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

NEB #E6440S/L

96/384 reactions

Version 9.0_7/22

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

The volumes provided are sufficient for preparation of up to 96 reactions (NEB #E6440S) and 384 reactions (NEB #E6440L). All reagents should be stored at –20°C.

NEBNext Adaptor for Illumina

USER® Enzyme

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

Overview

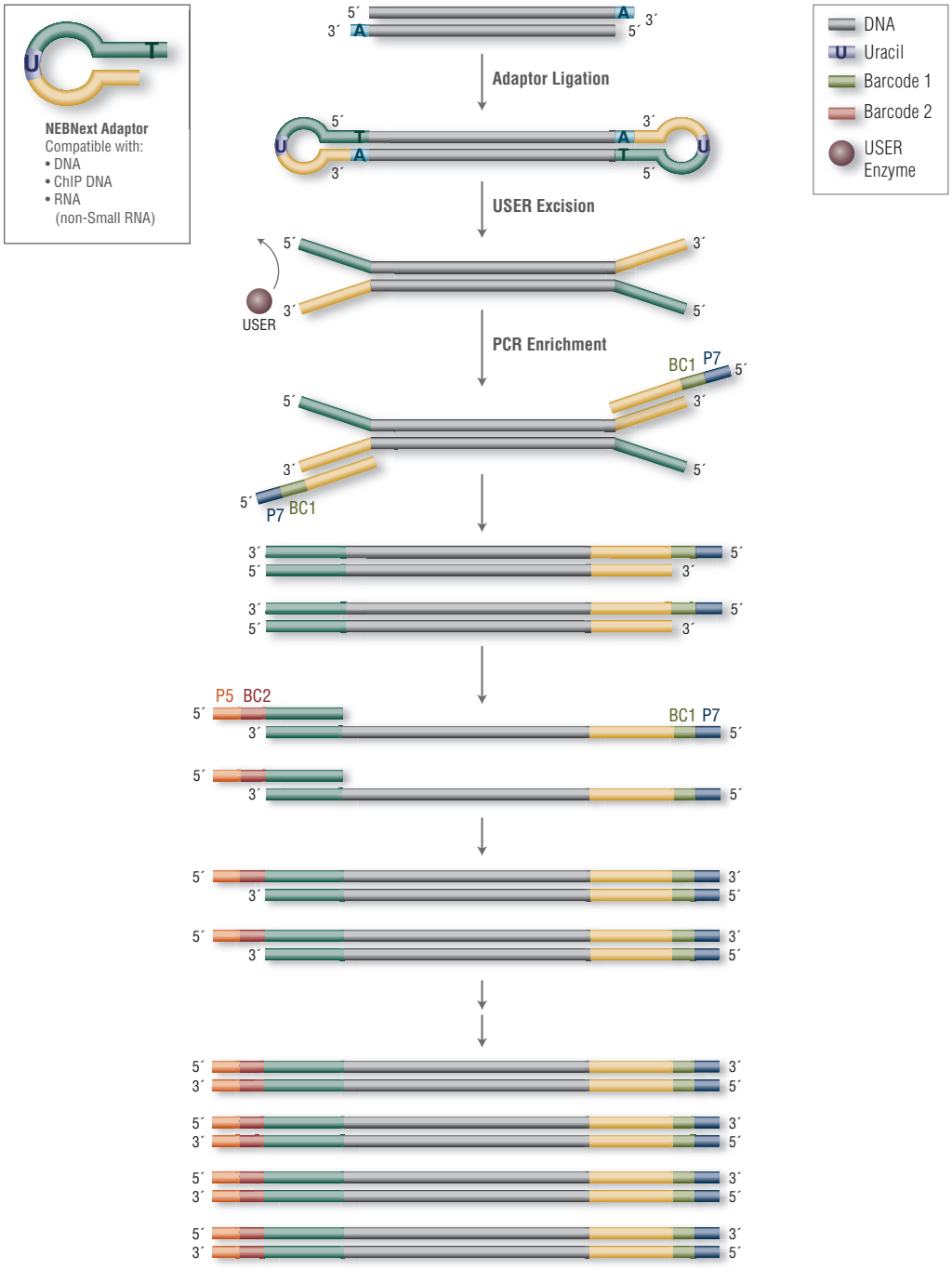
The NEBNext Multiplex Oligos for Illumina (96 Unique Dual Index Primer Pairs) contains adaptors and primers that are ideally suited for multiplex sample preparation for next-generation sequencing on the Illumina platform (Illumina, Inc.). Each kit component must pass rigorous quality control standards,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

