NEBNext® Multiplex Oligos for Illumina® (96 Unique Dual Index Primer Pairs Set 5)

NEB #E6448S/L

96/384 reactions

Version 2.0_7/22

Table of Contents

Workflow 2

Library Preparation Kits for use with NEBNext Multiplex Oligos for Illumina 3

NEBNext Adaptor for Illumina Overview 3

Section 1
Setting up the PCR Reactions 4

Section 2
Index Pooling Guidelines 5

Kit Components 9

Revision History 10

The NEBNext Multiplex Oligos for Illumina (96 Unique Dual Index Primer Pairs Set 5) Includes

The volumes provided are sufficient for preparation of up to 96 reactions (NEB #E6448S) and 384 reactions (NEB #E6448L). All reagents should be stored at –20°C.

NEBNext Adaptor for Illumina

USER® Enzyme

NEBNext 96 Unique Dual Index Primer Pairs Plate (Set 5)
Each well contains a unique pair of Index Primers (S size contains 1 plate, L size contains 4 plates)

Overview

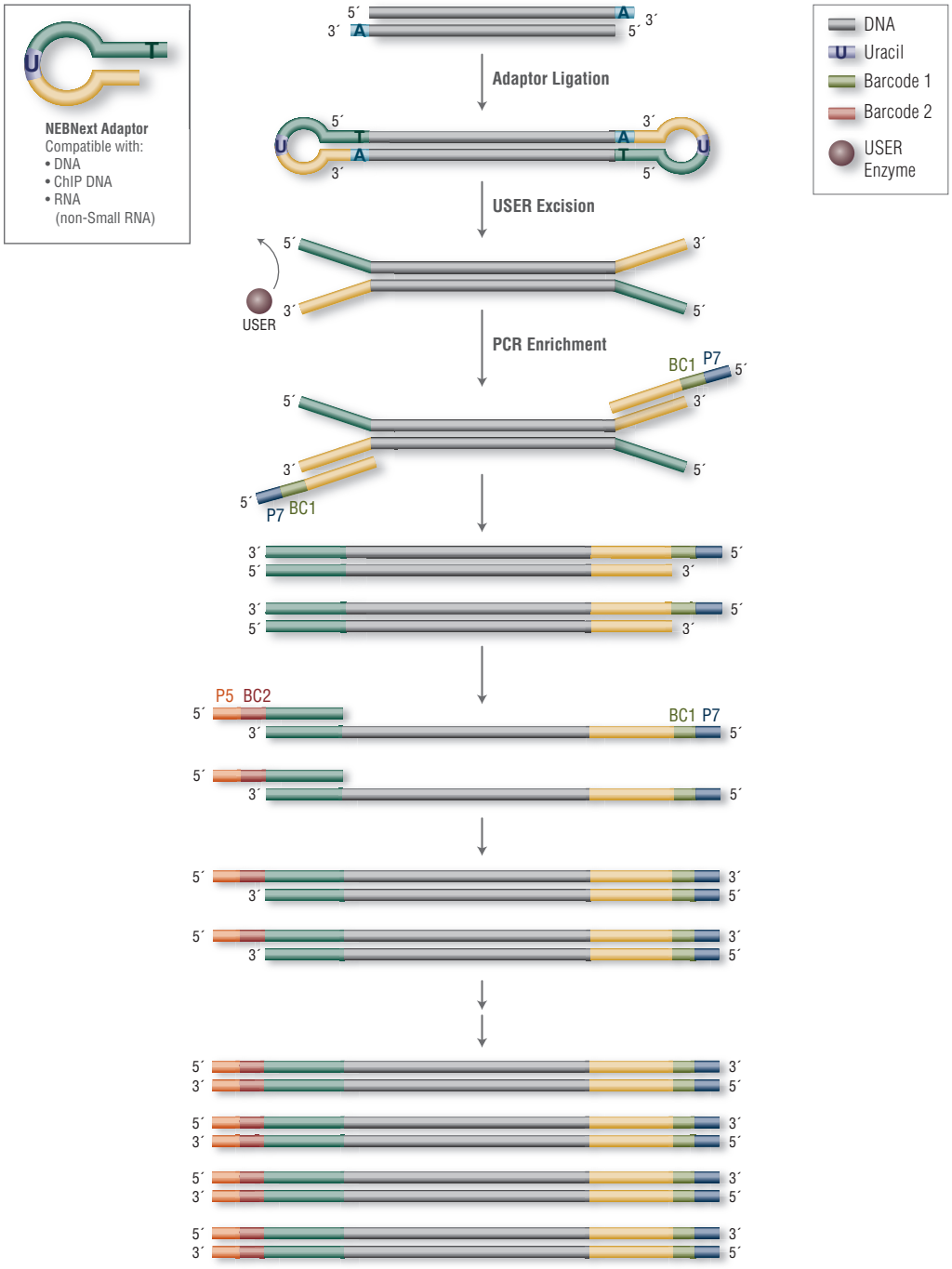
The NEBNext Multiplex Oligos for Illumina (96 Unique Dual Index Primer Pairs Set 5) contains adaptors and primers that are ideally suited for multiplex sample preparation for next-generation sequencing on the Illumina platform (Illumina, Inc.). Each kit component must pass rigorous quality control standards, and for each new lot the entire set of reagents is functionally validated together by construction and sequencing of indexed libraries on an Illumina sequencing platform.

