NEBNext® Multiplex Oligos for Illumina® (96 Unique Dual Index Primer Pairs Set 2)

NEB #E6442S/L

96/384 reactions

Version 4.0_7/22

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The NEBNext Multiplex Oligos for Illumina (96 Unique Dual Index Primer Pairs Set 2) Includes

The volumes provided are sufficient for preparation of up to 96 reactions (NEB #E6442S) and 384 reactions (NEB #E6442L). All reagents should be stored at –20°C.

NEBNext Adaptor for Illumina

USER® Enzyme

NEBNext 96 Unique Dual Index Primer Pairs Plate (Set 2)
Each well contains a unique pair of Index Primers (S size contains 1 plate, L size contains 4 plates)

Overview

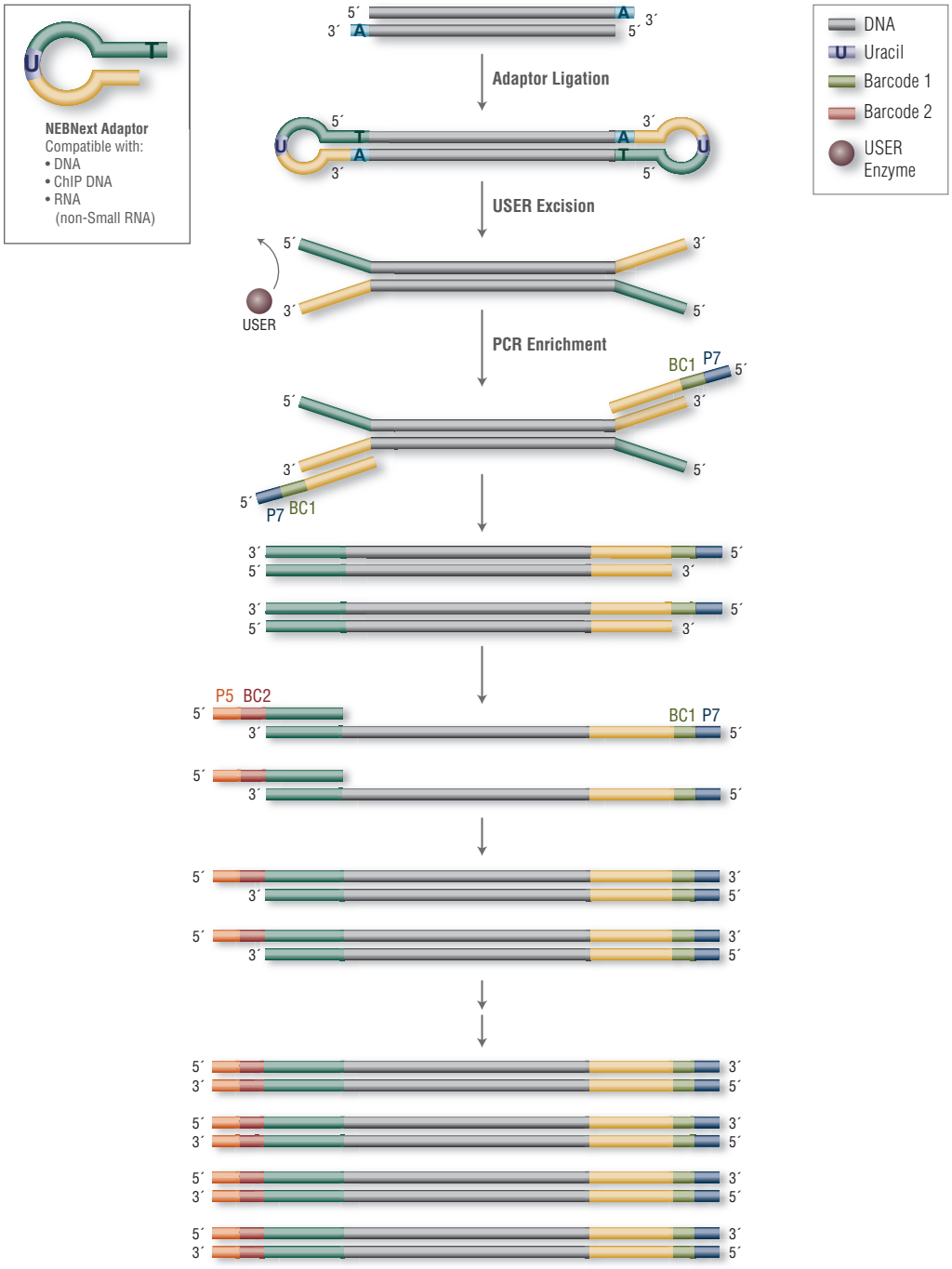
The NEBNext Multiplex Oligos for Illumina (96 Unique Dual Index Primer Pairs Set 2) contains adaptors and primers that are ideally suited for multiplex sample preparation for next-generation sequencing on the Illumina platform (Illumina, Inc.). Each kit component must pass rigorous quality control standards, 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.