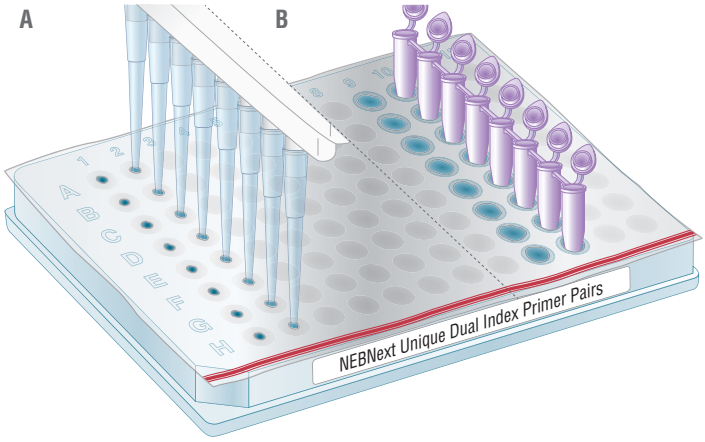
1.1A.4. Remove the hard plastic plate cover. Mix briefly by vortexing and then centrifuge the plate (280 × *g* for ~1 min) to collect all of the primer at the bottom of each well.

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



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 E6440 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.

Table 2.1.

PLEX	WELL POSITION
< 4	Not recommended
4	A6, B6, C6, and D6 A12, B12, C12, and D12 B6, C6, D6, and E6 B12, C12, D12, and E12 C1, D1, E1, and F1 C7, D7, E7, and F7 E4, F4, G4, and H4 E10, F10, G10, H10
5	A1, B1, C1, D1, E1 A6, B6, C6, D6, E6 A7, B7, C7, D7, E7 A12, B12, C12, D12, E12 B1, C1, D1, E1, F1 B6, C6, D6, E6, F6 B7, C7, D7, E7, F7 B12, C12, D12, E12, F12 C1, D1, E1, F1, G1 C2, D2, E2, F2, G2 C4, D4, E4, F4, G4 C7, D7, E7, F7, G7 C8, D8, E8, F8, G8 C10, D10, E10, F10, G10 D4, E4, F4, G4, H4 D10, E10, F10, G10, H10
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:

BAD																									
WELL POSITION	EXPECTED i7 INDEX READ								EXPECTED i5 INDEX READ																
									FORWARD STRAND WORKFLOW*								REVERSE STRAND WORKFLOW*							
E8	T	A	T	G	G	C	A	C	T	T	G	C	G	A	G	A	T	C	T	C	G	C	A	A
F8	G	A	A	T	C	A	C	C	G	A	A	C	G	A	A	G	C	T	T	C	G	T	T	C
G8	G	T	A	A	G	G	T	G	C	G	A	A	T	T	G	C	G	C	A	A	T	T	C	G
H8	C	G	A	G	A	G	A	A	G	G	A	A	G	A	G	A	T	C	T	C	T	T	C	C
	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	X	X	✓	✓	✓	✓	✓	✓	X	X	✓	✓	✓
A1	T	T	A	C	C	G	A	C	C	G	A	A	T	A	C	G	C	G	T	A	T	T	G	G
B1	T	C	G	T	C	T	G	A	G	T	C	C	T	T	G	A	T	C	A	A	G	G	A	C
C1	T	T	C	C	A	G	G	T	C	A	G	T	G	C	T	T	A	A	G	C	A	C	T	G
D1	T	A	C	G	G	T	C	T	T	C	C	A	T	T	G	C	G	C	A	A	T	G	G	A
	X	✓	✓	✓	✓	X	✓	✓	✓	✓	✓	✓	X	✓	✓	✓	✓	✓	✓	X	✓	✓	✓	✓

