

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

NEB #E6440S/L

96/384 reactions

Version 7.0_7/20

Table of Contents

Workflow	2
Library Preparation Kits for use with NEBNext Multiplex Oligos for Illumina.....	3
Section 1	
Setting up the PCR Reaction	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) 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

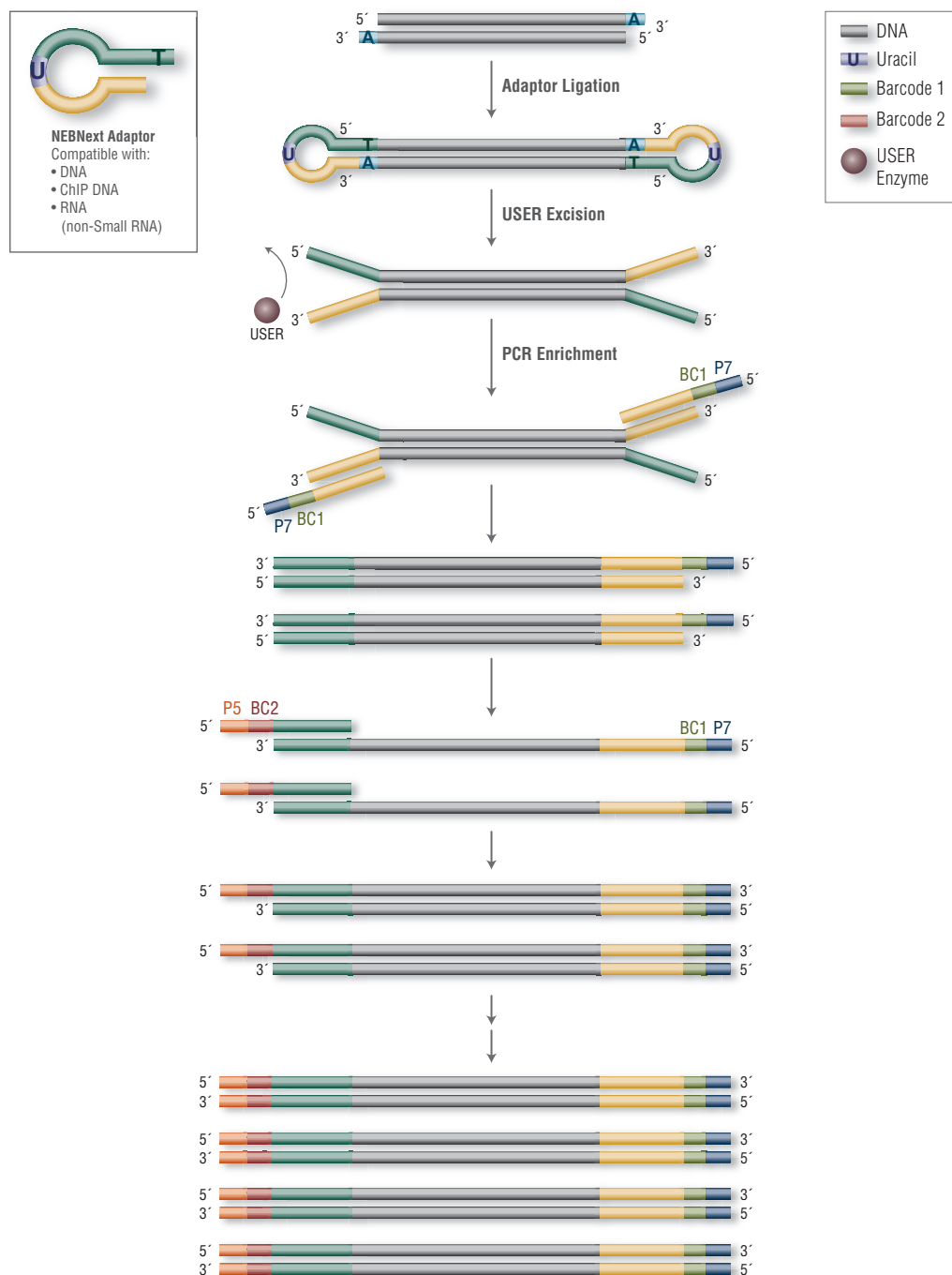
The NEBNext Multiplex Oligos for Illumina (96 Unique Dual Index Primer Pairs) contains adaptors and primers that are ideally suited for multiplex sample preparation for next-generation sequencing on the Illumina platform (Illumina, Inc.). Each kit component must pass rigorous quality control standards,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



