

Index Adapters

Pooling Guide

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Introduction

This guide provides recommendations for optimizing color balance across all Illumina systems when pooling indexed libraries. Pooling combines at least two libraries to sequence in one run.

The recommendations are designed to conveniently form low-plex, color-balanced pools. Higher plexity pools are inherently color-balanced so any index adapter combinations are acceptable. To develop alternative pools, review index adapter sequences in *Illumina Adapter Sequences (document # 1000000002694)* to ensure color balance for your system.



NOTE

Plexity is the number of libraries combined in a reaction or pool. For example, if 12 libraries are combined in one pool, the plexity is 12.

Use this guide as a reference to plan indexing and pooling for library prep. For library prep instructions, see the reference guide for your kit. All documentation is available on the Illumina website (support.illumina.com).

Dual Indexing Options

Combinatorial dual (CD) indexes have unique dual pairs, but the index adapters share some sequences. In contrast, unique dual (UD) indexes have distinct sequences so each index adapter is unique.

- ▶ UD indexes have 96 unique Index 1 (i7) adapters and 96 unique Index 2 (i5) adapters, preventing repeated sequences in a plate.
- ▶ CD indexes have 12 unique Index 1 (i7) adapters and 8 unique Index 2 (i5) adapters, so sequences are repeated down the rows and columns of a plate.

Plate layouts for IDT for Illumina UD Indexes are designed with the proper color balance. Achieving this balance with CD indexes requires additional planning.

Color Balance

Selecting index adapters with diverse sequences for pooled libraries optimizes color balance for successful sequencing and data analysis.

When index adapter sequences are recorded in Illumina Experiment Manager (IEM), Local Run Manager, or BaseSpace Sequence Hub Prep tab, the software confirms that libraries in a pool have unique index combinations. However, Local Run Manager and BaseSpace Sequence Hub do not check for color balance. IEM checks for color balance, but for HiSeq systems only.

Example Index Adapter Combinations

The following table shows good and bad index combinations to demonstrate color balance on a four-channel system. The good examples have signal in both channels (red and green). The bad examples are missing signal in one channel, as indicated with an X.

Good Examples				Bad Examples			
Index 1		Index 2		Index 1		Index 2	
N705	G G A C T C C T	S503	T A T C C T C T	N705	G G A C T C C T	S502	C T C T C T A T
N706	T A G G C A T G	S503	T A T C C T C T	N706	T A G G C A T G	S502	C T C T C T A T
N701	T A A G G C G A	S504	A G A G T A G A	N701	T A A G G C G A	S503	T A T C C T C T
N702	C G T A C T A G	S504	A G A G T A G A	N702	C G T A C T A G	S503	T A T C C T C T
	✓✓✓✓✓✓✓✓		✓✓✓✓✓✓✓✓		✓✓✓✓✓✓✓✓		✓✓✓✓XXXX

Sequencing Chemistry

Illumina sequencing systems use one-, two-, or four-channel chemistry to perform base calling. During sequencing, a separate read called the Index Read sequences the index. Dual-indexed sequencing includes Index Read 1 and Index Read 2.

Chemistry	Sequencing System
One-channel	iSeq 100 System
Two-channel	NovaSeq 6000, NextSeq 550, NextSeq 500, and MiniSeq Systems
Four-channel	The MiSeq System and all HiSeq Systems

For more information on sequencing chemistry and base calling, see the system guide for your instrument. See *Indexed Sequencing Overview (document # 15057455)* for indexing workflows on Illumina systems.

One-Channel Chemistry

One-channel chemistry requires one dye and two images to encode data for the four bases. Intensities extracted from one image and compared to a second image to result in four distinct populations, each corresponding to a nucleotide. Base calling determines which population each cluster belongs to.

When sequencing on a one-channel system, the first two cycles of the Index Read cannot start with two G bases. Otherwise, intensity is not generated.

- ▶ Make sure that **at least** one index sequence in a library pool does not start with two G bases.
- ▶ Select balanced index sequences so that signal is present in at least one image (preferably both) for every cycle.

Two-Channel Chemistry

Two-channel chemistry requires two dyes and two images, one from the green channel and one from the red channel, to encode data for the four bases. Intensities extracted from an image compared to another image result in four distinct populations, each corresponding to a base. Base calling determines which population each cluster belongs to.

When sequencing on a two-channel system, either of the first two cycles of the Index Read must start with at least one base other than G. If an Index Read starts with two G bases, signal intensity is not generated. Signal must be present in the first two cycles.

Combine index sequences so that signal is present in at least one channel (preferably both) for every cycle:

- ▶ **Red channel**—A or C
- ▶ **Green channel**—A or T

For more example index combinations for two-channel systems, see the *Library pooling guidelines for the NextSeq and MiniSeq systems* bulletin on the Illumina website.

Four-Channel Chemistry

Four-channel chemistry uses four dyes and four images per cycle to observe which dye is incorporated into a cluster. A green laser sequences G and T bases, while a red laser sequences A and C bases. To ensure proper image registration, each cycle must include at least one of two nucleotides per color channel.

When sequencing on a four-channel system, make sure that pooled libraries contain unique and color-compatible index combinations for each Index Read.

Pooling Guidelines for Nextera Kits

This section provides guidelines for pooling indexed Nextera libraries. The following index adapters are compatible with PCR extension-based Nextera library prep kits.

Index Adapters	Indexing Scheme	Format
IDT for Illumina Nextera DNA UD Indexes	Dual indexing	Plate
Nextera DNA CD Indexes (96 indexes, 96 samples)	Dual indexing	Plate
Nextera DNA CD Indexes (24 indexes, 24 samples)	Dual indexing	Tube
Nextera XT Index Kit v2	Single and dual indexing	Tube
Nextera Index Kit	Single and dual indexing	Tube

IDT for Illumina Nextera DNA UD Indexes

The following table depicts the plate layout for IDT for Illumina Nextera DNA UD Indexes, which are designed for dual-indexing.