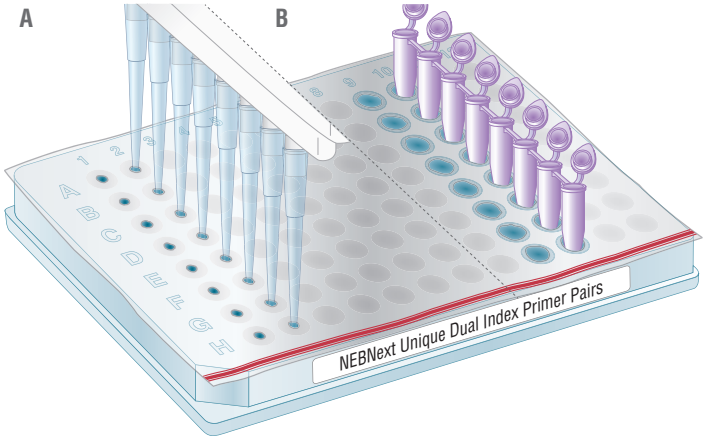
1.1A.4. Remove the hard plastic plate cover. Mix briefly by vortexing and then centrifuge the plate (280 × *g* for ~1 min) to collect all of the primer at the bottom of each well.

1.1A.5. Orient the 96 Unique Dual Index Primers Plate Set 2 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 2



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 E6442 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. Table 2.1 lists some valid combinations (up to 8-plex) that can be sequenced together. For combinations > 8 choose any column and add any plex combinations as needed.

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. Use Table 2.1 for some suggested combinations. Not all possible combinations are listed. Please confirm the color balance of the selected barcodes for low plex pooling. Please refer to Table 2.2 for examples.

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 pools containing 3-8 libraries, add any other primers from that column. For pools containing more than 8 libraries, choose any column and add any other primers as needed.

Table 2.1.

PLEX	WELL POSITION
2	A1, B1 A2, B2 A3, B3 A4, B4 (for additional combinations, confirm color balance according to examples in tables 2.2 and 2.3)
3	A1, B1, C1 A2, B2, C2 A3, B3, C3 A4, B4, C4 (for additional combinations, confirm color balance according to examples in tables 2.2 and 2.3)
4	A1, B1, C1, D1 A2, B2, C2, D2 A3, B3, C3, D3 A4, B4, C4, D4 A2, B2, G2, H2 A3, B3, G3, H3 A6, F6, G6, H6 A8, E8, F8, G8 B9, E9, F9, G9 A12, B12, C12, E12
5	A1, B1, C1, D1, E1 A2, B2, C2, D2, E2 A3, B3, C3, D3, E3 A4, B4, C4, D4, E4 A2, B2, C2, G2, H2 A3, B3, C3, G3, H3 A6, E6, F6, G6, H6 A8, E8, F8, G8, H8 A9, B9, E9, F9, G9 A12, B12, C12, D12, E12
6-7	Any 5 plex plus 1-2 adjacent wells from the same column
8	Any column

***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 2.2. 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	C	A	C	T	G	T	A	G	A	A	G	C	G	A	C	T	A	G	T	C	G	C	T	T
B1	G	T	G	C	A	C	G	A	T	G	A	T	A	G	G	C	G	C	C	T	A	T	C	A
C1	A	T	G	T	T	C	C	T	T	C	A	G	C	G	C	C	G	G	C	G	C	T	G	A
D1	C	A	T	T	A	T	G	G	A	G	T	C	A	C	A	T	A	T	G	T	G	A	C	T
	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓

