NEBNext® Multiplex Oligos for Illumina® (96 Unique Dual Index Primer Pairs Set 4)

NEB #E6446S/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 4) Includes

The volumes provided are sufficient for preparation of up to 96 reactions (NEB #E6446S) and 384 reactions (NEB #E6446L). All reagents should be stored at –20°C.

NEBNext Adaptor for Illumina

USER® Enzyme

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

Overview

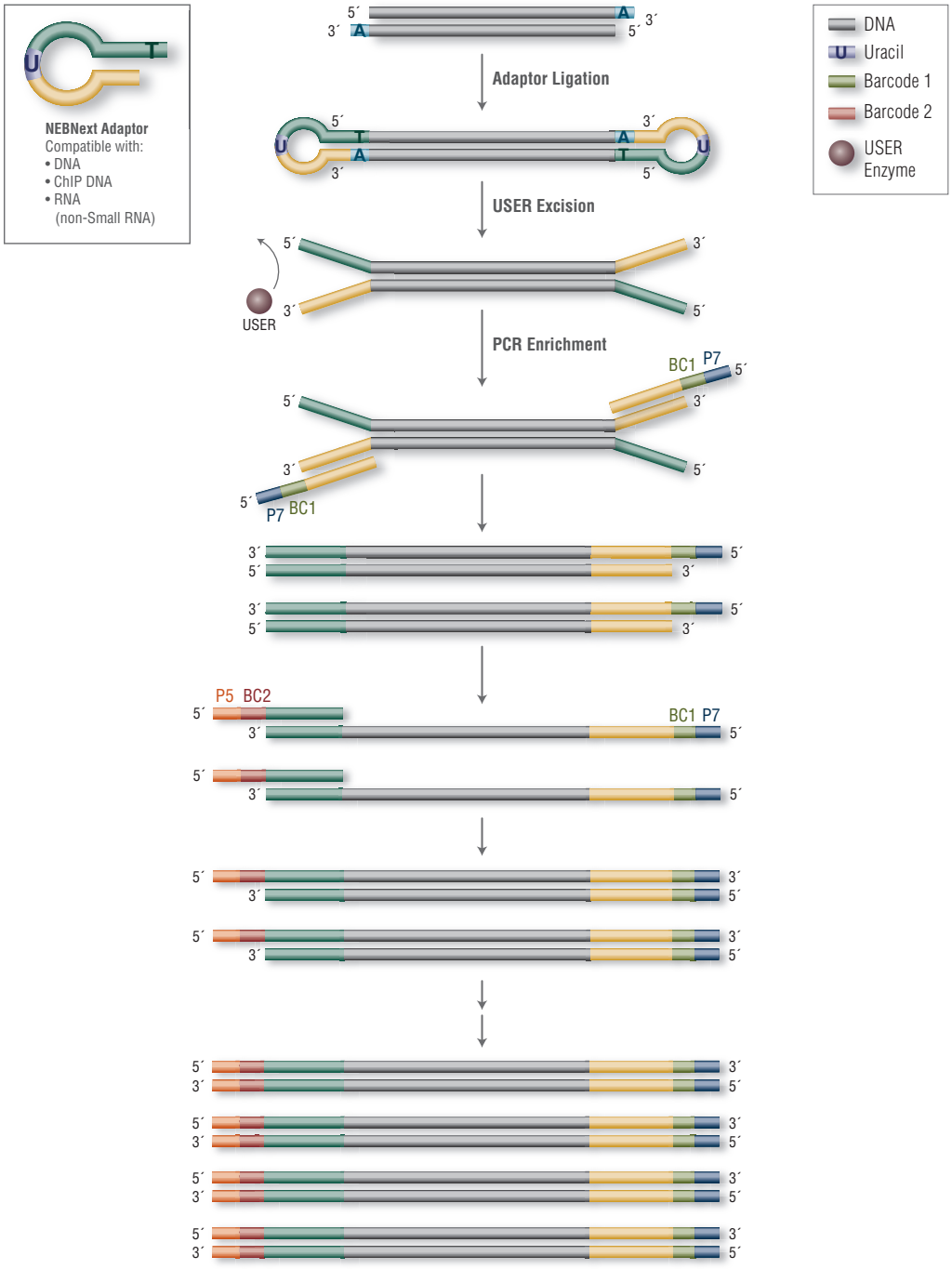
The NEBNext Multiplex Oligos for Illumina (96 Unique Dual Index Primer Pairs Set 4) contains adaptors and primers that are ideally suited for multiplex sample preparation for next-generation sequencing on the Illumina platform (Illumina, Inc.). Each kit component must pass rigorous quality control standards, 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.

