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Nextera Low Plex Pooling Guidelines

Introduction

Illumina uses a green laser to sequence G/T and a red laser to sequence A/C. At each cycle at least one of two nucleotides for each color channel need to 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. This Tech Note details information about the Dual Indexing strategy and important considerations for Low Plexity Pooling.

Dual Indexing Principle

The dual indexing strategy uses two 8 base indices, Index 1 (i7) adjacent to the P7 sequence, and Index 2 (i5) adjacent to the P5 sequence. Dual indexing is enabled by adding a unique Index 1 (i7) and Index 2 (i5) to each sample from 12 different Index 1 (i7) adapters (N701–N712) and 8 different Index 2 (i5) adapters (N501–N508) for the 96 sample Nextera Index Kit (FC-121–1012), and 6 different Index 1 (i7) adapters (N701–N706) and 4 different Index 2 (i5) adapters (N501–N504) for the 24 sample Nextera Index Kit (FC-121–1011). In the Index adapter name, the N refers to Nextera sample preparation, 7 or 5 refers to Index 1 (i7) or Index 2 (i5), respectively, and 01–12 refers to the Index number. A list of index sequences is provided for generating sample sheets to demultiplex the samples (Table 1).

Table 1: Sequences for Index 1 and Index 2 Adapters

Index 1 (i7)	Sequence	Index 2 (i5)	Sequence
N701	TAAGGCGA	N501	TAGATCGC
N702	CGTACTAG	N502	CTCTCTAT
N703	AGGCAGAA	N503	TATCCTCT
N704	TCCTGAGC	N504	AGAGTAGA
N705	GGACTCCT	N505	GTAAGGAG
N706	TAGGCATG	N506	ACTGCATA
N707	CTCTCTAC	N507	AAGGAGTA
N708	CAGAGAGG	N508	CTAAGCCT
N709	GCTACGCT		
N710	CGAGGCTG		
N711	AAGAGGCA		
N712	GTAGAGGA		

Low Plexity Pooling Guidelines

If you choose the dual index sequencing workflow always use at least two unique and compatible barcodes for each index (index 1 and index 2). Tables 2 and 3 illustrate possible pooling strategies.

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

Plex	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	

AGAATGATAACAGTAACACACTTCTGTTAACCTTAAGATTACTTGATCCACTGATTCAACGTACCGTAACGAACGTATCAATTGAGACTAAATATTAACGTACCATTAAGAGCTACCGTCTTCTGTTAACCTTAAGATTACTTGATCCACTGATTCAAC
TCAACGTACCGTAACGAACGTATCATTAAGATTACTTGATCCACTGATTCAACGTACCGTAACGAACGTATCAATTGAGACTAAATATTAACGTACCATTAAGAGCTACCGTGCAACGACGAAAAGAATGATAACAGTAACACACTTCTGTTAACCTT
CGACGAAAAGAATGATAACAGTAACACACTTCTGTTAACCTTAAGATTACTTGATCCACTGATTCAACGTACCGTAAAGATTACTTGATCCACTGATTCAACGTACCGTAACGAACGTATCAATTGAGACTAAATATTAACGTACCATTAAGAGCTACC
AACGTACCATTAAGAGCTACCGTGCAACAGTAACACACTTCTGTTAACCTTAAGATTACTTGATCCACTGATTCAACGTACCGTAACGAACGTATCAATTGAGACTAAATATTAACGTACCATTAAGAGCTACCGTGCAACGACGAAAAGAATGATAA
AGAATGATAACAGTAACACACTTCTGTTAACCTTAAGATTACTTGATCCACTGATTCAACGTACCGTAACGAACGTATCAATTGAGACTAAATATTAACGTACCATTAAGAGCTACCGTCTTCTGTTAACCTTAAGATTACTTGATCCACTGATTCAAC
GATTACTTGATCCACTGATTCAACGTTAAGATTACTTGATCCACTGATTCAACGTACCGTAACGAACGTATCAATTGAGCTTCTGTTAACCTTAAGATTACTTGATCCACTGATTCAACGTACCGTAACGAACGTATCAATTGAGACTAGCAACGAGC
CGTATCAATTGAGACTAAATATTAACGTACCATTAAGAGTCTGTTAACCTTAAGATTACTTGATCCACTGATTCAACGTACCGTAACGAACGTATCAATTGAGACTAAATATTAACGTACCATTAAGAGCTACCGTGCAACGAAAAGAATGATAACAGTA