Library Preparation Kits for use with NEBNext Multiplex Oligos for Illumina

The following kits are designed for use with the NEBNext Multiplex Oligos for Illumina:

- #E7760, NEBNext Ultra™ II Directional RNA Library Prep Kit for Illumina
- #E7765, NEBNext Ultra II Directional RNA Library Prep with Sample Purification Beads
- #E7770, NEBNext Ultra II RNA Library Prep Kit for Illumina
- #E7775, NEBNext Ultra II RNA Library Prep with Sample Purification Beads
- #E7805, NEBNext Ultra II FS DNA Library Prep Kit for Illumina
- #E6177, NEBNext Ultra II FS DNA Library Prep with Sample Purification Beads
- #E7645, NEBNext Ultra II DNA Library Prep Kit for Illumina
- #E7103, NEBNext Ultra II DNA Library Prep with Sample Purification Beads
- #E7595, NEBNext Ultra II Ligation Module
- #E7420, NEBNext Ultra Directional RNA Library Prep Kit for Illumina
- #E7530, NEBNext Ultra RNA Library Prep Kit for Illumina
- #E7370, NEBNext Ultra DNA Library Prep Kit for Illumina
- #E7445, NEBNext Ultra Ligation Module
- #E6040, NEBNext DNA Library Prep Master Mix Set for Illumina
- #E6240, NEBNext ChIP-Seq Library Prep Master Mix Set for Illumina
- #E6420, NEBNext Single Cell/Low Input RNA Library Prep Kit for Illumina
- #E6056, NEBNext Quick Ligation Module

Please refer to the library preparation kit manual for additional required materials that are not included.

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

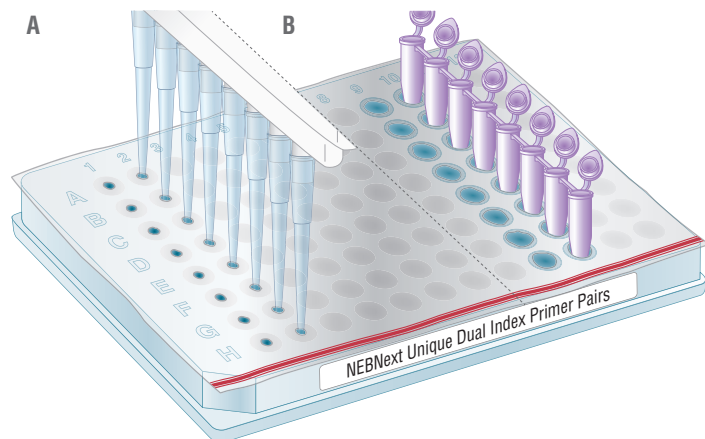


For < 96 samples, follow the protocol in Section 1.1A. For 96 samples, follow the protocol in Section 1.1B.

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 ($280 \times g$ 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 ($280 \times g$ 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 go to our FAQ's tab on www.neb.com/E6440 – NEBNext Multiplex Oligos for Illumina (96 Unique Dual Index Primer Pairs) (NEB #E6440).