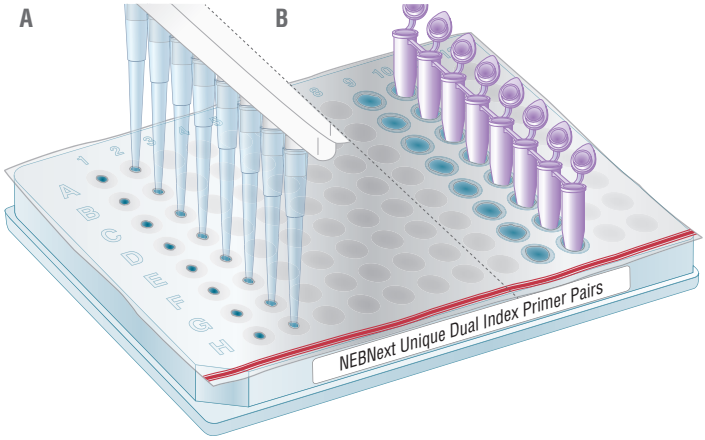
1.1A.4. Remove the hard plastic plate cover. Mix briefly by vortexing and then centrifuge the plate (280 × *g* for ~1 min) to collect all of the primer at the bottom of each well.

1.1A.5. Orient the 96 Unique Dual Index Primers Plate Set 4 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 4



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 E6446 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 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.

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

BAD																									
WELL POSITION	EXPECTED i7 INDEX READ								EXPECTED i5 INDEX READ																
									FORWARD STRAND WORKFLOW								REVERSE STRAND WORKFLOW							
E1	A	A	A	T	C	C	T	C	A	A	C	C	C	G	C	C	G	G	C	G	G	G	T	T
F1	T	A	C	A	G	A	T	G	C	G	A	A	C	G	T	G	C	A	C	G	T	T	C	G
G1	T	A	A	G	C	G	C	A	C	C	G	T	A	G	A	A	T	T	C	T	A	C	G	G
H1	C	A	A	C	G	G	A	A	C	A	T	C	T	A	C	T	A	G	T	A	G	A	T	G
	✓	X	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	C	T	T	C	T	G	C	A	G	T	C	C	C	G	G	C	C	G	G	G	A	C	T	
B1	T	T	A	A	G	C	A	G	T	C	C	T	G	G	A	C	G	T	C	C	A	G	G	A	
C1	A	T	C	A	A	A	T	C	C	T	A	C	A	T	G	A	T	C	A	T	G	T	A	G	
	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	

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

WELL POSITION	EXPECTED i7 INDEX READ		EXPECTED i5 INDEX READ		
	i7 INDEX ID		i5 INDEX ID	FORWARD STRAND WORKFLOW	REVERSE STRAND WORKFLOW
A1	7-297	ACTTCTGC	5-345	AGTCCCGG	CCGGGACT
B1	7-298	TTAAGCAG	5-346	TCCTGGAC	GTCCAGGA
C1	7-299	ATCAAATC	5-347	CTACATGA	TCATGTAG
D1	7-300	TTTGAGTC	5-348	CCGGATAG	CTATCCGG
E1	7-301	AAATCCTC	5-349	AACCCGCC	GGCGGGTT
F1	7-302	TACAGATG	5-350	CGAACGTG	CACGTTCG
G1	7-303	TAAGCGCA	5-351	CCGTAGAA	TTCTACGG
H1	7-304	CAACGGAA	5-352	CATCTACT	AGTAGATG
A2	7-305	AGCCTGGA	5-353	AGTCTGCT	AGCAGACT
B2	7-306	GAGGACAG	5-354	GCCGAATC	GATTCGGC
C2	7-307	CTATCGAA	5-355	ACTATGAT	ATCATAGT
D2	7-308	TCACTAAC	5-356	CCCTATCT	AGATAGGG
E2	7-309	TCGATAAG	5-357	CGTTGTCC	GGACAACG
F2	7-310	CTTTATTC	5-358	TGGAACGG	CCGTTCCA
G2	7-311	CTGCCTTC	5-359	CCCTTCGG	CCGAAGGG
H2	7-312	ACAACCAA	5-360	TGTCCAAA	TTTGGACA
A3	7-313	GCAATGGG	5-361	AGTACAAG	CTTGTACT
B3	7-314	CTGGACAC	5-362	TACTGTGA	TCACAGTA
C3	7-315	AAGTATGC	5-363	CCGGAATT	AATTCCGG
D3	7-316	TCCGATGG	5-364	TCGCTCGG	CCGAGCGA
E3	7-317	GACAACGG	5-365	AGTGCGGA	TCCGCACT
F3	7-318	TAGCTTTA	5-366	GCTTCACA	TGTGAAGC
G3	7-319	AAACAGTC	5-367	CCGATCGT	ACGATCGG
H3	7-320	ACCTCACT	5-368	CCGTAAGC	GCTTACGG
A4	7-321	GACATTAA	5-369	AGTTGGAT	ATCCAACT
B4	7-322	ATGTACGT	5-370	TAACACGC	GCGTGTTA
C4	7-323	ATGACAAA	5-371	AGACTCAC	GTGAGTCT
D4	7-324	CCACCTAC	5-372	CAGAGTGT	ACACTCTG
E4	7-325	TGCTGTTG	5-373	ACATTACG	CGTAATGT
F4	7-326	CATTTCAG	5-374	ATTACTAC	GTAGTAAT
G4	7-327	CGTCCCTA	5-375	TGGACCCT	AGGGTCCA
H4	7-328	TACGATTA	5-376	CGCTTGCA	TGCAAGCG
A5	7-329	AAAGATCG	5-377	GTTGCCTT	AAGGCAAC
B5	7-330	GTGAGCTA	5-378	CCAATGAA	TTCATTGG
C5	7-331	AACGCGGG	5-379	ATCAGTTG	CAACTGAT
D5	7-332	CTTGTGCT	5-380	ACATACAC	GTGTATGT
E5	7-333	GCTTACCC	5-381	CTCCATAT	ATATGGAG
F5	7-334	AGTAAACA	5-382	GCTTAAGT	ACTTAAGC
G5	7-335	CGATGTAA	5-383	ACACGTAT	ATACGTGT
H5	7-336	CCTAGTCG	5-384	CAAGAAGT	ACTTCTTG