GOOD																									
WELL POSITION	EXPECTED i7 INDEX READ								EXPECTED i5 INDEX READ																
									FORWARD STRAND WORKFLOW*								REVERSE STRAND WORKFLOW*							
C1	T	T	C	C	A	G	G	T	C	A	G	T	G	C	T	T	A	A	G	C	A	C	G	G
D1	T	A	C	G	G	T	C	T	T	C	C	A	T	T	G	C	G	C	A	A	T	G	G	A
E1	A	A	G	A	C	C	G	T	G	T	C	G	A	T	T	G	C	A	A	T	C	G	A	C
F1	C	A	G	G	T	T	C	A	A	T	A	A	C	G	C	C	G	G	C	G	T	T	A	T
	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
A12	C	G	G	C	A	T	T	A	G	T	C	A	G	T	C	A	T	G	A	C	T	G	A	C
B12	C	A	C	G	C	A	A	T	C	C	T	T	C	C	A	T	A	T	G	G	A	A	G	G
C12	G	G	A	A	T	G	T	C	A	G	G	A	A	C	A	C	G	T	G	T	T	C	C	T
D12	T	G	G	T	G	A	A	G	C	T	T	A	C	A	G	C	G	C	T	G	T	A	A	G
	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓

The index primer sequences, for different Illumina sequencer input sheets are indicated in Table 2.2.

Table 2.3 Index Sequences (Color coded based on HiSeq/MiSeq guidelines)

WELL POSITION	EXPECTED i7 INDEX READ		EXPECTED i5 INDEX READ		
	i7 INDEX ID		i5 INDEX ID	FORWARD STRAND WORKFLOW*	REVERSE STRAND WORKFLOW*
A1	S762	TTACCGAC	S512	CGAATACG	CGTATTCG
B1	S713	TCGTCTGA	S586	GTCCTTGA	TCAAGGAC
C1	S736	TTCCAGGT	S543	CAGTGCTT	AAGCACTG
D1	S709	TACGGTCT	S575	TCCATTGC	GCAATGGA
E1	S732	AAGACCGT	S550	GTCGATTG	CAATCGAC
F1	S774	CAGGTTCA	S506	ATAACGCC	GGCGTTAT
G1	S747	TAGGAGCT	S524	GCCTTAAC	GTTAAGGC
H1	S794	TACTCCAG	S590	GGTATAGG	CCTATACC
A2	S729	AGTGACCT	S591	TCTAGGAG	CTCCTAGA
B2	S777	AGCCTATC	S526	TGCGTAAC	GTTACGCA
C2	S772	TCATCTCC	S567	CTTGCTAG	CTAGCAAG
D2	S725	CCAGTATC	S538	AGCGAGAT	ATCTCGCT
E2	S755	TTGCGAGA	S566	TATGGCAC	GTGCCATA
F2	S760	GAACGAAG	S511	GAATCACC	GGTGATTC
G2	S716	CGAATTGC	S559	GTAAGGTG	CACCTTAC
H2	S708	GGAAGAGA	S521	CGAGAGAA	TTCTCTCG
A3	S702	TCGGATTC	S523	CGCAACTA	TAGTTGCG
B3	S796	CTGTACCA	S507	CACAGACT	AGTCTGTG
C3	S757	GAGAGTAC	S545	TGGAAGCA	TGCTTCCA
D3	S783	TCTACGCA	S546	CAATAGCC	GGCTATTG
E3	S722	GCAATTCC	S578	CTCGAACA	TGTTCGAG
F3	S710	CTCAGAAG	S581	GGCAAGTT	AACTTGCC
G3	S770	GTCCTAAG	S540	AGCTACCA	TGGTAGCT
H3	S734	GCGTTAGA	S592	CAGCATAC	GTATGCTG
A4	S763	CAAGGTAC	S505	CGTATCTC	GAGATACG
B4	S797	AGACCTTG	S501	TTACGTGC	GCACGTAA
C4	S735	GTCGTTAC	S554	AGCTAAGC	GCTTAGCT
D4	S727	GTAACCGA	S598	AAGACACC	GGTGTCTT
E4	S742	GAATCCGT	S551	CAACTCCA	TGGAGTTG
F4	S795	CATGAGCA	S517	GATCTTGC	GCAAGATC
G4	S749	CTTAGGAC	S565	CTTCACTG	CAGTGAAG
H4	S773	ATCTGACC	S593	CTCGACTT	AAGTCGAG
A5	S769	TCCTCATG	S519	GTACACCT	AGGTGTAC
B5	S752	AGGATAGC	S544	CCAAGGTT	AACCTTGG
C5	S704	GGAGGAAT	S585	GAACGGTT	AACCGTTC
D5	S715	GACGTCAT	S518	CCAGTTGA	TCAACTGG
E5	S753	CCGCTTAA	S548	GTCATCGT	ACGATGAC
F5	S758	GACGAACT	S568	CAATGCGA	TCGCATTG
G5	S784	TCCACGTT	S541	GGTTGAAC	GTTCAACC
H5	S714	AACCAGAG	S520	CTTCGGTT	AACCGAAG