BAD																									
WELL POSITION	EXPECTED i7 INDEX READ								EXPECTED i5 INDEX READ																
									FORWARD STRAND WORKFLOW								REVERSE STRAND WORKFLOW							
A11	A	A	G	G	A	A	G	G	A	C	C	G	G	A	G	T	A	C	T	C	C	G	G	T
B11	G	C	A	C	A	C	A	A	C	T	T	G	A	C	G	A	T	C	G	T	C	A	A	G
C11	G	T	C	A	G	T	A	T	A	G	A	A	G	C	C	T	A	G	G	C	T	T	C	T
D11	A	T	T	C	G	A	G	C	C	T	A	G	G	T	T	G	C	A	A	C	C	T	A	G
	✓	✓	✓	✓	✓	✓	✓	✓	X	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	X

The index primer sequences, for different Illumina sequencer input sheets are indicated in Table 2.4.

Table 2.3. 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	C	A	C	T	G	T	A	G	A	A	G	C	G	A	C	T	A	G	T	C	G	C	T	T	
B1	G	T	G	C	A	C	G	A	T	G	A	T	A	G	G	C	G	C	C	T	A	T	C	A	
C1	A	T	G	T	T	C	C	T	T	C	A	G	C	G	C	C	G	G	C	G	C	T	G	A	
	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	

BAD																									
WELL POSITION	EXPECTED i7 INDEX READ								EXPECTED i5 INDEX READ																
									FORWARD STRAND WORKFLOW								REVERSE STRAND WORKFLOW								
C1	A	T	G	T	T	C	C	T	T	C	A	G	C	G	C	C	G	G	C	G	C	T	G	A	
A2	A	A	G	C	G	A	C	T	A	C	G	A	A	T	C	C	G	G	A	T	T	C	G	T	
A10	A	G	G	T	A	G	G	A	T	G	T	T	C	G	C	C	G	G	C	G	A	A	C	A	
	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	X	X	✓	✓	✓	✓	✓	✓	

Table 2.4. 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	P7126	CACTGTAG	P5134	AAGCGACT	AGTCGCTT
B1	P7148	GTGCACGA	P5136	TGATAGGC	GCCTATCA
C1	P7133	ATGTTCCT	P5107	TCAGCGCC	GGCGCTGA
D1	P7141	CATTATGG	P5108	AGTCACAT	ATGTGACT
E1	P7142	TCTTGTTT	P5109	CCTTTCAC	GTGAAAGG
F1	P7143	GGCTTACT	P5111	CTTTCCCT	AGGGAAAG
G1	P7146	ACGATATG	P5117	GACAATTC	GAATTGTC
H1	P7152	ATCCGCAG	P5119	ACACGACT	AGTCGTGT
A2	P7134	AAGCGACT	P5135	ACGAATCC	GGATTCGT
B2	P7136	TGATAGGC	P5170	GTCTGAGT	ACTCAGAC
C2	P7153	AACACCAC	P5122	GGTGTGAG	CTCACACC
D2	P7154	ACCTCTTC	P5124	CTTGCATA	TATGCAAG
E2	P7155	GTCCGATC	P5125	GCCAATCC	GGATTGGC
F2	P7157	GAGGACCA	P5129	ATGCCGGT	ACCGGCAT
G2	P7158	CGCTCTTA	P5137	CATACCGT	ACGGTATG
H2	P7159	CTGAGCTC	P5138	ATCAGAGC	GCTCTGAT
A3	P7135	ACGAATCC	P5127	ATTACCCA	TGGGTAAT
B3	P7170	GTCTGAGT	P5169	GACTTGTG	CACAAGTC
C3	P7160	CCTAAACT	P5139	ACGAGGAG	CTCCTCGT
D3	P7162	TGTCACAC	P5140	TAATCTCG	CGAGATTA
E3	P7165	GATATGAA	P5144	TACGGCAG	CTGCCGTA
F3	P7166	AAGTGTGG	P5145	TGCCCATC	GATGGGCA
G3	P7174	GTTGGCGT	P5147	CAGCAGTC	GACTGCTG
H3	P7176	TAGCTGGC	P5149	TACCGGCT	AGCCGGTA
A4	P7127	ATTACCCA	P5126	CACTGTAG	CTACAGTG
B4	P7169	GACTTGTG	P5148	GTGCACGA	TCGTGCAC
C4	P7177	CAGGTAAG	P5150	CTCGAAAT	ATTTCGAG
D4	P7181	AAGGAGAC	P5151	CTCACAAC	GTTGTGAG
E4	P7182	AGTCAGGT	P5156	GTAACCAC	GTGGTTAC
F4	P7184	ACCGTAAG	P5161	CATATCCA	TGGATATG
G4	P7185	TATGACGT	P5163	CGCTAATC	GATTAGCG
H4	P7186	TTGGGTAC	P5164	CTTCCAAC	GTTGGAAG
A5	P7101	TTCAATAG	P5115	TCCCACGA	TCGTGGGA
B5	P7116	GTTTGCTC	P5132	ACCAACAG	CTGTTGGT
C5	P7187	AGAAGCCT	P5167	GTCAGTAT	ATACTGAC
D5	P7188	CTAGGTTG	P5168	ATTCGAGC	GCTCGAAT
E5	P7190	TGTGTCAG	P5171	CACCTGTA	TACAGGTG
F5	P7191	AGAACCAG	P5172	CCGACTCT	AGAGTCGG
G5	P7192	ATTGGACA	P5173	TTGCTGGA	TCCAGCAA
H5	P7385	ACCCGTTG	P5175	CAGCTTCG	CGAAGCTG

