Low-Plex Pooling Guidelines for Enrichment Protocols

Introduction

This document provides important guidelines for low-plex pooling with enrichment protocols (TruSeq® Rapid Enrichment). If using the dual-indexed sequencing workflow for enrichment protocols, always use at least 2 unique and compatible barcodes for each index (Index 1 and Index 2).

Each enrichment reaction supports pooling up to 12 samples together for enrichment. There are 1, 2, 4, and 8 enrichment reactions worth of reagents in the 8, 24, 48, and 96 sample kits, respectively (Table 1). There are 24 enrichment reactions worth of reagents in the 288 sample custom kit.

There is sufficient volume in each Index 1 (i7) tube for 16 reactions and in each Index 2 (i5) tube for 24 reactions. Illumina recommends planning experiments accordingly to avoid running out of index primers.

Table 1: Enrichment Reactions Per Kit

Samples Per Kit	Enrichment Reactions
8	8
24	8
48	8
72	8
96	8
288	24

HiSeq® and MiSeq® System Guidelines

The HiSeq and MiSeq Systems use a green laser to sequence G/T and a red laser to sequence A/C. At each cycle at least 1 of 2 nucleotides for each color channel must be read to ensure proper registration. It is important to maintain color balance for each base of the Index Read being sequenced, otherwise Index Read sequencing could fail due to registration failure. Follow the instructions described here to determine which libraries are pooled pre-enrichment for HiSeq and MiSeq sequencing.

The enrichment kits support pre-enrichment pooling of up to 12 different indexed samples. Illumina recommends the following:

- For pooling 12 samples pre-enrichment, pool samples with Index 1 (i7) 701–712 (any Index 2 (i5)), followed by a single-index sequencing run.
- For pooling < 12 samples, set up a single index workflow sequencing run using different Index 1 (i7) indexes (any Index 2 (i5)). Illumina provides compatible i5 indexes in this kit, but when pooling fewer than 6 i7 indexes, use the combinations in Table 2 for proper color balancing.
- For pooling details when using both Index 1 (i7) and Index 2 (i5) followed by a dual-indexed sequencing run, see Table 3.

Tables 4 and 5 provide examples of the acceptable combinations for 4 pooled samples. For single-index runs, Table 4 provides combination examples. The E505 and E506 indexes are compatible with each other. For dual-index runs with the Nextera® Rapid Capture Custom Enrichment Kit (288 Samples), Table 6 provides additional combination examples.

Table 2: Libraries Pooled: 6 or Fewer; Sequencing Workflow: Single Index

Plexity	Index 1 (i7) Selection	Index 2 (i5) Selection
1 plex (no pooling)	Any Index 1 adapter	Any Index 2 adapter
2 plex	• Option 1: N702 and N701 • Option 2: N702 and N704	
3 plex	• Option 1: N701, N702, and N704 • Option 2: N703, N705, and N706	
4 or 5 plex	• Option 1: N701, N702, N704, and any other Index 1 adapter • Option 2: N703, N705, N706, and any other Index 1 adapter	
6 plex	N701, N702, N703, N704, N705, and N706	

Table 3: Libraries Pooled: 7 or More; Sequencing Workflow: Single or Dual Index

Plexity	Index 1 (i7) Selection	Index 2 (i5) Selection
7–12 plex, dual index	• Option 1: N701, N702, N704, and any other Index 1 adapter (as needed) • Option 2: N703, N705, N706, and any other Index 1 adapter (as needed)	With 24-, 48-, and 96-sample kits: • E505 and E506 With the custom 288-sample kit: • Option 2: E503 and E504 • Option 3: E505 and E506
7–12 plex, single index	N701–N706 and any other Index 1 adapter (as needed)	Any Index 2 (i5) adapter
Greater than 12 plex	N701–N706, and any other Index 1 adapter (as needed)	• Option 2: E503, E504, and any other Index 2 adapter (as needed) • Option 3: E505, E506, and any other Index 2 adapter (as needed)

Table 4: Example Combinations for Single-Index Runs

Sample	Good Index 1								Good Index 2							
1	701	T	A	A	G	G	C	G	A	702	C	G	T	A	C	T	A	G
2	702	C	G	T	A	C	T	A	G	703	A	G	G	C	A	G	A	A
3	704	T	C	C	T	G	A	G	C	711	A	A	G	A	G	G	C	A
		✓	✓	✓	✓	✓	✓	✓	✓		X	✓	X	X	✓	X	X	✓

✓= signal in both colors
X = signal missing in 1 color channel

Table 5: Example Combinations for Dual-Index Runs With Custom 288-Sample Kit

Good Examples				Bad Examples			
Index 1		Index 2		Index 1		Index 2	
705	G G A C T C C T	503	T A T C C T C T	705	G G A C T C C T	502	C T C T C T A T
706	T A G G C A T G	503	T A T C C T C T	706	T A G G C A T G	502	C T C T C T A T
701	T A A G G C G A	504	A G A G T A G A	701	T A A G G C G A	503	T A T C C T C T
702	C G T A C T A G	504	A G A G T A G A	702	C G T A C T A G	503	T A T C C T C T
	✓ ✓ ✓ ✓ ✓ ✓ ✓ ✓		✓ ✓ ✓ ✓ ✓ ✓ ✓ ✓		✓ ✓ ✓ ✓ ✓ ✓ ✓ ✓		✓ ✓ ✓ ✓ X X X X

✓= signal in both colors
X = signal missing in 1 color channel

NextSeq® System Guidelines

NextSeq Systems use 2-channel sequencing, which requires only 2 images to encode the data for 4 DNA bases: 1 red channel and 1 green channel. The NextSeq also uses a new implementation of Real-Time Analysis (RTA) called RTA2.0, which includes important architecture differences from RTA on other Illumina sequencing systems. For any index sequences, RTA2.0 requires that there is at least one base other than G in the first 2 cycles. This requirement for index diversity allows the use of any Illumina index selection for single-plex indexing except index 1 (i7) 705, which uses the sequence GGACTCCT. Use the combinations in Table 6 for proper color balancing on NextSeq Systems.

Table 6: Combinations for NextSeq Systems

Plexity	Index 1 (i7) Selection	Index 2 (i5) Selection
3 plex	N702, N704, N705	Fixed Index 2 adapter
6 plex	N701, N702, N703, N704, N705, N707	
9 plex	N701, N702, N703, N704, N705, N707, N710, N711, N712	
12 plex	N701, N702, N703, N704, N705, N706, N707, N708, N709, N710, N711, N712	

When pooling:
a. 3–6 plex—Use the indexes required for a 3-plex pool and select from the indexes required for a 6-plex pool.
b. 6–9 plex—Use the indexes required for a 6-plex pool and select from the indexes required for a 9-plex pool.
c. 9–12 plex—Use the indexes required for a 9-plex pool and select from the indexes required for a 12-plex pool.

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AGAATGATAACAGTAACACACTTCTGTTAACCTTAAGATTACTTGATCCACTGATTCAACGTACCGTAACGAACGTATCAATTGAGACTAAATATTAACGTACCATTAAGAGCTACCGTCTTCTGTTAACCTTAAGATTACTTGATCCACTGATTCAAC
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