	1	2	3	4	5	6	7	8	9	10	11	12
A	UDP0001	UDP0009	UDP0017	UDP0025	UDP0033	UDP0041	UDP0049	UDP0057	UDP0065	UDP0073	UDP0081	UDP0089
B	UDP0002	UDP0010	UDP0018	UDP0026	UDP0034	UDP0042	UDP0050	UDP0058	UDP0066	UDP0074	UDP0082	UDP0090
C	UDP0003	UDP0011	UDP0019	UDP0027	UDP0035	UDP0043	UDP0051	UDP0059	UDP0067	UDP0075	UDP0083	UDP0091
D	UDP0004	UDP0012	UDP0020	UDP0028	UDP0036	UDP0044	UDP0052	UDP0060	UDP0068	UDP0076	UDP0084	UDP0092
E	UDP0005	UDP0013	UDP0021	UDP0029	UDP0037	UDP0045	UDP0053	UDP0061	UDP0069	UDP0077	UDP0085	UDP0093
F	UDP0006	UDP0014	UDP0022	UDP0030	UDP0038	UDP0046	UDP0054	UDP0062	UDP0070	UDP0078	UDP0086	UDP0094
G	UDP0007	UDP0015	UDP0023	UDP0031	UDP0039	UDP0047	UDP0055	UDP0063	UDP0071	UDP0079	UDP0087	UDP0095
H	UDP0008	UDP0016	UDP0024	UDP0032	UDP0040	UDP0048	UDP0056	UDP0064	UDP0072	UDP0080	UDP0088	UDP0096

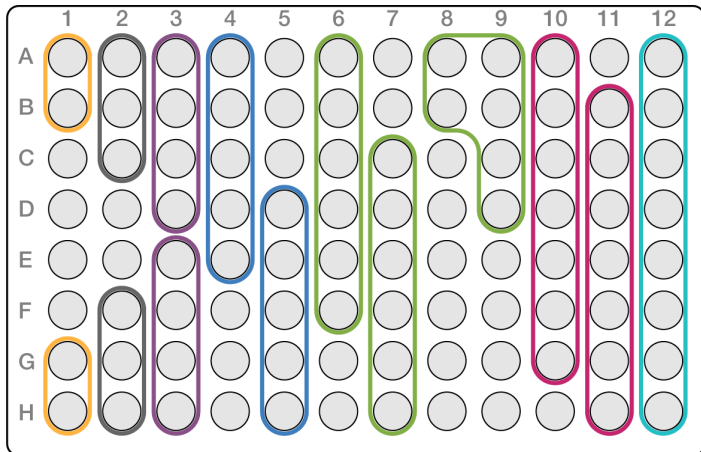
Two-Plex Through Eight-Plex Pooling Strategies

The following table shows index adapters (wells) that can be combined in a 2–8-plex pool, while the color-coded figure illustrates each combination.

Pool any plexity ≥ 2 from the top or bottom of a column. Do not pool across a row.

Plexity	Combinations	Color in Figure
2	The first two or last two wells in a column: <ul style="list-style-type: none"> • A and B • G and H Rows C–F are not used.	Orange
3	The first three or last three wells in a column: <ul style="list-style-type: none"> • A–C • F–H Rows D and E are not used.	Gray

Plexity	Combinations	Color in Figure
4	The first four or last four wells in a column: <ul style="list-style-type: none"> • A–D • E–H 	Purple
5	The first five or last five wells in a column: <ul style="list-style-type: none"> • A–E • D–H 	Blue
6	[Option 1] The first six or last six wells in a column: <ul style="list-style-type: none"> • A–F • D–H [Option 2] The first two wells (A and B) or last two wells (G and H) in one column and any four wells in an adjacent column.	Green
7	The first seven or last seven wells in a column: <ul style="list-style-type: none"> • A–G • B–H 	Pink
8	The entire column.	Teal

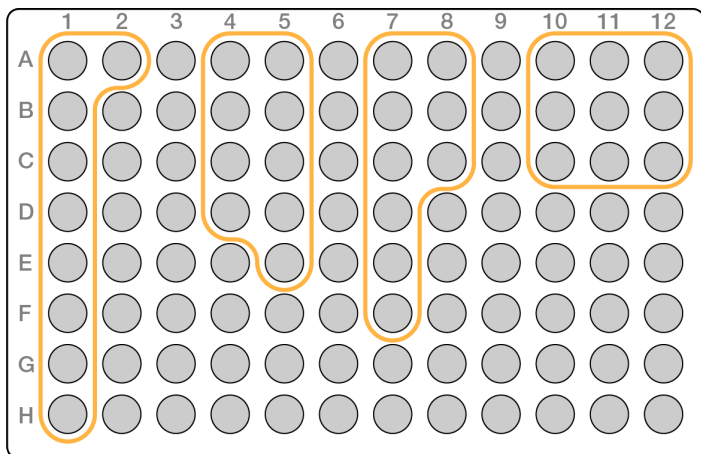


Nine-Plex Pooling Strategies

Use index adapters from any wells that optimize color balance in a sequencing run, for example:

- ▶ A1–H1 and A2
- ▶ A4–D4 and A5–E5
- ▶ A7–F7 and A8–C8
- ▶ A10–C10, A11–C11, and A12–C12

The following figure depicts all four examples.



Nextera DNA CD Indexes (96 Indexes, 96 Samples)

The following table depicts the plate layout for Nextera DNA CD Indexes (96 indexes, 96 samples), which are designed for dual-indexing.

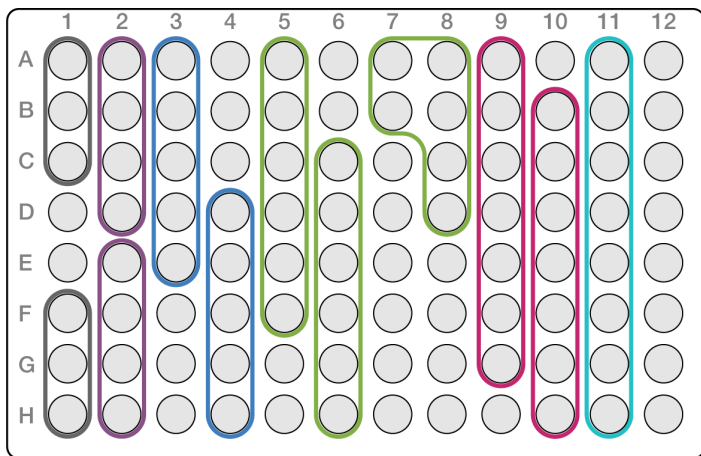
	1	2	3	4	5	6	7	8	9	10	11	12
A	H701 H505	H702 H506	H703 H517	H705 H505	H707 H506	H723 H517	H706 H505	H712 H506	H720 H517	H710 H505	H711 H506	H714 H517
B	H702 H517	H703 H505	H701 H506	H707 H517	H723 H505	H705 H506	H712 H517	H720 H505	H706 H506	H711 H517	H714 H505	H710 H506
C	H703 H506	H701 H517	H702 H505	H723 H506	H705 H517	H707 H505	H720 H506	H706 H517	H712 H505	H714 H506	H710 H517	H711 H505
D	H705 H503	H707 H503	H723 H503	H706 H503	H712 H503	H720 H503	H710 H503	H711 H503	H714 H503	H701 H503	H702 H503	H703 H503
E	H706 H516	H712 H516	H720 H516	H710 H516	H711 H516	H714 H516	H701 H516	H702 H516	H703 H516	H705 H516	H707 H516	H723 H516
F	H710 H522	H711 H510	H714 H513	H701 H522	H702 H510	H703 H513	H705 H522	H707 H510	H723 H513	H706 H522	H712 H510	H720 H513
G	H711 H513	H714 H522	H710 H510	H702 H513	H703 H522	H701 H510	H707 H513	H723 H522	H705 H510	H712 H513	H720 H522	H706 H510
H	H714 H510	H710 H513	H711 H522	H703 H510	H701 H513	H702 H522	H723 H510	H705 H513	H707 H522	H720 H510	H706 H513	H712 H522

Three-Plex Through Eight-Plex Pooling Strategies

The following table shows index adapters (wells) that can be combined in a 3–8-plex pool, while the color-coded figure illustrates each combination.

