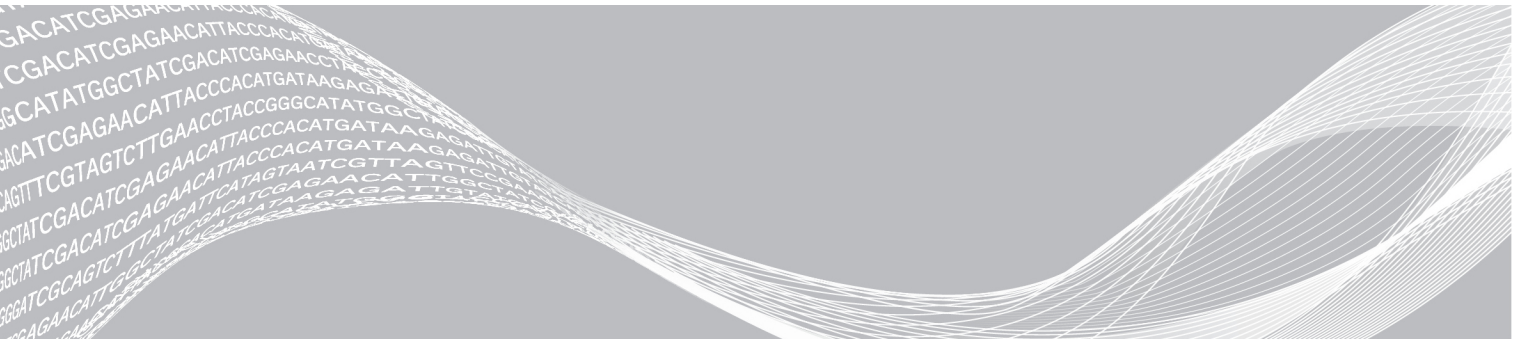
illumina®

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.

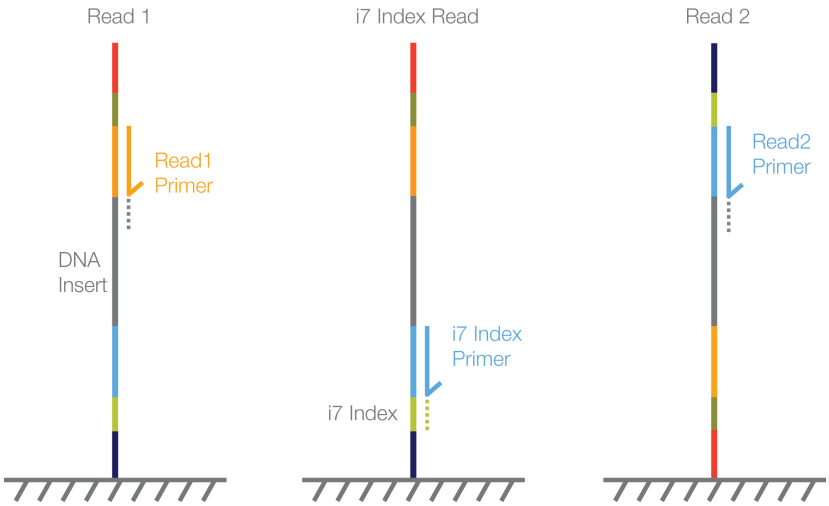
- **Single-indexed libraries**—Adds up to 48 unique six-base Index 1 (i7) sequences to generate up to 48 uniquely tagged libraries.
- **Dual-indexed libraries**—Adds up to 24 unique eight-base Index 1 (i7) sequences and up to 16 unique eight-base Index 2 (i5) sequences, generating up to 384 uniquely 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

