

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

NEB #E6442S/L

96/384 reactions

Version 2.0_7/20

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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

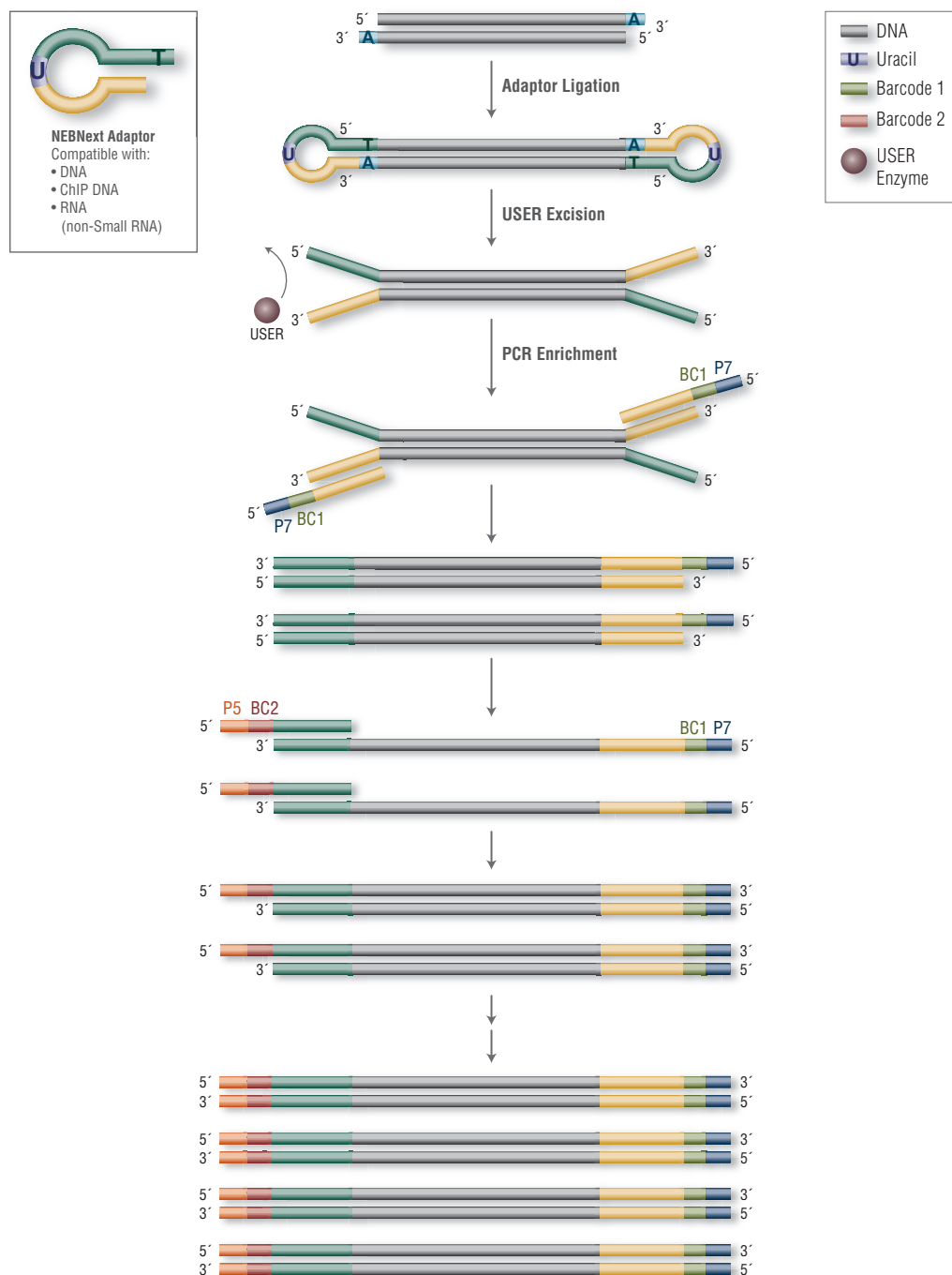
The NEBNext Multiplex Oligos for Illumina (96 Unique Dual Index Primer Pairs Set 2) contains adaptors and primers that are ideally suited for multiplex sample preparation for next-generation sequencing on the Illumina platform (Illumina, Inc.). Each kit component must pass rigorous quality control standards,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