A minimum plexity of three ensures that libraries are color balanced for sequencing on any Illumina system. To create a two-plex pool, review the index adapter sequences to ensure color balance on your system.

Plexity	Combinations	Color in Figure
3	The first three or last three wells in a column: <ul style="list-style-type: none"> • A-C • F-H Rows D and E are not used.	Gray
4	The first four or last four wells in a column: <ul style="list-style-type: none"> • A-D • E-H 	Purple
5	The first five or last five wells in a column: <ul style="list-style-type: none"> • A-E • D-H 	Blue
6	[Option 1] The first six or last six wells in a column: <ul style="list-style-type: none"> • A-F • D-H [Option 2] The first two wells (A and B) or last two wells (G and H) in one column and any four wells in an adjacent column.	Green
7	The first seven or last seven wells in a column: <ul style="list-style-type: none"> • A-G • B-H 	Pink
8	The entire column.	Teal

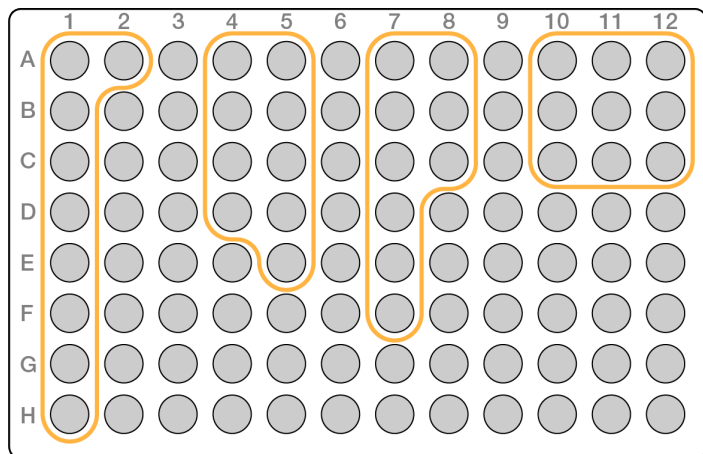


Nine-Plex Pooling Strategies

Use index adapters from any wells that optimize color balance in a sequencing run, for example:

- ▶ A1-H1 and A2
- ▶ A4-D4 and A5-E5
- ▶ A7-F7 and A8-C8
- ▶ A10-C10, A11-C11, and A12-C12

The following figure depicts all four examples.



Nextera DNA CD Indexes (24 Indexes, 24 Samples)

Nextera DNA CD Indexes (24 indexes, 24 samples) contain six Index 1 (i7) adapters and four Index 2 (i5) adapters packaged in tubes. The following table shows strategies for pooling 3–8 dual-indexed libraries prepared with these indexes.

A minimum plexity of three ensures that libraries are color balanced for sequencing on any Illumina system. To create a two-plex pool, review the index adapter sequences to ensure color balance on your system.

Plexity	Index 1 (i7) Adapters	Index 2 (i5) Adapters
3	One of the following combinations: <ul style="list-style-type: none"> • H705, H706, and H707 • H710, H711, and H714 	H505, H506, and H517
4	One of the following combinations: <ul style="list-style-type: none"> • H705, H706, H707, and any other i7 adapter • H710, H711, H714, and any other i7 adapter 	H503, H505, H506, and H517
5	One of the following combinations: <ul style="list-style-type: none"> • H705, H706, H707, and any two i7 adapters • H710, H711, H714, and any two i7 adapters 	H503, H505, H506, H517, and any other i5 adapter
6	One of the following combinations: <ul style="list-style-type: none"> • H705, H706, H707, H710, H711, H714 • Two 3-plex pools 	One of the following combinations: <ul style="list-style-type: none"> • H503, H505, H506, H517, and any two additional i5 adapters • Two 3-plex pools
7	One of the following combinations: <ul style="list-style-type: none"> • H705, H706, H707, H710, H711, H714, and any other i7 adapter • Two 3-plex pools and any other i7 adapter 	One of the following combinations: <ul style="list-style-type: none"> • H503, H505, H506, H517, and any three additional i5 adapters • Two 3-plex pools and any other i5 adapter
8	H705, H706, H707, H710, H711, H714, and two other i7 adapters	H503, H505, H506, and H517, used two times each

Four-Plex Example

Libraries with the following Index 1 and Index 2 adapter pairs can be combined in a four-plex pool:

- ▶ H705-H503
- ▶ H706-H505
- ▶ H707-H506
- ▶ H710-H517

Five-Plex Example

Libraries with the following Index 1 and Index 2 adapter pairs can be combined in a five-plex pool:

- ▶ H503-H705
- ▶ H505-H706
- ▶ H506-H707
- ▶ H517-H710
- ▶ H503-H711

Six-Plex Example

Libraries with the following Index 1 and Index 2 adapter pairs can be combined in a six-plex pool:

- ▶ H705-H503
- ▶ H706-H505
- ▶ H707-H506
- ▶ H710-H517
- ▶ H711-H503
- ▶ H714-H505

Nextera XT Index Kit v2 and Nextera Index Kit

The Nextera XT Index Kit v2 is available in Sets A–D. Each set contains 12 Index 1 (i7) and eight Index 2 (i5) adapters packaged in tubes.

The Nextera Index Kit also packages Index 1 and Index 2 adapters in tubes, and is available in two sizes:

- ▶ The 24 indexes, 96 samples size contains six Index 1 and four Index 2 adapters.
- ▶ The 96 indexes, 384 samples size contains 12 Index 1 adapters and eight Index 2 adapters.

Dual Indexing

The following table shows strategies for pooling dual-indexed libraries for dual-indexed sequencing, which sequences Index 1 and Index 2.

Plexity	Index 1 (i7) Adapters	Index 2 (i5) Adapters
2–6	At least two unique i7 adapters	At least two unique i5 adapters
7–12	One of the following combinations: <ul style="list-style-type: none"> • N701, N702, N704, and any other i7 adapter • N703, N705, N706, and any other i7 adapter 	One of the following combinations: <ul style="list-style-type: none"> • N503 and N504 • N505 and N506
> 12	N701–N706 and any other i7 adapter	One of the following combinations: <ul style="list-style-type: none"> • N503, N504, and any other i5 adapter • N505, N506, and any other i5 adapter

Single Indexing With Dual Index Adapters

The following table shows strategies for creating 2–12-plex pools of dual-indexed libraries for single-indexed sequencing, which sequences Index 1 only.