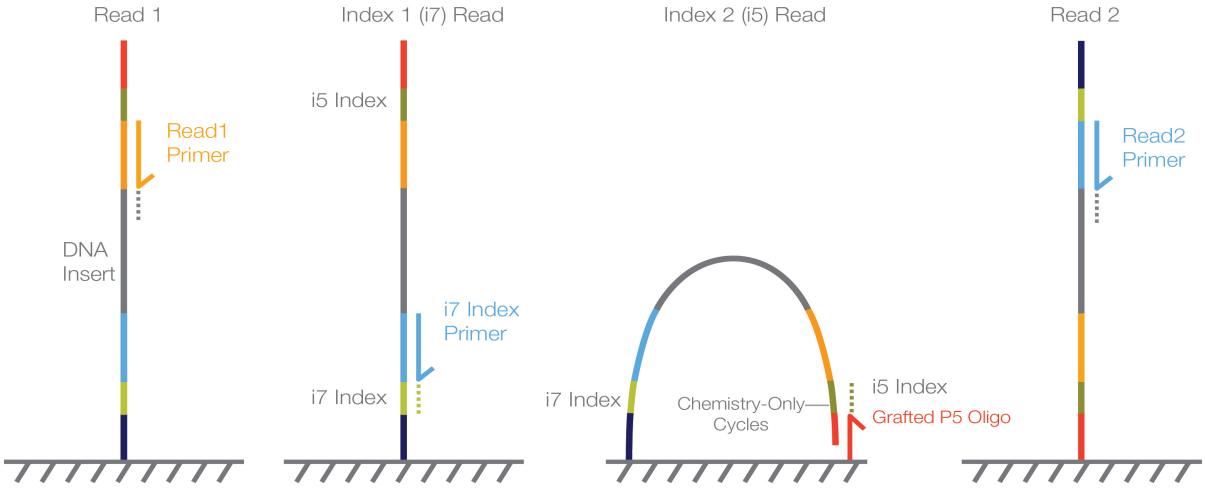
Dual-index sequencing on a paired-end flow cell follows one of two workflows, depending on the system:

- 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.

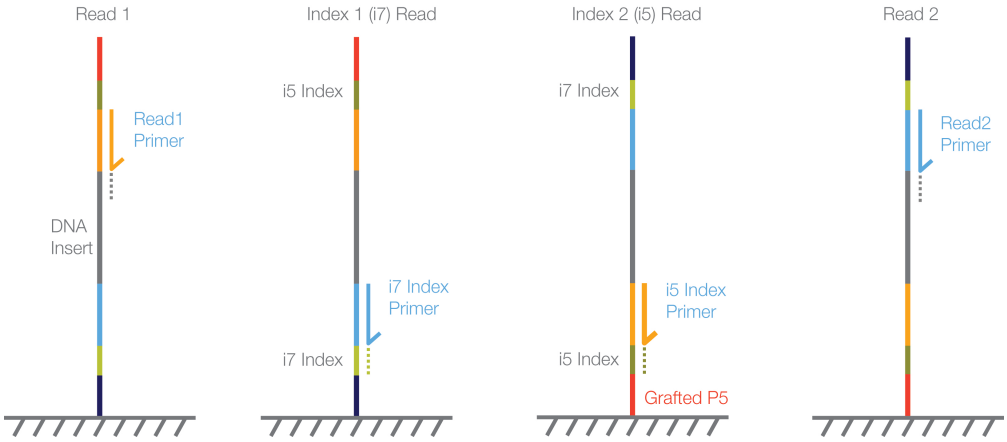
6 **Read 2**—Read 2 follows the standard paired-end sequencing protocol using SBS reagents.

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.

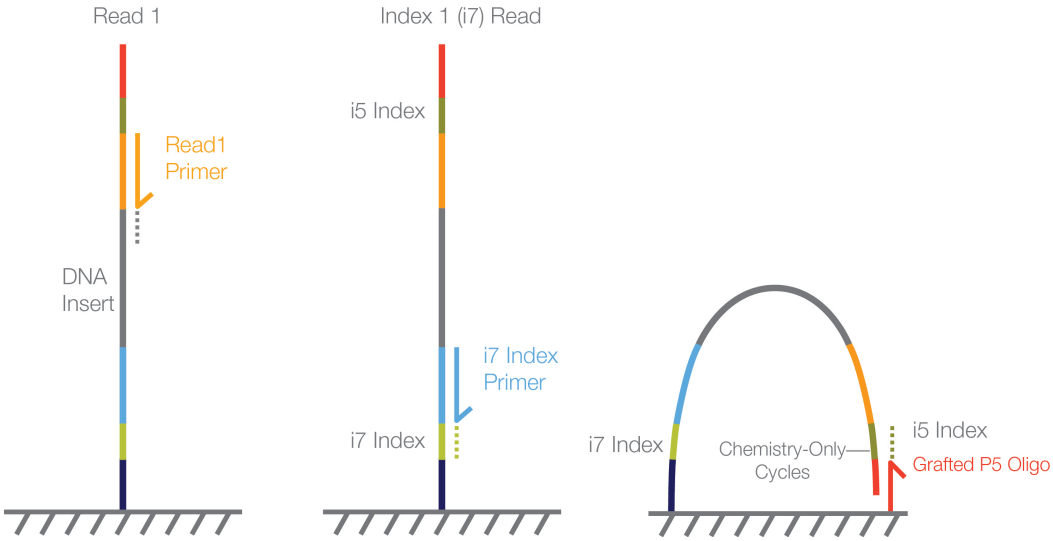
Dual-Indexed Workflow on a Single-Read Flow Cell