WELL POSITION	EXPECTED i7 INDEX READ		EXPECTED i5 INDEX READ		
	i7 INDEX ID		i5 INDEX ID	FORWARD STRAND WORKFLOW	REVERSE STRAND WORKFLOW
A6	P7105	ACCGGAGT	P5114	AAGGAAGG	CCTTCCTT
B6	P7118	CTTGACGA	P5131	GCACACAA	TTGTGTGC
C6	P7998	GCCACGAC	P5178	CCTCGGGT	ACCCGAGG
D6	P7099	TCTGGAAC	P5179	TAGCACCT	AGGTGCTA
E6	P7100	CACTAGAC	P5180	TGAGGACT	AGTCCTCA
F6	P7102	TTGCGTTA	P5183	TTCCCGAA	TTCGGGAA
G6	P7103	CCTATGCA	P5189	GAGTCGAT	ATCGACTC
H6	P7104	CAACCGAG	P5997	TACCTGTG	CACAGGTA
A7	P7106	TGTTCGCC	P5113	AGGTAGGA	TCCTACCT
B7	P7121	ACAAGGCA	P5130	TCGCGCAA	TTGCGCGA
C7	P7107	TCAGCGCC	P5133	ATGTTCCT	AGGAACAT
D7	P7108	AGTCACAT	P5141	CATTATGG	CCATAATG
E7	P7109	CCTTTCAC	P5142	TCTTGTTT	AAACAAGA
F7	P7111	CTTTCCCT	P5143	GGCTTACT	AGTAAGCC
G7	P7117	GACAATTC	P5146	ACGATATG	CATATCGT
H7	P7119	ACACGACT	P5152	ATCCGCAG	CTGCGGAT
A8	P7110	CCTGTCAA	P5112	ATGGCTGT	ACAGCCAT
B8	P7123	CCATCCGC	P5128	AAGGCGTA	TACGCCTT
C8	P7122	GGTGTGAG	P5153	AACACCAC	GTGGTGTT
D8	P7124	CTTGCATA	P5154	ACCTCTTC	GAAGAGGT
E8	P7125	GCCAATCC	P5155	GTCCGATC	GATCGGAC
F8	P7129	ATGCCGGT	P5157	GAGGACCA	TGGTCCTC
G8	P7137	CATACCGT	P5158	CGCTCTTA	TAAGAGCG
H8	P7138	ATCAGAGC	P5159	CTGAGCTC	GAGCTCAG
A9	P7112	ATGGCTGT	P5110	CCTGTCAA	TTGACAGG
B9	P7128	AAGGCGTA	P5123	CCATCCGC	GCGGATGG
C9	P7139	ACGAGGAG	P5160	CCTAAACT	AGTTTAGG
D9	P7140	TAATCTCG	P5162	TGTCACAC	GTGTGACA
E9	P7144	TACGGCAG	P5165	GATATGAA	TTCATATC
F9	P7145	TGCCCATC	P5166	AAGTGTGG	CCACACTT
G9	P7147	CAGCAGTC	P5174	GTTGGCGT	ACGCCAAC
H9	P7149	TACCGGCT	P5176	TAGCTGGC	GCCAGCTA
A10	P7113	AGGTAGGA	P5106	TGTTCGCC	GGCGAACA
B10	P7130	TCGCGCAA	P5121	ACAAGGCA	TGCCTTGT
C10	P7150	CTCGAAAT	P5177	CAGGTAAG	CTTACCTG
D10	P7151	CTCACAAC	P5181	AAGGAGAC	GTCTCCTT
E10	P7156	GTAACCAC	P5182	AGTCAGGT	ACCTGACT
F10	P7161	CATATCCA	P5184	ACCGTAAG	CTTACGGT
G10	P7163	CGCTAATC	P5185	TATGACGT	ACGTCATA
H10	P7164	CTTCCAAC	P5186	TTGGGTAC	GTACCCAA