Plexity	Index 1 (i7) Adapters	Index 2 (i5) Adapters
2	One of the following combinations: <ul style="list-style-type: none"> • N701 and N702 • N702 and N704 	Any i5 adapters
3	One of the following combinations: <ul style="list-style-type: none"> • N701, N702, and N704 • N703, N705, and N706 	Any i5 adapters
4 or 5	One of the following combinations: <ul style="list-style-type: none"> • N701, N702, N704, and any other i7 adapter • N703, N705, N706, and any other i7 adapter 	Any i5 adapters
6	N701, N702, N703, N704, N705, and N706	Any i5 adapters
7–12	N701–N706 and any other i7 adapter	Any i5 adapters

Pooling Guidelines for AmpliSeq for Illumina Kits

This section provides guidelines for pooling AmpliSeq for Illumina libraries ligated with AmpliSeq CD Indexes for Illumina. These index adapters are designed for dual-indexing and use with all AmpliSeq for Illumina panels.

The following table depicts the index plate layout. Use a column- or row-based pooling strategy to create 2–8-plex pools.

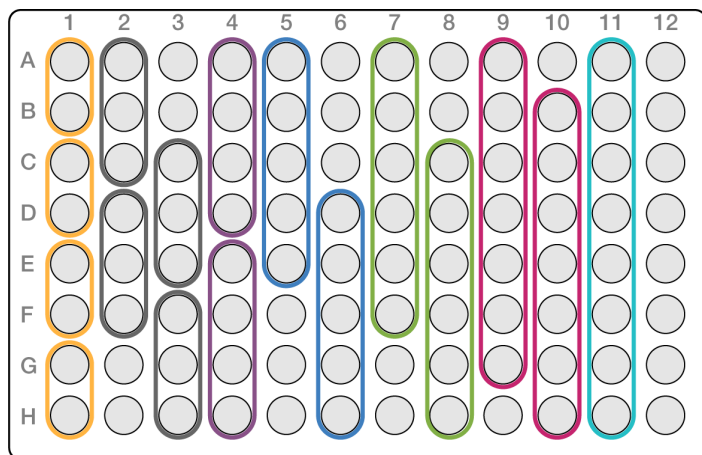
	1	2	3	4	5	6	7	8	9	10	11	12
A	Q7005 Q5001	Q7015 Q5002	Q7006 Q5007	Q7007 Q5008	Q7016 Q5009	Q7008 Q5010	Q7018 Q5001	Q7023 Q5002	Q7017 Q5007	Q7025 Q5008	Q7024 Q5009	Q7026 Q5010
B	Q7006 Q5002	Q7016 Q5001	Q7005 Q5008	Q7008 Q5007	Q7015 Q5010	Q7007 Q5009	Q7017 Q5002	Q7024 Q5001	Q7018 Q5008	Q7026 Q5007	Q7023 Q5010	Q7025 Q5009
C	Q7016 Q5007	Q7008 Q5008	Q7015 Q5009	Q7006 Q5010	Q7007 Q5013	Q7005 Q5014	Q7024 Q5007	Q7026 Q5008	Q7023 Q5009	Q7017 Q5010	Q7025 Q5013	Q7018 Q5014
D	Q7015 Q5008	Q7007 Q5007	Q7016 Q5010	Q7005 Q5009	Q7008 Q5014	Q7006 Q5013	Q7023 Q5008	Q7025 Q5007	Q7024 Q5010	Q7018 Q5009	Q7026 Q5014	Q7017 Q5013
E	Q7017 Q5009	Q7025 Q5010	Q7018 Q5013	Q7023 Q5014	Q7026 Q5001	Q7024 Q5002	Q7006 Q5009	Q7007 Q5010	Q7005 Q5013	Q7015 Q5014	Q7008 Q5001	Q7016 Q5002
F	Q7018 Q5010	Q7026 Q5009	Q7017 Q5014	Q7024 Q5013	Q7025 Q5002	Q7023 Q5001	Q7005 Q5010	Q7008 Q5009	Q7006 Q5014	Q7016 Q5013	Q7007 Q5002	Q7015 Q5001
G	Q7026 Q5013	Q7024 Q5014	Q7025 Q5001	Q7018 Q5002	Q7023 Q5007	Q7017 Q5008	Q7008 Q5013	Q7016 Q5014	Q7007 Q5001	Q7005 Q5002	Q7015 Q5007	Q7006 Q5008
H	Q7025 Q5014	Q7023 Q5013	Q7026 Q5002	Q7017 Q5001	Q7024 Q5008	Q7018 Q5007	Q7007 Q5014	Q7015 Q5013	Q7008 Q5002	Q7006 Q5001	Q7016 Q5008	Q7005 Q5007

Column-Based Strategies

The following table shows index adapters (wells) that can be combined in a 2–8-plex pool, while the color-coded figure illustrates each combination.

All combinations apply to any column on the plate. The minimum plexity for a column is two.

Plexity	Combinations	Color in Figure
2	Two consecutive wells in a column: <ul style="list-style-type: none"> • A and B • C and D • E and F • G and H 	Orange
3	Three consecutive wells in a column: <ul style="list-style-type: none"> • A-C • D-F • C-E • F-H 	Gray
4	Four consecutive wells in a column: <ul style="list-style-type: none"> • A-D • E-H 	Purple
5	Five consecutive wells in a column: <ul style="list-style-type: none"> • A-E • D-H 	Blue
6	Six consecutive wells in a column: <ul style="list-style-type: none"> • A-F • C-H 	Green
7	Seven consecutive wells in a column: <ul style="list-style-type: none"> • A-G • B-H 	Pink
8	The entire column. All eight combinations are unique.	Teal

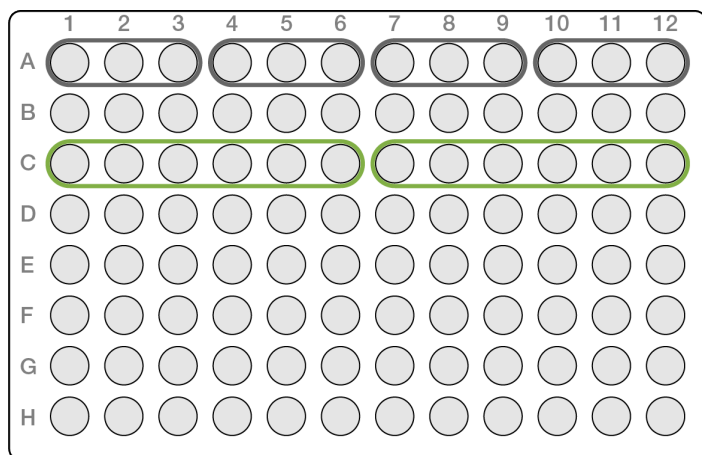


Row-Based Strategies

The following table shows index adapters (wells) that can be combined in 3- or 6-plex pool, while the color-coded figure illustrates each well combination.