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)

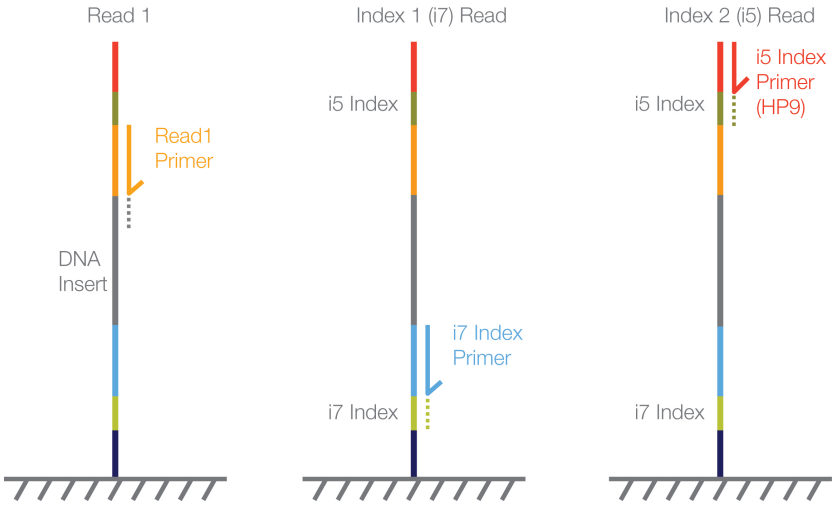


1. **Read 1**—Read 1 follows the standard Read 1 sequencing protocol using SBS reagents. The Read 1 sequencing primer is annealed to the template strand during the cluster generation step.
2. **Index Read preparation**—The Read 1 product is removed and the Index 1 (i7) sequencing primer is annealed to 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.

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 3000/4000 SR Cluster Kit	HP10	HP14	--[1]	--
HiSeq PE Cluster Kit v4	HP10	HP14	--[1]	HP11
HiSeq SR Cluster Kit v4	HP10	HP14	HP9	--
TruSeq PE Cluster Kit v3[2]	HP6	HP8	--[1]	HP7
TruSeq SR Cluster Kit v3[2]	HP6	HP8	--[3]	--

[1] The resynthesis mix is used to perform the Index 2 Read.

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

Additional Primers for TruSeq Cluster Kit v3

The TruSeq Dual Index Sequencing Primer Box is required when sequencing Nextera libraries (except Nextera Mate Pair libraries) using TruSeq Cluster Kit v3, regardless of run type. 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.

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.

Revision History

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

Technical Assistance

For technical assistance, contact Illumina Technical Support.

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

Illumina Customer Support Telephone Numbers

Region	Toll Free	Regional
North America	+1.800.809.4566	
Australia	+1.800.775.688	
Austria	+43 800006249	+43 19286540
Belgium	+32 80077160	+32 34002973
China	400.066.5835	
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Hong Kong	800960230	
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Italy	+39 800985513	+39 236003759
Japan	0800.111.5011	
Netherlands	+31 8000222493	+31 207132960
New Zealand	0800.451.650	
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Spain	+34 911899417	+34 800300143
Sweden	+46 850619671	+46 200883979
Switzerland	+41 565800000	+41 800200442
Taiwan	00806651752	
United Kingdom	+44 8000126019	+44 2073057197
Other countries	+44.1799.534000	

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

Product documentation—Available for download in PDF from the Illumina website. Go to support.illumina.com, select a product, then select **Documentation & Literature**.

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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

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