For larger volume requirements, customized and bulk packaging is available by purchasing through the OEM/Bulks department at NEB. Please contact OEM@neb.com for further information.

Workflow

Designed for use in library prep for DNA, ChIP DNA and RNA (but not Small RNA), the NEBNext Adaptors enable high-efficiency adaptor ligation and high library yields, with minimized adaptor-dimer formation. Incorporating a novel hairpin loop structure, the NEBNext Adaptor ligates with increased efficiency to end-repaired, dA-tailed DNA. The loop contains a U, which is removed by treatment with USER Enzyme (a combination of UDG and Endo VIII), to open up the loop and make it available as a substrate for PCR. During PCR, barcodes can be incorporated by use of the NEBNext index primers, thereby enabling multiplexing. The 96 8-base index primer pairs included in this kit are pre-mixed and are packaged in a single-use 96-well plate with a pierceable foil seal. NEBNext Oligos can be used with NEBNext products, and with other standard Illumina-compatible library preparation protocols.

Figure 1. Workflow demonstrating the use of NEBNext Multiplex Oligos for Illumina (96 Unique Dual Index Primer Pairs).



Please Refer to the Kit Specific Protocol for using the NEBNext Multiplex Oligos for Illumina

For compatibility of NEBNext Multiplex Oligos please refer to the NEBNext Multiplex Oligos Selection Chart at **neb.com/oligos**

NEBNext Adaptor for Illumina Overview

NEBNext Adaptor for Illumina sequence:

5´-/5Phos/GAT CGG AAG AGC ACA CGT CTG AAC TCC AGT CdUA CAC TCT TTC CCT ACA CGA CGC TCT TCC GAT C-s-T-3´

The following sequences are used for adaptor trimming of NEBNext adaptors for Illumina.

Read 1 AGATCGGAAGAGCACACGTCTGAACTCCAGTCA

Read 2 AGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT

Section 1
Setting up the PCR Reactions

Symbols

This caution sign signifies a step in the protocol that has multiple paths leading to the same end point but is dependent on a user variable, like the amount of input DNA.

1.1. PCR Amplification

For < 96 samples, follow the protocol in Section 1.1A. For 96 samples, follow the protocol in Section 1.1B.

1.1A. Setting up the PCR reactions (< 96 samples)

1.1A.1. Determine the number of libraries that will be amplified and pooled for subsequent sequencing.

1.1A.2. Ensure that you choose a valid combination of barcode primers based on color balance guidelines in Section 2.