All combinations apply to any row on the plate. A row can contain any set of pools confined to columns 1–6 or columns 7–12. The minimum plexity for a row is three.

Plexity	Combinations	Color in Figure
3	Three consecutive wells in a row. <ul style="list-style-type: none"> • 1-3 • 4-6 • 7-9 • 10-12 	Gray
6	Six consecutive wells in a row: <ul style="list-style-type: none"> • 1-6 • 7-12 	Green



Pooling Guidelines for TruSeq Kits

This section provides guidelines for pooling indexed TruSeq libraries. The following index adapters are compatible with ligation-based TruSeq library prep kits.

Index Adapters	Indexing Scheme	Format
IDT for Illumina TruSeq DNA UD Indexes IDT for Illumina TruSeq RNA UD Indexes	Dual indexing	Plate
TruSeq DNA CD Indexes TruSeq RNA CD Indexes	Single and dual indexing	Plate
TruSeq DNA Single Indexes TruSeq RNA Single Indexes	Single indexing	Tubes

IDT for Illumina TruSeq UD Indexes

The following table depicts the plate layout for IDT for Illumina TruSeq DNA UD Indexes and IDT for Illumina TruSeq RNA UD Indexes. These indexes are designed for select TruSeq libraries.

Pool any plexity ≥ 2 down a column. Do not pool across a row.

	1	2	3	4	5	6	7	8	9	10	11	12
A	UD0001	UD0009	UD0017	UD0025	UD0033	UD0041	UD0049	UD0057	UD0065	UD0073	UD0081	UD0089
B	UD0002	UD0010	UD0018	UD0026	UD0034	UD0042	UD0050	UD0058	UD0066	UD0074	UD0082	UD0090
C	UD0003	UD0011	UD0019	UD0027	UD0035	UD0043	UD0051	UD0059	UD0067	UD0075	UD0083	UD0091
D	UD0004	UD0012	UD0020	UD0028	UD0036	UD0044	UD0052	UD0060	UD0068	UD0076	UD0084	UD0092
E	UD0005	UD0013	UD0021	UD0029	UD0037	UD0045	UD0053	UD0061	UD0069	UD0077	UD0085	UD0093
F	UD0006	UD0014	UD0022	UD0030	UD0038	UD0046	UD0054	UD0062	UD0070	UD0078	UD0086	UD0094
G	UD0007	UD0015	UD0023	UD0031	UD0039	UD0047	UD0055	UD0063	UD0071	UD0079	UD0087	UD0095
H	UD0008	UD0016	UD0024	UD0032	UD0040	UD0048	UD0056	UD0064	UD0072	UD0080	UD0088	UD0096

TruSeq CD Indexes (Dual Indexing)

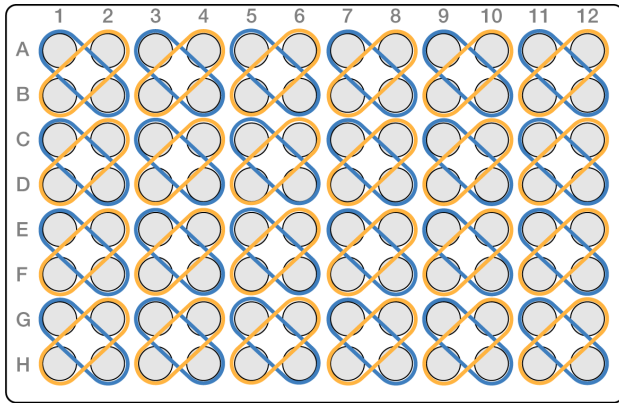
The following table depicts the plate layout for TruSeq DNA CD Indexes and TruSeq RNA CD Indexes (formerly TruSeq HT Indexes). Subsequent figures illustrate dual indexing strategies for combining these indexes in 2–16-plex pools:

- ▶ Circles indicate index adapter combinations that can be combined in a pool.
- ▶ Dark gray indicates wells that are not used for pool plexity ≥ 2 , but can be used for one-plex pools (single libraries).

	1	2	3	4	5	6	7	8	9	10	11	12
A	D701 D501	D702 D501	D703 D501	D704 D501	D705 D501	D706 D501	D707 D501	D708 D501	D709 D501	D710 D501	D711 D501	D712 D501
B	D701 D502	D702 D502	D703 D502	D704 D502	D705 D502	D706 D502	D707 D502	D708 D502	D709 D502	D710 D502	D711 D502	D712 D502
C	D701 D503	D702 D503	D703 D503	D704 D503	D705 D503	D706 D503	D707 D503	D708 D503	D709 D503	D710 D503	D711 D503	D712 D503
D	D701 D504	D702 D504	D703 D504	D704 D504	D705 D504	D706 D504	D707 D504	D708 D504	D709 D504	D710 D504	D711 D504	D712 D504
E	D701 D505	D702 D505	D703 D505	D704 D505	D705 D505	D706 D505	D707 D505	D708 D505	D709 D505	D710 D505	D711 D505	D712 D505
F	D701 D506	D702 D506	D703 D506	D704 D506	D705 D506	D706 D506	D707 D506	D708 D506	D709 D506	D710 D506	D711 D506	D712 D506
G	D701 D507	D702 D507	D703 D507	D704 D507	D705 D507	D706 D507	D707 D507	D708 D507	D709 D507	D710 D507	D711 D507	D712 D507
H	D701 D508	D702 D508	D703 D508	D704 D508	D705 D508	D706 D508	D707 D508	D708 D508	D709 D508	D710 D508	D711 D508	D712 D508

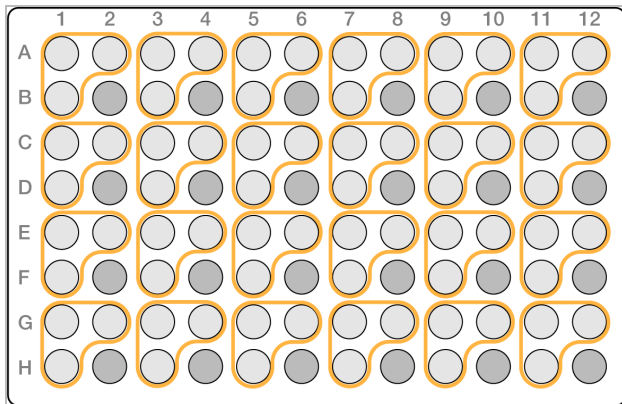
Two-Plex, Dual-Index

Pool diagonally across adjacent columns and rows. For example: A1 and B2 or A2 and B1.