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
- #E7120, NEBNext Enzymatic Methyl-seq Kit

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

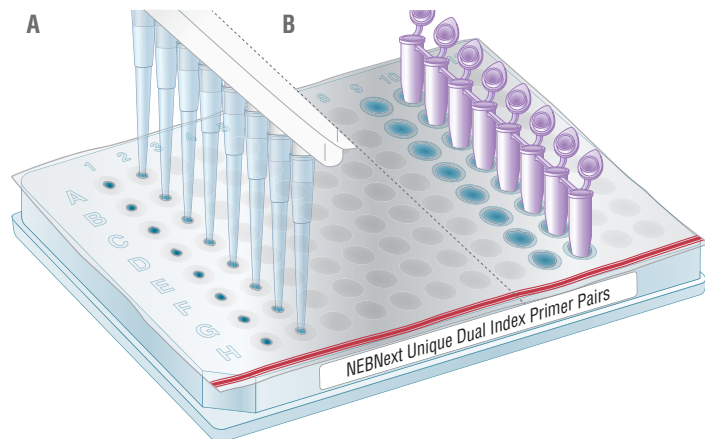


For < 96 samples, follow the protocol in Section 1.1A. For 96 samples, follow the protocol in Section 1.1B.

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 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 ($280 \times g$ 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/E6442 – NEBNext Multiplex Oligos for Illumina (96 Unique Dual Index Primer Pairs Set 2) (NEB #E6442).

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.

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

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															
									MiSeq, HiSeq 2000/2500								HiSeq 3000/4000, HiSeq X							
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															
									MiSeq, HiSeq 2000/2500								HiSeq 3000/4000, HiSeq X							
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															
									NovaSeq								MiniSeq, NextSeq							
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															
									NovaSeq								MiniSeq, NextSeq							
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.

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	CACTGTAG	AAGCGACT	AGTCGCTT
B1	GTGCACGA	TGATAGGC	GCCTATCA
C1	ATGTTCTT	TCAGCGCC	GGCGCTGA
D1	CATTATGG	AGTCACAT	ATGTGACT
E1	TCTTGTTT	CCTTTCAC	GTGAAAGG
F1	GGCTTACT	CTTTCCTT	AGGGAAAG
G1	ACGATATG	GACAATTC	GAATTGTC
H1	ATCCGCAG	ACACGACT	AGTCGTGT
A2	AAGCGACT	ACGAATCC	GGATTCGT
B2	TGATAGGC	GTCTGAGT	ACTCAGAC
C2	AACACCAC	GGTGTGAG	CTCACACC
D2	ACCTCTTC	CTTGCATA	TATGCAAG
E2	GTCCGATC	GCCAATCC	GGATTGGC
F2	GAGGACCA	ATGCCGGT	ACCGGCAT
G2	CGCTCTTA	CATACCGT	ACGGTATG
H2	CTGAGCTC	ATCAGAGC	GCTCTGAT
A3	ACGAATCC	ATTACCCA	TGGGTAAT
B3	GTCTGAGT	GACTTGTG	CACAAGTC
C3	CCTAAACT	ACGAGGAG	CTCCTCGT
D3	TGTCACAC	TAATCTCG	CGAGATTA
E3	GATATGAA	TACGGCAG	CTGCCGTA
F3	AAGTGTGG	TGCCCATC	GATGGGCA
G3	GTTGGCGT	CAGCAGTC	GA CTGCTG
H3	TAGCTGGC	TACCGGCT	AGCCGGTA
A4	ATTACCCA	CACTGTAG	CTACAGTG
B4	GACTTGTG	GTGCACGA	TCGTGCAC
C4	CAGGTAAG	CTCGAAAT	ATTTTCGAG
D4	AAGGAGAC	CTCACAAC	GTTGTGAG
E4	AGTCAGGT	GTAACCAC	GTGGTTAC
F4	ACCGTAAG	CATATCCA	TGGATATG
G4	TATGACGT	CGCTAATC	GATTAGCG
H4	TTGGGTAC	CTTCCAAC	GTTGGAAG
A5	TTCAATAG	TCCCACGA	TCGTGGGA
B5	GTTTGCTC	ACCAACAG	CTGTTGGT
C5	AGAAGCCT	GTCAGTAT	ATACTGAC
D5	CTAGGTTG	ATTCGAGC	GCTCGAAT
E5	TGTGTCAG	CACCTGTA	TACAGGTG
F5	AGAACCAG	CCGACTCT	AGAGTCGG
G5	ATTGGACA	TTGCTGGA	TCCAGCAA
H5	ACCCGTTG	CAGCTTCG	CGAAGCTG