1.1A.3. Thaw the 96 Unique Dual Index Primers Plate for 10-15 minutes at room temperature.

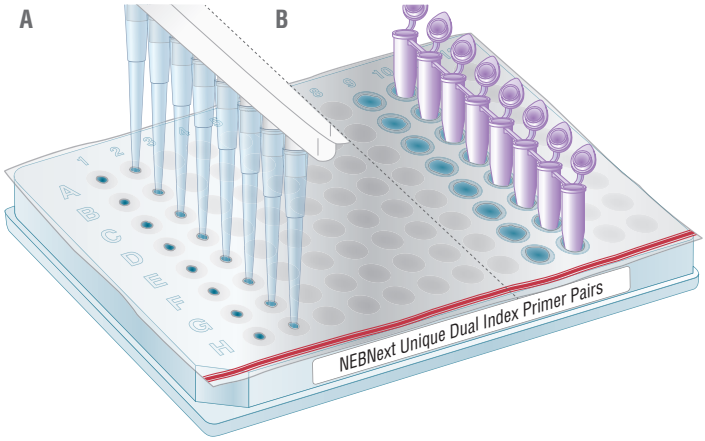
1.1A.4. Remove the hard plastic plate cover. Mix briefly by vortexing and then centrifuge the plate (280 × *g* for ~1 min) to collect all of the primer at the bottom of each well.

1.1A.5. Orient the 96 Unique Dual Index Primers Plate Set 5 as indicated in Figure 1.1 (red stripe towards the user). With a pipette tip, pierce the desired well(s) (Figure 1.1A) and transfer the volume of primer mix required for the PCR reaction to the PCR plate/tubes (see specific library construction manual for protocol). It is important to change pipette tips before piercing a new well to avoid cross contamination of indexed primers. Alternatively, the wells can be pierced using the bottom of clean PCR strip tubes (see Figure 1.1B) prior to pipetting the primer mix. Use a new, clean strip tube for each new well to be pierced.

Note: Each well contains a unique pair of index primers. There is enough primer in each well for one PCR reaction. Do not reuse primer if the seal has been previously pierced to avoid contamination with other indexed primers.

1.1A.6. Proceed with the PCR reaction according to the specific library construction manual.

Figure 1.1. NEBNext Unique Dual Index Pairs Plate Set 5



1.1B Setting up the PCR reactions (96 samples)

1.1B.1. Thaw the 96 Unique Dual Index Primer Pairs plate for 10-15 minutes at room temperature.

1.1B.2. Remove the hard plastic plate cover. Mix briefly by vortexing and then centrifuge the plate (280 × *g* for ~1 min) to collect all of the primer at the bottom of each well.

1.1B.3. Orient the 96 Unique Dual Index Primer Pairs plate as indicated in Figure 1.1 (red stripe towards the user). With a pipette tip, pierce the wells (Figure 1.1A) and transfer the volume of primer mix required for the PCR reaction to the PCR plate (see specific library construction manual for protocol). It is important to change pipette tips before piercing a new well to avoid cross contamination of indexed primers. Alternatively, the wells can be pierced using the bottom of clean PCR strip tubes (see Figure 1.1B) prior to pipetting the primer mix. Use a new, clean strip tube for each new well to be pierced.

Note: Each well contains a unique pair of index primers. There is enough primer in each well for one PCR reaction. Do not reuse primer if the seal has been previously pierced to avoid contamination with other indexed primers.

1.1B.4. Proceed with the PCR reaction according to the specific library construction manual.

Section 2
Index Pooling Guidelines: 96 Reaction Kit

For a link to download a sample sheet with the index sequences for use with the Illumina Experiment Manager (IEM) please visit the "Usage Guidelines" sub tab located under the "protocols, manuals and usage" tab on the E6448 product page.

For all HiSeq®/MiSeq® sequencers, Illumina uses a red laser/LED to sequence bases A and C and a green laser/LED to sequence bases G and T. For each cycle, both the red and the green channel need to be read to ensure proper image registration (i.e., A or C must be in each cycle, and G or T must be in each cycle). If this color balance is not maintained, sequencing the index read could fail. See Table 2.1 for examples of Good and Bad Index combinations.