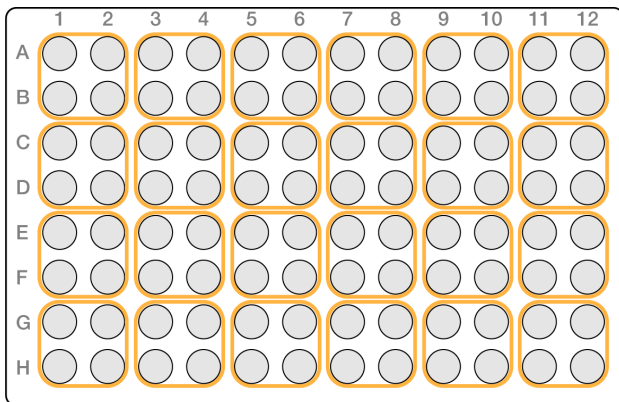
Three-Plex, Dual-Index

Start at an odd-numbered column and rows A, C, E, and G. Use all wells in a 2 x 2 square except the lower-right well, which is depicted in dark gray.



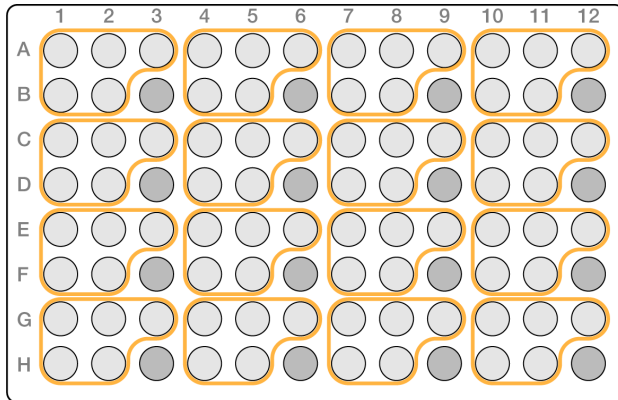
Four-Plex, Dual-Index

Start at odd-numbered columns and rows A, C, E, and G. Use all four wells in a 2 x 2 square.



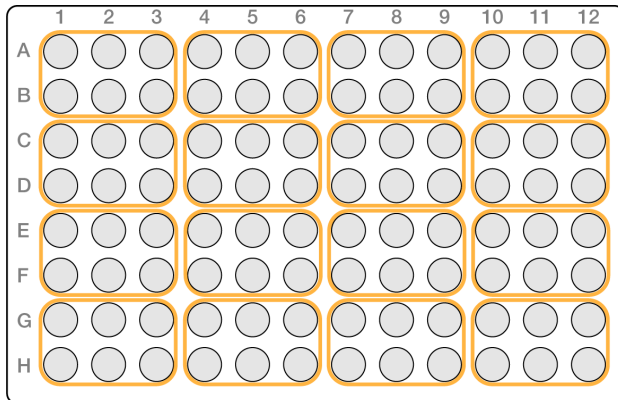
Five-Plex, Dual-Index

Start at columns 1, 4, 7, and 10 and rows A, C, E, and G. Use all wells in a 2 × 3 rectangle except the lower-right well, which is depicted in dark gray.



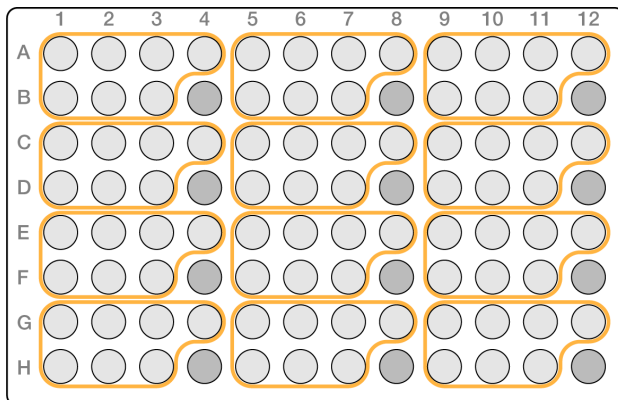
Six-Plex, Dual-Index

Start at columns 1, 4, 7, and 10 and rows A, C, E, and G. Use all six wells in a 2 × 3 rectangle.



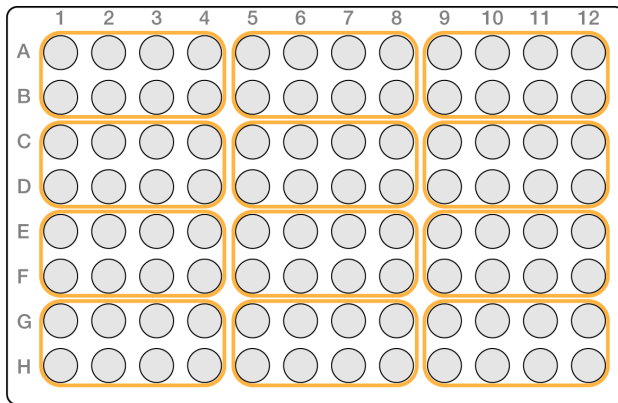
Seven-Plex, Dual-Index

Start at columns 1, 5, and 9 and rows A, C, E, and G. Use all wells in a 2 × 4 rectangle except the lower-right well, which is depicted in dark gray.



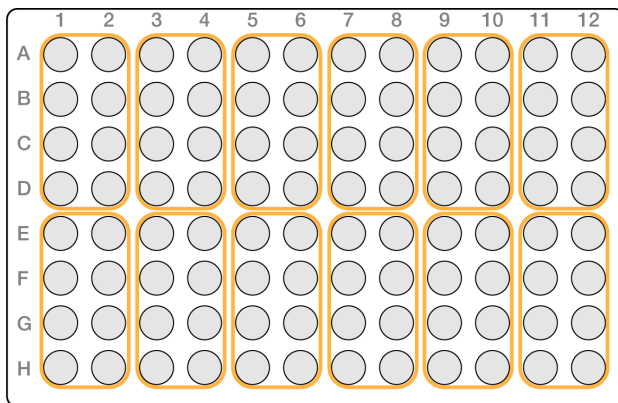
Eight-Plex, Dual-Index (Option 1)

Start at columns 1, 5, or 9 and rows A, C, E, and G. Use all eight wells in a 2 × 4 rectangle.



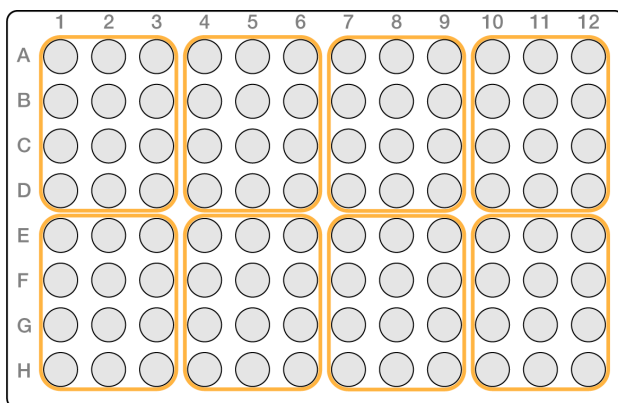
Eight-Plex, Dual-Index (Option 2)

Start at odd-numbered columns and rows A and E. Use all eight wells in a 4 × 2 rectangle.