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

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															
									NovaSeq, MiSeq, HiSeq 2000/2500								MiniSeq, NextSeq, HiSeq 3000/4000, HiSeq X							
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															
									NovaSeq, MiSeq, HiSeq 2000/2500								MiniSeq, NextSeq, HiSeq 3000/4000, HiSeq X							
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	C	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	
		NovaSeq, MiSeq, HiSeq 2000/2500 (PE Flow Cell), HiSeq 3000/4000 (Single Read Flow Cell)	NextSeq, MiniSeq, HiSeq 2000/2500 (Single Read Flow Cell), HiSeq 3000/4000/HiSeq X (PE Flow Cell)
A1	TTACCGAC	CGAATACG	CGTATTCTG
B1	TCGTCTGA	GTCCTTGA	TCAAGGAC
C1	TTCCAGGT	CAGTGCTT	AAGCACTG
D1	TACGGTCT	TCCATTGC	GCAATGGA
E1	AAGACCGT	GTCGATTG	CAATCGAC
F1	CAGGTTCA	ATAACGCC	GGCGTTAT
G1	TAGGAGCT	GCCTTAAC	GTTAAGGC
H1	TACTCCAG	GGTATAGG	CCTATACC
A2	AGTGACCT	TCTAGGAG	CTCCTAGA
B2	AGCCTATC	TGCGTAAC	GTTACGCA
C2	TCATCTCC	CTTGCTAG	CTAGCAAG
D2	CCAGTATC	AGCGAGAT	ATCTCGCT
E2	TTGCGAGA	TATGGCAC	GTGCCATA
F2	GAACGAAG	GAATCACC	GGTGATTCT
G2	CGAATTGC	GTAAGGTG	CACCTTAC
H2	GGAAGAGA	CGAGAGAA	TTCTCTCG
A3	TCGGATTCT	CGCAACTA	TAGTTGCTG
B3	CTGTACCA	CACAGACT	AGTCTGTG
C3	GAGAGTAC	TGGAAGCA	TGCTTCCA
D3	TCTACGCA	CAATAGCC	GGCTATTG
E3	GCAATTCC	CTCGAACA	TGTTCTGAG
F3	CTCAGAAG	GGCAAGTT	AACTTGCC
G3	GTCCTAAG	AGCTACCA	TGGTAGCT
H3	GCGTTAGA	CAGCATACT	GTATGCTG
A4	CAAGGTAC	CGTATCTCT	GAGATACG
B4	AGACCTTG	TTACGTGC	GCACGTAA
C4	GTCGTTAC	AGCTAAGC	GCTTAGCT
D4	GTAACCGA	AAGACACC	GGTGTCTT
E4	GAATCCGT	CAACTCCA	TGGAGTTG
F4	CATGAGCA	GATCTTGC	GCAAGATCT
G4	CTTAGGAC	CTTCACTG	CAGTGAAG
H4	ATCTGACC	CTCGACTT	AAGTCGAG
A5	TCCTCATG	GTACACCT	AGGTGTAC
B5	AGGATAGC	CCAAGGTT	AACCTTGG
C5	GGAGGAAT	GAACGGTT	AACCGTTC
D5	GACGTCAT	CCAGTTGA	TCAACTGG
E5	CCGCTTAA	GTCATCGT	ACGATGAC
F5	GACGAACT	CAATGCGA	TCGCATTG
G5	TCCACGTT	GGTTGAAC	GTTCAACC
H5	AACCAGAG	CTTCGGTT	AACCGAAG