WELL POSITION	EXPECTED i7 INDEX READ		EXPECTED i5 INDEX READ		
	i7 INDEX ID		i5 INDEX ID	FORWARD STRAND WORKFLOW*	REVERSE STRAND WORKFLOW*
A6	S771	GTCAGTCA	S531	CGGCATTA	TAATGCCG
B6	S779	CCTTCCAT	S589	CACGCAAT	ATTGCGTG
C6	S788	AGGAACAC	S587	GGAATGTC	GACATTCC
D6	S739	CTTACAGC	S503	TGGTGAAG	CTTCACCA
E6	S737	TACCTGCA	S576	GGACATCA	TGATGTCC
F6	S728	AGACGCTA	S582	GGTGTACA	TGTACACC
G6	S780	CAACACAG	S530	GATAGCCA	TGGCTATC
H6	S761	GTACCACA	S533	CCACAACA	TGTTGTGG
A7	S712	CGAATACG	S562	TTACCGAC	GTCGGTAA
B7	S786	GTCCTTGA	S513	TCGTCTGA	TCAGACGA
C7	S743	CAGTGCTT	S536	TTCCAGGT	ACCTGGAA
D7	S775	TCCATTGC	S509	TACGGTCT	AGACCGTA
E7	S750	GTCGATTG	S532	AAGACCGT	ACGGTCTT
F7	S706	ATAACGCC	S574	CAGGTTCA	TGAACCTG
G7	S724	GCCTTAAC	S547	TAGGAGCT	AGCTCCTA
H7	S790	GGTATAGG	S594	TACTCCAG	CTGGAGTA
A8	S791	TCTAGGAG	S529	AGTGACCT	AGGTCACT
B8	S726	TGCGTAAC	S577	AGCCTATC	GATAGGCT
C8	S767	CTTGCTAG	S572	TCATCTCC	GGAGATGA
D8	S738	AGCGAGAT	S525	CCAGTATC	GATACTGG
E8	S766	TATGGCAC	S555	TTGCGAGA	TCTCGCAA
F8	S711	GAATCACC	S560	GAACGAAG	CTTCGTTC
G8	S759	GTAAGGTG	S516	CGAATTGC	GCAATTCG
H8	S721	CGAGAGAA	S508	GGAAGAGA	TCTCTTCC
A9	S723	CGCAACTA	S502	TCGGATTC	GAATCCGA
B9	S707	CACAGACT	S596	CTGTACCA	TGGTACAG
C9	S745	TGGAAGCA	S557	GAGAGTAC	GTACTCTC
D9	S746	CAATAGCC	S583	TCTACGCA	TGCGTAGA
E9	S778	CTCGAACA	S522	GCAATTCC	GGAATTGC
F9	S781	GGCAAGTT	S510	CTCAGAAG	CTTCTGAG
G9	S740	AGCTACCA	S570	GTCCTAAG	CTTAGGAC
H9	S792	CAGCATAC	S534	GCGTTAGA	TCTAACGC
A10	S705	CGTATCTC	S563	CAAGGTAC	GTACCTTG
B10	S701	TTACGTGC	S597	AGACCTTG	CAAGGTCT
C10	S754	AGCTAAGC	S535	GTCGTTAC	GTAACGAC
D10	S798	AAGACACC	S527	GTAACCGA	TCGGTTAC
E10	S751	CAACTCCA	S542	GAATCCGT	ACGGATTC
F10	S717	GATCTTGC	S595	CATGAGCA	TGCTCATG
G10	S765	CTTCACTG	S549	CTTAGGAC	GTCCTAAG
H10	S793	CTCGACTT	S573	ATCTGACC	GGTCAGAT