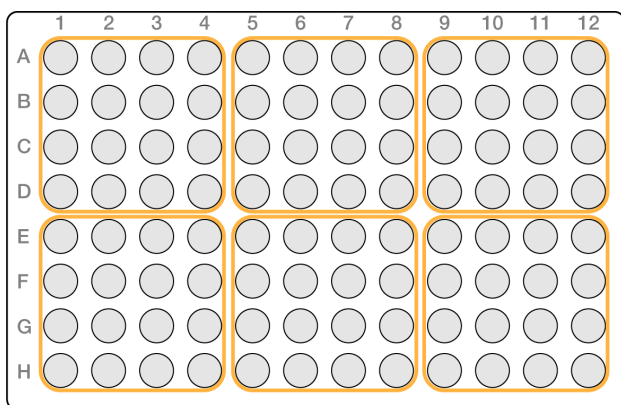
Twelve-Plex, Dual-Index

Start at columns 1, 4, 7, and 10 and rows A and E. Use all 12 wells in a 4 × 3 rectangle.



Sixteen-Plex, Dual-Index

Start at columns 1, 5, and 9 and rows A and E. Use all 16 wells in a 4 × 4 square.



TruSeq CD Indexes (Single Indexing With Dual Index Adapters)

The following table depicts the Index 1 portion of the plate layout for TruSeq DNA CD Indexes and TruSeq RNA CD Indexes (formerly TruSeq HT Indexes). Subsequent figures illustrate pooling strategies for libraries ligated with the Index 1 (i7) and Index 2 (i5) adapters, but only Index 1 is sequenced:

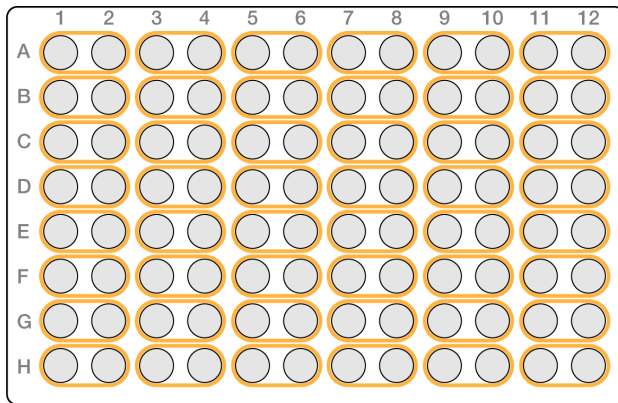
- ▶ Circles indicate index adapter combinations that can be combined in a pool.
- ▶ Dark gray indicates wells that are not used for pool plexity ≥ 2 , but can be used for one-plex pools (single libraries).

These strategies are designed for 2–12-plex pools. For 7–11-plex pools, combine any of the 2–6-plex pools.

1	2	3	4	5	6	7	8	9	10	11	12
D701	D702	D703	D704	D705	D706	D707	D708	D709	D710	D711	D712
D701	D702	D703	D704	D705	D706	D707	D708	D709	D710	D711	D712
D701	D702	D703	D704	D705	D706	D707	D708	D709	D710	D711	D712
D701	D702	D703	D704	D705	D706	D707	D708	D709	D710	D711	D712
D701	D702	D703	D704	D705	D706	D707	D708	D709	D710	D711	D712
D701	D702	D703	D704	D705	D706	D707	D708	D709	D710	D711	D712
D701	D702	D703	D704	D705	D706	D707	D708	D709	D710	D711	D712
D701	D702	D703	D704	D705	D706	D707	D708	D709	D710	D711	D712

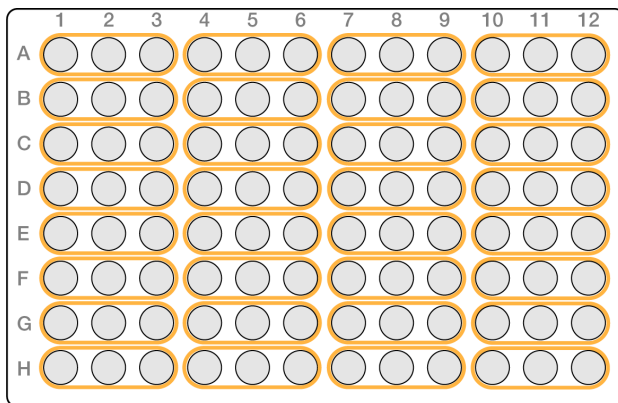
Two-Plex, Single-Index

Starting at an odd-numbered column, use two consecutive wells across a row.



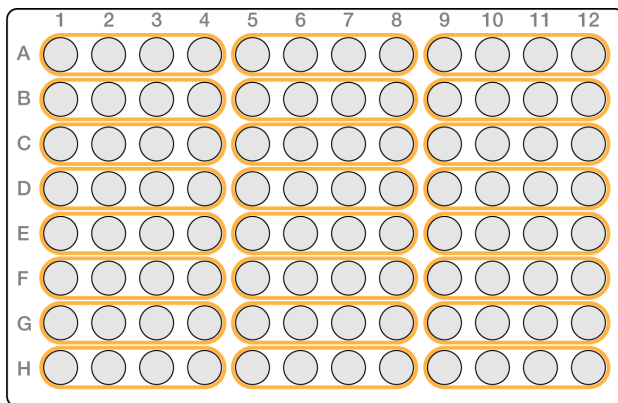
Three-Plex, Single-Index

Starting at column 1, 4, 7, or 10, use three consecutive wells across a row.



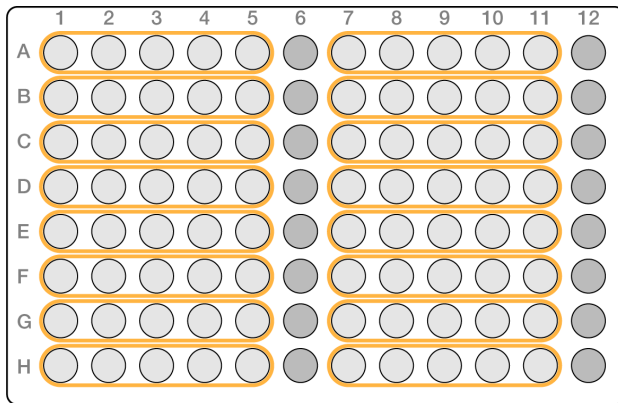
Four-Plex, Single-Index

Starting a column 1, 5, or 9, use four consecutive wells across a row.



