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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 dualindexed 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	
2 plex	 Option 1: N702 and N701 Option 2: N702 and N704	
3 plex	Option 1: N701, N702, and N704Option 2: N703, N705, and N706	Any Index 2
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 	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

		·								0								
Sample	Good	d In	de>	(1						Good	d In	de>	(2					
1	701	Т	А	А	G	G	С	G	А	702	С	G	Т	А	С	Т	А	G
2	702	С	G	Т	А	С	Т	А	G	703	А	G	G	С	А	G	А	А
3	704	Т	С	С	Т	G	А	G	С	711	А	А	G	А	G	G	С	А
		\checkmark		Х	\checkmark	Х	Х	\checkmark	Х	Х	\checkmark							

✓= signal in both colors

X = signal missing in 1 color channel

	Good E	kamples	Bad Examples						
Inde	<1	Index 2	Index 1	Index 2					
705	GGACTCCT	503 T A T C C T C T	705 GGACTCCT	502 C T C T C T A T					
706	тассатс	503 ТАТССТСТ	706 TAGGCATG	502 CTCTCTAT					
701	TAAGGCGA	504 AGAGTAGA	701 TAAGGCGA	503 TATCCTCT					
702	CGTACTAG	504 AGAGTAGA	702 CGTACTAG	503 T A T C C T C T					
	$\checkmark \checkmark \checkmark \checkmark \checkmark \checkmark \checkmark \checkmark \checkmark$	$\checkmark \checkmark \checkmark \checkmark \checkmark \checkmark \checkmark \checkmark$	$\checkmark \checkmark \checkmark \checkmark \checkmark \checkmark \checkmark \checkmark$						

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

✓= 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	
6 plex	N701, N702, N703, N704, N705, N707	
9 plex	N701, N702, N703, N704, N705, N707, N710, N711, N712	Fixed Index 2 adapter
12 plex	N701, N702, N703, N704, N705, N706, N707, N708, N709, N710, N711, N712	

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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