WELL POSITION	EXPECTED i7 INDEX READ		EXPECTED i5 INDEX READ		
	i7 INDEX ID		i5 INDEX ID	FORWARD STRAND WORKFLOW*	REVERSE STRAND WORKFLOW*
A11	S719	GTACACCT	S569	TCCTCATG	CATGAGGA
B11	S744	CCAAGGTT	S552	AGGATAGC	GCTATCCT
C11	S785	GAACGGTT	S504	GGAGGAAT	ATTCCTCC
D11	S718	CCAGTTGA	S515	GACGTCAT	ATGACGTC
E11	S748	GTCATCGT	S553	CCGCTTAA	TTAAGCGG
F11	S768	CAATGCGA	S558	GACGAACT	AGTTCGTC
G11	S741	GGTTGAAC	S584	TCCACGTT	AACGTGGA
H11	S720	CTTCGGTT	S514	AACCAGAG	CTCTGGTT
A12	S731	CGGCATTA	S571	GTCAGTCA	TGACTGAC
B12	S789	CACGCAAT	S579	CCTTCCAT	ATGGAAGG
C12	S787	GGAATGTC	S588	AGGAACAC	GTGTTCCT
D12	S703	TGGTGAAG	S539	CTTACAGC	GCTGTAAG
E12	S776	GGACATCA	S537	TACCTGCA	TGCAGGTA
F12	S782	GGTGTACA	S528	AGACGCTA	TAGCGTCT
G12	S730	GATAGCCA	S580	CAACACAG	CTGTGTTG
H12	S733	CCACAACA	S561	GTACCACA	TGTGGTAC

Kit Components

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

NEB #E6440S Table of Components

NEB #	CONCENTRATION	PRODUCT	VOLUME
E6612A	15 µM	NEBNext Adaptor for Illumina	0.96 ml
E6610A		USER Enzyme	0.288 ml
E6441A	5 µM each	NEBNext 96 Unique Dual Index Primer Pairs Plate	1 plate (10 µl/well)

NEB #E6440L 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
E6441A	5 µM each	NEBNext 96 Unique Dual Index Primer Pairs Plate	4 plates (10 µl/well)

Revision History

REVISION #	DESCRIPTION	DATE
1.0	N/A	
2.0	Add concentration column to table of components.	12/18
3.0	Add new column heading text to Table 2.2.	4/19
4.0	Placed manual into a new format	7/19
5.0	Corrected Kit Components tables	8/19
6.0	Updated to new manual format.	2/20
7.0	Update Table 2.3 header of fourth column	7/20
8.0	Updating tables to have the most current Illumina instrument information and removed HiSeqX.	2/21
9.0	Update Protocol and Tables	7/22

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