For the NovaSeq®/NextSeq®/MiniSeq® which utilize 2 color chemistry, valid index combinations must include some indices that do not start with GG in the first two cycles. See Table 2.2 for examples of Good and Bad Index combinations.

The barcoded primers are organized on the plate such that including the primers in rows A and B from any column will produce a color balanced pool. For example, if preparing 2 libraries, choose primer wells A1 and B1. For larger pools, add any other primers from column 1.

***Forward Strand Workflow** for the following instruments: NovaSeq 6000 with v1.0 reagents kits, MiniSeq with rapid reagent kits, MiSeq®, HiSeq® 2000/2500 (pair-end flow cell), HiSeq 3000/4000 (single-read flow cell).

Reverse Strand Workflow for the following instruments: iSeq 100, MiniSeq with standard reagent kits, NextSeq Systems, NovaSeq 6000 with v1.5 reagent kits, HiSeq 2000/5000 (single-read flow cell), HiSeq 3000/4000 (paired-end flow cell).

Table 2.1. lists each index sequence color coded to correspond to the red/green channel. For combinations of valid indices, ensure that you will have signal in both the red and green channels in each cycle. See below for examples of Good and Bad index combinations based on HiSeq/MiSeq guidelines:

GOOD																									
WELL POSITION	EXPECTED i7 INDEX READ								EXPECTED i5 INDEX READ																
									FORWARD STRAND WORKFLOW*								REVERSE STRAND WORKFLOW*							
A1	A	G	T	T	T	C	G	A	A	T	C	G	T	A	G	G	C	C	T	A	C	G	A	T
B1	G	A	A	C	C	T	C	T	G	C	G	C	A	G	A	C	G	T	C	T	G	C	G	C
C1	G	C	C	C	A	G	T	G	A	A	T	T	G	C	G	G	C	C	G	C	A	A	T	T
D1	T	G	A	C	A	G	C	T	C	C	T	A	C	G	G	G	C	C	C	G	T	A	G	G
	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓

BAD																									
WELL POSITION	EXPECTED i7 INDEX READ								EXPECTED i5 INDEX READ																
									FORWARD STRAND WORKFLOW								REVERSE STRAND WORKFLOW							
E1	C	A	T	C	A	C	C	C	T	G	C	T	A	T	A	T	A	T	A	T	A	G	C	A
F1	C	T	G	G	A	G	T	A	A	A	C	C	G	A	A	C	G	T	T	C	G	G	T	T
G1	G	A	T	C	C	G	G	G	A	C	C	T	G	C	T	T	A	A	G	C	A	G	G	T
H1	A	A	C	A	C	C	T	G	C	C	C	A	T	G	C	G	C	G	C	A	T	G	G	G
	✓	✓	✓	✓	X	✓	✓	✓	✓	✓	X	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	X	✓	✓

The index primer sequences, for different Illumina sequencer input sheets are indicated in Table 2.3.

Table 2.2. NovaSeq, NextSeq and MiniSeq use 2 color channel sequencing to simplify nucleotide detection. Clusters only in red or green are interpreted as C or T, respectively. Clusters in both red and green are read as A, while unlabeled clusters are G bases. For multiplexing a small number of samples, make sure the final index pool contains some indices that do not start with GG in the first two cycles. Listed here are some examples of good (signal in at least one channel for the first 2 cycles) and bad (the index read begins with GG) index combinations.