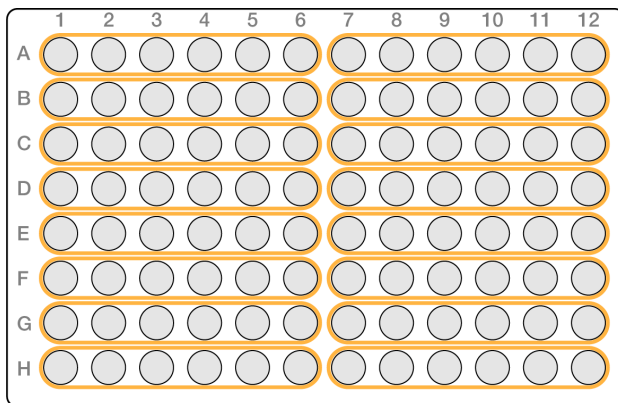
Five-Plex, Single-Index

Starting at column 1 or 7, use five consecutive wells across a row.



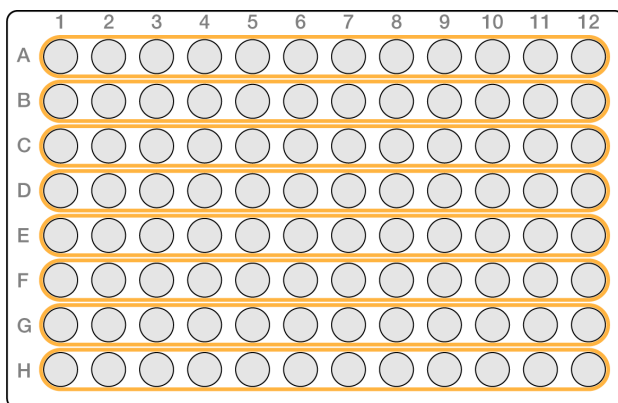
Six-Plex, Single-Index

Starting at column 1 or 7, use six consecutive wells across a row.



Twelve-Plex, Single-Index

Use an entire row.



TruSeq Single Indexes

TruSeq Single Indexes are available in Set A and Set B. Each set contains 12 index adapters packaged in tubes.

The following tables show strategies for 2–12-plex pooling when using either set. For 5–11-plex pools, start with a 4-plex option and add any other index adapters from the same set.

Table 1 TruSeq DNA Single Indexes

Plexity	Option	Set A Index Adapters Only	Set B Index Adapters Only
2	1	AD006 and AD012	Not recommended
	2	AD005 and AD019	Not recommended
3	1	AD002, AD007, and AD019	AD001, AD010, and AD020
	2	AD005, AD006, and AD015	AD003, AD009, and AD025
	3	Two-plex options plus another index adapter from Set A	AD008, AD011, and AD022
4	1	AD005, AD006, AD012, and AD019	AD001, AD008, AD010, and AD011
	2	AD002, AD004, AD007, and AD016	AD003, AD009, AD022, and AD027
	3	Three-plex options plus another adapter from Set A	Three-plex options plus another index adapter from Set B

Table 2 TruSeq RNA Single Indexes

Plexity	Option	Set A Index Adapters Only	Set B Index Adapters Only
2	1	AR006 and AR012	Not recommended
	2	AR005 and AR019	Not recommended
3	1	AR002, AR007, and AR019	AR001, AR010, and AR020
	2	AR005, AR006, and AR015	AR003, AR009, and AR025
	3	Two-plex options plus another index adapter from Set A	AR008, AR011, and AR022
4	1	AR005, AR006, AR012, and AR019	AR001, AR008, AR010, and AR011
	2	AR002, AR004, AR007, and AR016	AR003, AR009, AR022, and AR027
	3	Three-plex options plus another index adapter from Set A	Three-plex options plus another index adapter from Set A

Revision History

Document	Date	Description of Change
Document #1000000041074 v05	December 2018	Corrected the Index 1 (i7) index adapter in well C2 of the AmpliSeq CD Indexes for Illumina plate to Q7008.
Document #1000000041074 v04	November 2018	<p>Combined, renamed, and reorganized all sections to improve continuity, facilitate navigation, and eliminate redundancy.</p> <p>Consolidated graphics depicting pooling strategies for IDT for Illumina Nextera DNA UD Indexes and Nextera DNA CD Indexes (96 Indexes, 96 Samples).</p> <p>Added pooling guidelines for Nextera XT Index Kit v2 and Nextera Index Kit.</p> <p>Added information on Illumina Experiment Manager, Local Run Manager, and BaseSpace Sequence Hub.</p> <p>Added good and bad examples of color balance on a four-channel system.</p> <p>Identified all Index 1 (i7) and all Index 2 (i5) adapters for Nextera DNA CD Indexes (24 Indexes, 24 Samples).</p> <p>Updated plate layouts to remove duplicate index adapter names and show the Index 1 (i7) adapters first.</p> <p>Updated descriptions of one-, two-, and four-dye chemistry.</p> <p>Described dual indexing options: combinatorial dual (CD) and unique dual (UD) indexes.</p> <p>Described pooling strategies for TruSeq CD Indexes and updated style of the graphics (the strategies have not changed).</p> <p>Referenced <i>Indexed Sequencing Overview</i> (document # 15057455) for indexing workflows on Illumina sequencing systems.</p> <p>Clarified the following points:</p> <ul style="list-style-type: none"> • TruSeq CD Indexes replaced TruSeq HT Indexes. • For two-plex, dual-indexing with TruSeq CD Indexes, pool diagonally. • Single-indexing with TruSeq CD Indexes requires dual index adapters. <p>Removed the following sequences, which are available in <i>Illumina Adapter Sequences</i> (document # 100000002694):</p> <ul style="list-style-type: none"> • Index adapter sequences for Nextera DNA CD Indexes (for Nextera DNA Flex). • Index adapter and adapter trimming sequences for AmpliSeq for Illumina. <p>Corrected column-based, three-plex pooling strategy for AmpliSeq for Illumina.</p> <p>Corrected names of the following items:</p> <ul style="list-style-type: none"> • IDT for Illumina kits. • Index adapters UDP0001–UDP0009 to include four digits.
Document #1000000041074 v03	October 2018	<p>Added information on IDT for Illumina Nextera DNA UD Indexes.</p> <p>Consolidated low-plexity guidelines.</p> <p>Corrected tables for Nextera DNA Flex 96 CD Indexes.</p> <p>Removed single indexing information for IDT for Illumina TruSeq DNA UD Indexes, which is compatible with dual indexing only.</p> <p>Removed incorrect guidelines for:</p> <ul style="list-style-type: none"> • Low plexity sequencing on the NovaSeq 6000 System. • Low throughput for Nextera DNA Flex combinatorial dual indexes.
Document #1000000041074 v02	May 2018	Added information on one-channel sequencing for the iSeq 100 System.
Document #1000000041074 v01	January 2018	Added pooling guidelines for AmpliSeq for Illumina and Nextera DNA Flex libraries.
Document #1000000041074 v00	October 2017	Initial release.

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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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