WELL POSITION	EXPECTED i7 INDEX READ	EXPECTED i5 INDEX READ	
		NovaSeq, MiSeq, HiSeq 2000/2500 (PE Flow Cell), HiSeq 3000/4000 (Single Read Flow Cell)	NextSeq, MiniSeq, HiSeq 2000/2500 (Single Read Flow Cell), HiSeq 3000/4000/HiSeq X (PE Flow Cell)
A6	GTCA GTCA	CGGC ATTA	TAATGCCG
B6	CCTTCCAT	CACGCAAT	ATTGCGTG
C6	AGGAACAC	GGAATGTC	GACATTCC
D6	CTTACAGC	TGGTGAAG	CTTCACCA
E6	TACCTGCA	GGACATCA	TGATGTCC
F6	AGACGCTA	GGTGTACA	TGTACACC
G6	CAACACAG	GATAGCCA	TGGCTATC
H6	GTACCACA	CCACAACA	TGTTGTGG
A7	CGAATACG	TTACCGAC	GTCCGTAA
B7	GTCTTGA	TCGTCTGA	TCAGACGA
C7	CAGTGCTT	TTCCAGGT	ACCTGGAA
D7	TCCATTGC	TACGGTCT	AGACCGTA
E7	GTGATTG	AAGACCGT	ACGGTCTT
F7	ATAACGCC	CAGGTTCA	TGAACCTG
G7	GCCTTAAC	TAGGAGCT	AGCTCCTA
H7	GGTATAGG	TACTCCAG	CTGGAGTA
A8	TCTAGGAG	AGTGACCT	AGGTCACT
B8	TGCGTAAC	AGCCTATC	GATAGGCT
C8	CTTGCTAG	TCATCTCC	GGAGATGA
D8	AGCGAGAT	CCAGTATC	GATACTGG
E8	TATGGCAC	TTGCGAGA	TCTCGCAA
F8	GAATCACC	GAACGAAG	CTTCGTTC
G8	GTAAGGTG	CGAATTGC	GCAATTCC
H8	CGAGAGAA	GGAAGAGA	TCTCTTCC
A9	CGCAACTA	TCGGATTG	GAATCCGA
B9	CACAGACT	CTGTACCA	TGGTACAG
C9	TGGAAGCA	GAGAGTAC	GTA CTCTC
D9	CAATAGCC	TCTACGCA	TGCGTAGA
E9	CTCGAACA	GCAATTCC	GGAATTGC
F9	GGCAAGTT	CTCAGAAG	CTTCTGAG
G9	AGCTACCA	GTCCTAAG	CTTAGGAC
H9	CAGCATAC	GCGTTAGA	TCTAACGC
A10	CGTATCTC	CAAGGTAC	GTACCTTG
B10	TTACGTGC	AGACCTTG	CAAGGTCT
C10	AGCTAAGC	GTCGTTAC	GTAACGAC
D10	AAGACACC	GTAACCGA	TCGGTTAC
E10	CAACTCCA	GAATCCGT	ACGGATTG
F10	GATCTTGC	CATGAGCA	TGCTCATG
G10	CTTCACTG	CTTAGGAC	GTCCTAAG
H10	CTCGACTT	ATCTGACC	GGTCAGAT

WELL POSITION	EXPECTED i7 INDEX READ	EXPECTED i5 INDEX READ	
		NovaSeq, MiSeq, HiSeq 2000/2500 (PE Flow Cell), HiSeq 3000/4000 (Single Read Flow Cell)	NextSeq, MiniSeq, HiSeq 2000/2500 (Single Read Flow Cell), HiSeq 3000/4000/HiSeq X (PE Flow Cell)
A11	GTACACCT	TCCTCATG	CATGAGGA
B11	CCAAGGTT	AGGATAGC	GCTATCCT
C11	GAACGGTT	GGAGGAAT	ATTCCTCC
D11	CCAGTTGA	GACGTCAT	ATGACGTC
E11	GTCATCGT	CCGCTTAA	TTAAGCGG
F11	CAATGCGA	GACGAACT	AGTTCGTC
G11	GGTTGAAC	TCCACGTT	AACGTGGA
H11	CTTCGGTT	AACCAGAG	CTCTGGTT
A12	CGGCATTA	GTCAGTCA	TGACTGAC
B12	CACGCAAT	CCTTCCAT	ATGGAAGG
C12	GAATGTC	AGGAACAC	GTGTTCTT
D12	TGGTGAAG	CTTACAGC	GCTGTAAG
E12	GGACATCA	TACCTGCA	TGCAGGTA
F12	GGTGTACA	AGACGCTA	TAGCGTCT
G12	GATAGCCA	CAACACAG	CTGTGTTG
H12	CCACAACA	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)

Note :

For the NEBNext Adaptor for Illumina sequence, please see NEBNext Multiplex Oligos for Illumina (Index Primers Set 1), NEB #E7355, Manual.

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

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be INSPIRED
drive DISCOVERY
stay GENUINE