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	A CCGGAGT	A AGGAAGG	C CTTCCTT
B6	C TTGACGA	G CACACAA	T TGTGTGC
C6	G CCACGAC	C CTCGGGT	A CCCGAGG
D6	T CTGGAAC	T AGCACCT	A GGTGCTA
E6	C ACTAGAC	T GAGGACT	A GTCCTCA
F6	T TGCGTTA	T TCCCGAA	T TCGGGAA
G6	C CTATGCA	G AGTCGAT	A TCGACTC
H6	C AACCGAG	T ACCTGTG	C ACAGGTA
A7	T GTTTCGCC	A GGTAGGA	T CCTACCT
B7	A CAAGGCA	T CGCGCAA	T TGCGCGA
C7	T CAGCGCC	A TGTTCTT	A GGAACAT
D7	A GTACACAT	C ATTATGG	C CATAATG
E7	C CTTTTAC	T CTTGTTT	A AACAAGA
F7	C TTTCCCT	G GCTTACT	A GTAAGCC
G7	G ACAATTC	A CGATATG	C ATATCGT
H7	A CACGACT	A TCCGCAg	C TGCGGAT
A8	C CTGTCAA	A TGGCTGT	A CAGCCAT
B8	C CATCCGC	A AGGCGTA	T ACGCCTT
C8	G GTGTGAG	A ACACCAC	G TGGTGTT
D8	C TTGCATA	A CCTCTTC	G AAGAGGT
E8	G CCAATCC	G TCCGATC	G ATCGGAC
F8	A TGCCGGT	G AGGACCA	T GGTCCTC
G8	C ATACCGT	C GCTCTTA	T AAGAGCG
H8	A TCAGAGC	C TGAGCTC	G AGCTCAG
A9	A TGGCTGT	C CTGTCAA	T TGACAGG
B9	A AGGCGTA	C CATCCGC	G CGGATGG
C9	A CGAGGAG	C CTAAACT	A GTTTAGG
D9	T AATCTCG	T GTACACAC	G TGTGACA
E9	T ACGGCAG	G ATATGAA	T TCATATC
F9	T GCCCATC	A AGTGTGG	C CACACTT
G9	C AGCAGTC	G TTGGCGT	A CGCCAAC
H9	T ACCGGCT	T AGCTGGC	G CCAGCTA
A10	A GGTAGGA	T GTTTCGCC	G GC GAACA
B10	T CGCGCAA	A CAAGGCA	T GCCTTGT
C10	C TCGAAAT	C AGGTAAG	C TTACCTG
D10	C TCACAAC	A AGGAGAC	G TCTCCTT
E10	G TAACCAC	A GTCAAGT	A CCTGACT
F10	C ATATCCA	A CCGTAAG	C TTACGGT
G10	C GCTAATC	T ATGACGT	A CGTCATA
H10	C TTCCAAC	T TGGGTAC	G TACCCAA

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	AAGGAAGG	ACCGGAGT	ACTCCGGT
B11	GCACACAA	CTTGACGA	TCGTCAAG
C11	GTCAGTAT	AGAAGCCT	AGGCTTCT
D11	ATTCGAGC	CTAGGTTG	CAACCTAG
E11	CACCTGTA	TGTGTCAG	CTGACACA
F11	CCGACTCT	AGAACCAG	CTGGTTCT
G11	TTGCTGGA	ATTGGACA	TGTCCAAT
H11	CAGCTTCG	ACCCGTTG	CAACGGGT
A12	TCCCACGA	TTCAATAG	CTATTGAA
B12	ACCAACAG	GTTTGCTC	GAGCAAAC
C12	CCTCGGGT	GCCACGAC	GTCGTGGC
D12	TAGCACCT	TCTGGAAC	GTTCCAGA
E12	TGAGGACT	CACTAGAC	GTCTAGTG
F12	TTCCCGAA	TTGCGTTA	TAACGCAA
G12	GAGTCGAT	CCTATGCA	TGCATAGG
H12	TACCTGTG	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

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be INSPIRED
drive DISCOVERY
stay GENUINE

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