GOOD																								
WELL POSITION	EXPECTED i7 INDEX READ								EXPECTED i5 INDEX READ															
									FORWARD STRAND WORKFLOW								REVERSE STRAND WORKFLOW							
A1	A	G	T	T	T	C	G	A	A	T	C	G	T	A	G	G	C	C	T	A	C	G	A	T
B1	G	A	A	C	C	T	C	T	G	C	G	C	A	G	A	C	G	T	C	T	G	C	G	C
C1	G	C	C	C	A	G	T	G	A	A	T	T	G	C	G	G	C	C	G	C	A	A	T	T
	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓

BAD																								
WELL POSITION	EXPECTED i7 INDEX READ								EXPECTED i5 INDEX READ															
									FORWARD STRAND WORKFLOW								REVERSE STRAND WORKFLOW							
E2	A	G	A	A	C	G	A	T	T	A	G	G	G	A	C	C	G	G	T	C	C	C	T	A
G6	A	T	C	C	C	G	T	A	A	G	A	A	G	A	C	C	G	G	T	C	T	T	C	T
E11	T	G	C	T	A	T	A	T	C	A	T	C	A	C	C	C	G	G	G	T	G	A	T	G
	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	X	X	✓	✓	✓	✓	✓	✓

Table 2.3. Lists each index sequence color coded to correspond to the red/green channel. For combinations of valid indices, ensure that you will have signal in both the red and green channels in each cycle.

	EXPECTED i7 INDEX READ		EXPECTED i5 INDEX READ		
WELL POSITION	i7 INDEX ID		i5 INDEX ID	FORWARD STRAND WORKFLOW	REVERSE STRAND WORKFLOW
A1	7-396	AGTTTCGA	5-444	ATCGTAGG	CCTACGAT
B1	7-397	GAACCTCT	5-445	GCGCAGAC	GTCTGCGC
C1	7-398	GCCCAGTG	5-446	AATTGCGG	CCGCAATT
D1	7-399	TGACAGCT	5-447	CCTACGGG	CCCGTAGG
E1	7-400	CATCACCC	5-448	TGCTATAT	ATATAGCA
F1	7-401	CTGGAGTA	5-449	AACCGAAC	GTTCGGTT
G1	7-402	GATCCGGG	5-450	ACCTGCTT	AAGCAGGT
H1	7-403	AACACCTG	5-451	CCCATGCG	CGCATGGG
A2	7-404	GTGACGTT	5-452	ATCTGGGA	TCCCAGAT
B2	7-405	ACAGGAAA	5-453	TAGACAAT	ATTGTCTA
C2	7-406	GTGCTCTG	5-454	TTCTTCCT	AGGAAGAA
D2	7-407	GTACCTGG	5-455	CACCTAAA	TTTAGGTG
E2	7-408	AGCGCAAA	5-456	GTACTCGC	GCGAGTAC
F2	7-409	AACGCCCT	5-457	TAACCAGT	ACTGGTTA
G2	7-410	GCGTACGG	5-458	CGGAAACT	AGTTTCCG
H2	7-411	GCCAGATT	5-459	GCTGAGAA	TTCTCAGC
A3	7-412	ATAGCAGA	5-460	ATGTCTTA	TAAGACAT
B3	7-413	GAGATGAT	5-461	TCACGCCT	AGGCGTGA
C3	7-414	GCCCGTCT	5-462	GCAACAGC	GCTGTTGC
D3	7-415	TTCGCCTG	5-463	ATCGTCTC	GAGACGAT
E3	7-416	CTAGCTCC	5-464	CCTTGTGA	TCACAAGG
F3	7-417	TATGCCGG	5-465	CTCACCAT	ATGGTGAG
G3	7-418	AATGTTGG	5-466	CAAATTCT	AGAATTTG
H3	7-419	ATCGGATA	5-467	CTCCTCAC	GTGAGGAG
A4	7-420	ATAGTGAC	5-468	ATGTGCAA	TTGCACAT
B4	7-421	GCTCCCTG	5-469	GCAAATGT	ACATTTGC
C4	7-422	AGTCAATT	5-470	ACGCATGG	CCATGCGT
D4	7-423	AATACGCT	5-471	ACACCACC	GGTGGTGT
E4	7-424	AACTTCGT	5-472	CACGCTGA	TCAGCGTG
F4	7-425	GAACTGCC	5-473	TCCCAGCC	GGCTGGGA
G4	7-426	AGCATTGT	5-474	CTATTCGT	ACGAATAG
H4	7-427	GACCAGGA	5-475	GAGCCATT	AATGGCTC
A5	7-428	ACAGCATT	5-476	ATTAATCG	CGATTAAT
B5	7-429	GTTCTGCA	5-477	GAAGGAGC	GCTCCTTC
C5	7-430	CTGTCCGG	5-478	GATTACAA	TTGTAATC
D5	7-431	GTCAAGCG	5-479	ATTTGAAG	CTTCAAAT
E5	7-432	TTCGCTCA	5-480	ACTTGCCA	TGGCAAGT
F5	7-433	TCTTAGTT	5-481	AGGAATTG	CAATTCCT
G5	7-434	ACCACTCG	5-482	CTATTACA	TGTAATAG
H5	7-435	AGAACGAT	5-483	TAGGGACC	GGTCCCTA

