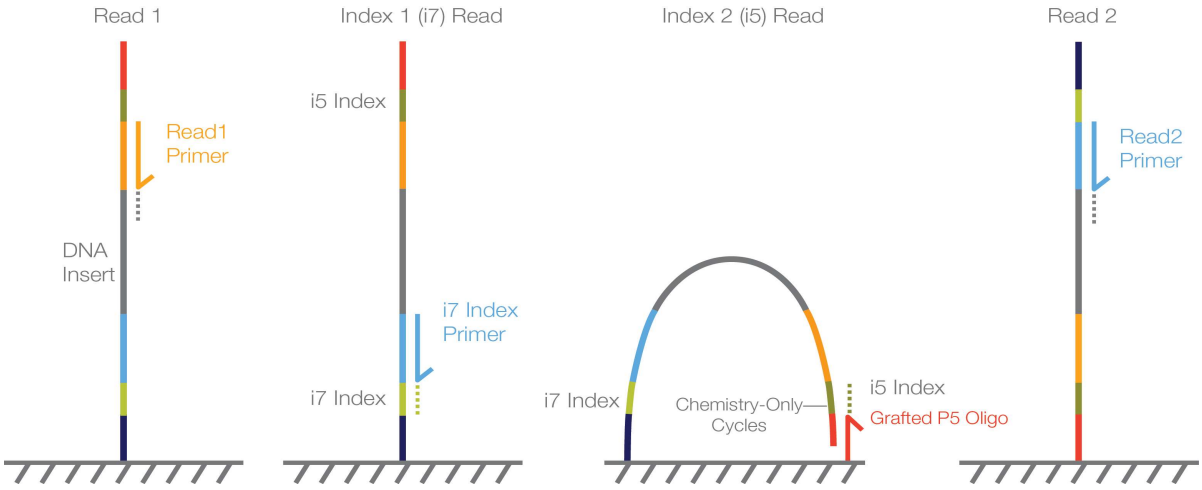
Dual-index sequencing on a paired-end flow cell follows one of two workflows, depending on the system:

- Workflow A is performed on the NovaSeq™ 6000, MiSeq™, HiSeq 2500, and HiSeq 2000.
- Workflow B is performed on the iSeq™ 100, MiniSeq™, NextSeq™, HiSeq X, HiSeq 4000, and HiSeq 3000.

Workflow A

The chemistry applied to the Index 2 Read during a paired-end dual-indexed run on the NovaSeq 6000, MiSeq, HiSeq 2500, or HiSeq 2000 is specific to the paired-end flow cell. Seven additional chemistry-only cycles are required to read the i5 index. This step uses the resynthesis mix, a paired-end reagent, during the Index 2 Read process.

Figure 2 Dual-Indexed Sequencing on a Paired-End Flow Cell (Workflow A)



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.

> **NOTE**
> The 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 7 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. 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.

Workflow B

A dual-indexed sequencing run on the iSeq 100, MiniSeq, NextSeq, HiSeq X, 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 the dual-indexing primer mix for iSeq 100, MiniSeq, and NextSeq. For HiSeq X, HiSeq 4000, and HiSeq 3000, the Index 2 sequencing primer is part of HP14, an indexing primer mix that contains primers for both index reads.

Figure 3 Dual-Indexed Sequencing on a Paired-End Flow Cell (Workflow B)

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 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 cycles of sequencing.

   NOTE

   Workflow B 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.

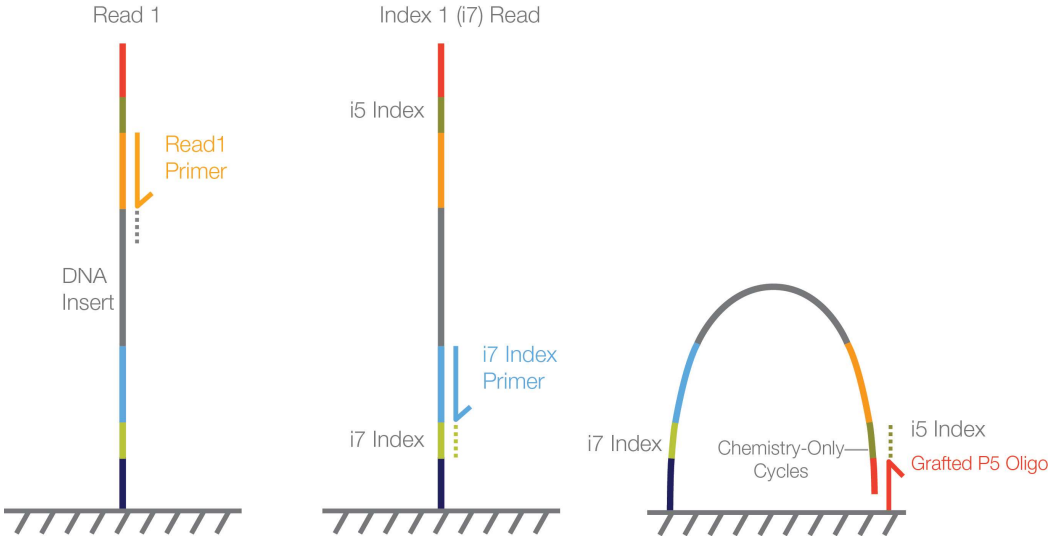
Dual-Indexed Workflow on a Single-Read Flow Cell

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

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

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 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.

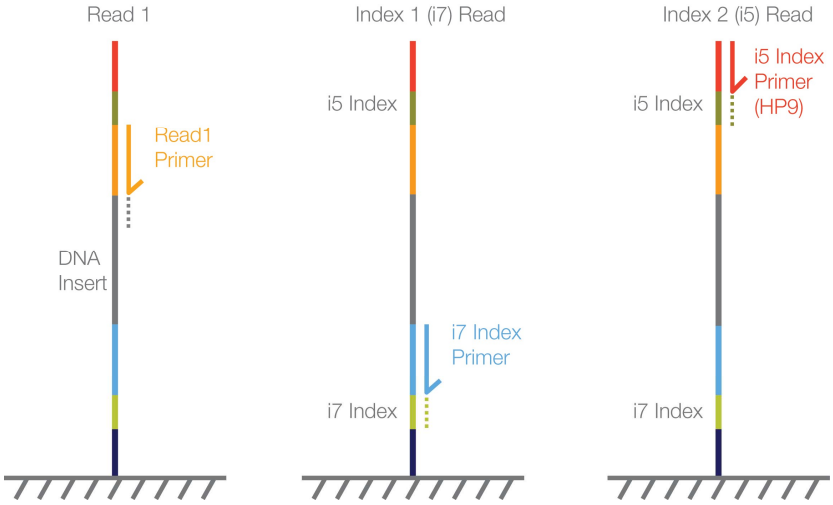
3 **Index 1 (i7) Read**—Following Index Read preparation, the Index 1 (i7) Read performs 8 cycles of sequencing.

4 **Index 2 (i5) Read**—The Index 1 (i7) Read product is removed and the template anneals to the grafted P5 oligo 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.

HiSeq 2500 and HiSeq 2000

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 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 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 cycles of sequencing.

NOTE

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 sequencing kits and the associated sequencing primers, which are used with each step of an indexed run.

NOTE

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