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

Plex	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)	[option 1] N501 and N502 [option 2] N503 and N504 [option 3] N505 and N506
7–12 plex, Single Index (96 sampleNextera Index adapter kit)	N701–N706 and any other Index 1 adapter (as needed)	Any Index 2 (i5) adapter
Greater than 12-plex	N701, N702, N703, N704, N705, N706, and any other Index 1 adapter	[option 1] N501, N502, and any other Index 2 adapter (as needed) [option 2] N503, N504, and any other Index 2 adapter (as needed) [option 3] N505, N506, and any other Index 2 adapter (as needed)

Tables 2 and 3 represent only some of the acceptable combinations. Alternatively, check the real sequences of each index in Table 1 above to make sure each base position will have signal in both color channels for the index read (Table 4).

Table 4: Examples of Good and Bad Index Combinations

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 color

X= signal missing in one color channel

Illumina Experiment Manager

The Illumina Experiment Manager (IEM) can be used to create your sample sheet using a wizard-based application. The IEM guides you through the steps to create your sample sheet based on the analysis workflow for your run. IEM will notify you if improper index combinations are used when creating a sample sheet for use with CASAVA, so it is highly recommended to create your sample sheet prior to performing sample prep/pooling. The IEM tool can be run on any Windows platform. You can download the Experiment Manager from the Illumina website at http://www.illumina.com. Go to the Nextera DNA Sample Preparation support page and click Downloads. A MyIllumina account is required.

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AGAATGATAACAGTAACACACTTCTGTTAACCTTAAGATTACTTGATCCACTGATTCAACGTACCGTAACGAACGTATCAATTGAGACTAAATATTAACGTACCATTAAGAGCTACCGTCTTCTGTTAACCTTAAGATTACTTGATCCACTGATTCAAC
TCAACGTACCGTAACGAACGTATCATTAAGATTACTTGATCCACTGATTCAACGTACCGTAACGAACGTATCAATTGAGACTAAATATTAACGTACCATTAAGAGCTACCGTGCAACGACGAAAAGAATGATAACAGTAACACACTTCTGTTAACCTT
CGACGAAAAGAATGATAACAGTAACACACTTCTGTTAACCTTAAGATTACTTGATCCACTGATTCAACGTACCGTAAAGATTACTTGATCCACTGATTCAACGTACCGTAACGAACGTATCAATTGAGACTAAATATTAACGTACCATTAAGAGCTACC
AACGTACCATTAAGAGCTACCGTGCAACAGTAACACACTTCTGTTAACCTTAAGATTACTTGATCCACTGATTCAACGTACCGTAACGAACGTATCAATTGAGACTAAATATTAACGTACCATTAAGAGCTACCGTGCAACGACGAAAAGAATGATAA
AGAATGATAACAGTAACACACTTCTGTTAACCTTAAGATTACTTGATCCACTGATTCAACGTACCGTAACGAACGTATCAATTGAGACTAAATATTAACGTACCATTAAGAGCTACCGTCTTCTGTTAACCTTAAGATTACTTGATCCACTGATTCAAC
GATTACTTGATCCACTGATTCAACGTTAAGATTACTTGATCCACTGATTCAACGTACCGTAACGAACGTATCAATTGAGCTTCTGTTAACCTTAAGATTACTTGATCCACTGATTCAACGTACCGTAACGAACGTATCAATTGAGACTAGCAACGACG
CGTATCAATTGAGACTAAATATTAACGTACCATTAAGAGTCTGTTAACCTTAAGATTACTTGATCCACTGATTCAACGTACCGTAACGAACGTATCAATTGAGACTAAATATTAACGTACCATTAAGAGCTACCGTGCAACGAAAAGAATGATAACAGTA