WELL POSITION	EXPECTED i7 INDEX READ		EXPECTED i5 INDEX READ		
	i7 INDEX ID		i5 INDEX ID	FORWARD STRAND WORKFLOW	REVERSE STRAND WORKFLOW
A6	7-436	ATCCCGTA	5-484	AGAAGACC	GGTCTTCT
B6	7-437	GCTGGACG	5-485	CGTAACAG	CTGTTACG
C6	7-438	TGATTGAT	5-486	ACCCATAA	TTATGGGT
D6	7-439	CCCAAATG	5-487	CACCATTG	CAATGGTG
E6	7-440	ATTCGCAT	5-488	TTAGCTAT	ATAGCTAA
F6	7-441	CGTCAAGA	5-489	AACAACCC	GGGTTGTT
G6	7-442	CTTCGACC	5-490	GTTACTGT	ACAGTAAC
H6	7-443	CAGACGAC	5-491	CCGAGCAC	GTGCTCGG
A7	7-444	ATCGTAGG	5-396	AGTTTCGA	TCGAAACT
B7	7-445	GCGCAGAC	5-397	GAACCTCT	AGAGGTTC
C7	7-446	AATTGCGG	5-398	GCCCAGTG	CACTGGGC
D7	7-447	CCTACGGG	5-399	TGACAGCT	AGCTGTCA
E7	7-448	TGCTATAT	5-400	CATCACCC	GGGTGATG
F7	7-449	AACCGAAC	5-401	CTGGAGTA	TACTCCAG
G7	7-450	ACCTGCTT	5-402	GATCCGGG	CCCGGATC
H7	7-451	CCCATGCG	5-403	AACACCTG	CAGGTGTT
A8	7-452	ATCTGGGA	5-404	GTGACGTT	AACGTCAC
B8	7-453	TAGACAAT	5-405	ACAGGAAA	TTTCCTGT
C8	7-454	TTCTTCCT	5-406	GTGCTCTG	CAGAGCAC
D8	7-455	CACCTAAA	5-407	GTACCTGG	CCAGGTAC
E8	7-456	GTACTCGC	5-408	AGCGCAAA	TTTGCGCT
F8	7-457	TAACCAGT	5-409	AACGCCCT	AGGGCGTT
G8	7-458	CGGAAACT	5-410	GCGTACGG	CCGTACGC
H8	7-459	GCTGAGAA	5-411	GCCAGATT	AATCTGGC
A9	7-460	ATGTCTTA	5-412	ATAGCAGA	TCTGCTAT
B9	7-461	TCACGCCT	5-413	GAGATGAT	ATCATCTC
C9	7-462	GCAACAGC	5-414	GCCCGTCT	AGACGGGC
D9	7-463	ATCGTCTC	5-415	TTCGCCTG	CAGGCGAA
E9	7-464	CCTTGTGA	5-416	CTAGCTCC	GGAGCTAG
F9	7-465	CTCACCAT	5-417	TATGCCGG	CCGGCATA
G9	7-466	CAAATTCT	5-418	AATGTTGG	CCAACATT
H9	7-467	CTCCTCAC	5-419	ATCGGATA	TATCCGAT
A10	7-468	ATGTGCAA	5-420	ATAGTGAC	GTCACTAT
B10	7-469	GCAAATGT	5-421	GCTCCCTG	CAGGGAGC
C10	7-470	ACGCATGG	5-422	AGTCAATT	AATTGACT
D10	7-471	ACACCACC	5-423	AATACGCT	AGCGTATT
E10	7-472	CACGCTGA	5-424	AACTTCGT	ACGAAGTT
F10	7-473	TCCCAGCC	5-425	GAACTGCC	GGCAGTTC
G10	7-474	CTATTCGT	5-426	AGCATTGT	ACAATGCT
H10	7-475	GAGCCATT	5-427	GACCAGGA	TCCTGGTC