WELL POSITION	EXPECTED i7 INDEX READ		EXPECTED i5 INDEX READ		
	i7 INDEX ID		i5 INDEX ID	FORWARD STRAND WORKFLOW	REVERSE STRAND WORKFLOW
A11	P7114	AAGGAAGG	P5105	ACCGGAGT	ACTCCGGT
B11	P7131	GCACACAA	P5118	CTTGACGA	TCGTCAAG
C11	P7167	GTCAGTAT	P5187	AGAAGCCT	AGGCTTCT
D11	P7168	ATTCGAGC	P5188	CTAGGTTG	CAACCTAG
E11	P7171	CACCTGTA	P5190	TGTGTCAG	CTGACACA
F11	P7172	CCGACTCT	P5191	AGAACCAG	CTGGTTCT
G11	P7173	TTGCTGGA	P5192	ATTGGACA	TGTCCAAT
H11	P7175	CAGCTTCG	P5385	ACCCGTTG	CAACGGGT
A12	P7115	TCCCACGA	P5101	TTCAATAG	CTATTGAA
B12	P7132	ACCAACAG	P5116	GTTTGCTC	GAGCAAAC
C12	P7178	CCTCGGGT	P5998	GCCACGAC	GTCGTGGC
D12	P7179	TAGCACCT	P5099	TCTGGAAC	GTTCCAGA
E12	P7180	TGAGGACT	P5100	CACTAGAC	GTCTAGTG
F12	P7183	TTCCCGAA	P5102	TTGCGTTA	TAACGCAA
G12	P7189	GAGTCGAT	P5103	CCTATGCA	TGCATAGG
H12	P7997	TACCTGTG	P5104	CAACCGAG	CTCGGTTG

Kit Components

The NEBNext Multiplex Oligos for Illumina (96 Unique Dual Index Primer Pairs Set 2) are functionally validated through library preparation using the NEBNext Library Prep Kits and sequencing on the Illumina platforms.

NEB #E6442S Table of Components

NEB #	CONCENTRATION	PRODUCT	VOLUME
E6612A	15 µM	NEBNext Adaptor for Illumina	0.96 ml
E6610A		USER Enzyme	0.288 ml
E6443A	5 µM each	NEBNext 96 Unique Dual Index Primer Pairs Plate (Set 2)	1 plate (10 µl/well)

NEB #E6442L 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
E6443A	5 µM each	NEBNext 96 Unique Dual Index Primer Pairs Plate (Set 2)	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	9/19
2.0	Update concentration of E6443A in Table of Components	7/20
3.0	Updating tables to have the most current Illumina instrument information and removed HiSeqX.	2/21
4.0	Update Protocol and Tables	7/22

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