WELL POSITION	EXPECTED i7 INDEX READ		EXPECTED i5 INDEX READ		
	i7 INDEX ID		i5 INDEX ID	FORWARD STRAND WORKFLOW	REVERSE STRAND WORKFLOW
A11	7-476	ATTAATCG	5-428	ACAGCATT	AATGCTGT
B11	7-477	GAAGGAGC	5-429	GTTCTGCA	TGCAGAAC
C11	7-478	GATTACAA	5-430	CTGTCCGG	CCGGACAG
D11	7-479	ATTTGAAG	5-431	GTCAAGCG	CGCTTGAC
E11	7-480	ACTTGCCA	5-432	TTCGCTCA	TGAGCGAA
F11	7-481	AGGAATTG	5-433	TCTTAGTT	AACTAAGA
G11	7-482	CTATTACA	5-434	ACCACTCG	CGAGTGGT
H11	7-483	TAGGGACC	5-435	AGAACGAT	ATCGTTCT
A12	7-484	AGAAGACC	5-436	ATCCCGTA	TACGGGAT
B12	7-485	CGTAACAG	5-437	GCTGGACG	CGTCCAGC
C12	7-486	ACCCATAA	5-438	TGATTGAT	ATCAATCA
D12	7-487	CACCATTG	5-439	CCCAAATG	CATTTGGG
E12	7-488	TTAGCTAT	5-440	ATTCGCAT	ATGCGAAT
F12	7-489	AACAACCC	5-441	CGTCAAGA	TCTTGACG
G12	7-490	GTTACTGT	5-442	CTTCGACC	GGTCGAAG
H12	7-491	CCGAGCAC	5-443	CAGACGAC	GTCGTCTG

Kit Components

The NEBNext Multiplex Oligos for Illumina (96 Unique Dual Index Primer Pairs Set 5) are functionally validated through library preparation using the NEBNext Library Prep Kits and sequencing on the Illumina platforms.

NEB #E6448S Table of Components

NEB #	CONCENTRATION	PRODUCT	VOLUME
E6612A	15 µM	NEBNext Adaptor for Illumina	0.96 ml
E6610A		USER Enzyme	0.288 ml
E6449A	10 µM each	NEBNext 96 Unique Dual Index Primer Pairs Plate (Set 5)	1 plate (10 µl/well)

NEB #E6446L Table of Components

NEB #	CONCENTRATION	PRODUCT	VOLUME
E6612A	15 µM	NEBNext Adaptor for Illumina	4 x 0.96 ml
E6610AA		USER Enzyme	2 x 0.576 ml
E6449A	10 µM each	NEBNext 96 Unique Dual Index Primer Pairs Plate (Set 5)	4 plates (10 µl/well)

Note :
For the NEBNext Adaptor for Illumina sequence, please see NEBNext Multiplex Oligos for Illumina (Index Primers Set 1), NEB #E7335, Manual.

Revision History

REVISION #	DESCRIPTION	DATE
1.0	N/A	1/22
2.0	Update Protocol and Tables	7/22

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