WELL POSITION	EXPECTED i7 INDEX READ		EXPECTED i5 INDEX READ		
	i7 INDEX ID		i5 INDEX ID	FORWARD STRAND WORKFLOW	REVERSE STRAND WORKFLOW
A6	7-337	AGTAGTAA	5-386	TATCAGTA	TACTGATA
B6	7-338	TACTAAGG	5-387	CGAGTCAG	CTGACTCG
C6	7-339	CATTCGGA	5-388	TTTCCATC	GATGGAAA
D6	7-340	AATCGTCA	5-389	CTTTAACT	AGTTAAAG
E6	7-341	GCTGATTT	5-390	GCCTCTAT	ATAGAGGC
F6	7-342	CGCGAAAG	5-391	CCTCCTTT	AAAGGAGG
G6	7-343	TTGCCACT	5-392	CAGTTCCC	GGGAACTG
H6	7-344	TTCGTGGA	5-393	TACCTTGT	ACAAGGTA
A7	7-345	AGTCCCGG	5-297	ACTTCTGC	GCAGAAGT
B7	7-346	TCCTGGAC	5-298	TTAAGCAG	CTGCTTAA
C7	7-347	CTACATGA	5-299	ATCAAATC	GATTTGAT
D7	7-348	CCGGATAG	5-300	TTTGAGTC	GACTCAAA
E7	7-349	AACCCGCC	5-301	AAATCCTC	GAGGATTT
F7	7-350	CGAACGTG	5-302	TACAGATG	CATCTGTA
G7	7-351	CCGTAGAA	5-303	TAAGCGCA	TGCGCTTA
H7	7-352	CATCTACT	5-304	CAACGGAA	TTCCGTTG
A8	7-353	AGTCTGCT	5-305	AGCCTGGA	TCCAGGCT
B8	7-354	GCCGAATC	5-306	GAGGACAG	CTGTCCTC
C8	7-355	ACTATGAT	5-307	CTATCGAA	TTCGATAG
D8	7-356	CCCTATCT	5-308	TCACTAAC	GTTAGTGA
E8	7-357	CGTTGTCC	5-309	TCGATAAG	CTTATCGA
F8	7-358	TGGAACGG	5-310	CTTTATTC	GAATAAAG
G8	7-359	CCCTTCGG	5-311	CTGCCTTC	GAAGGCAG
H8	7-360	TGTCCAAA	5-312	ACAACCAA	TTGGTTGT
A9	7-361	AGTACAAG	5-313	GCAATGGG	CCCATTGC
B9	7-362	TACTGTGA	5-314	CTGGACAC	GTGTCCAG
C9	7-363	CCGGAATT	5-315	AAGTATGC	GCATACTT
D9	7-364	TCGCTCGG	5-316	TCCGATGG	CCATCGGA
E9	7-365	AGTGCGGA	5-317	GACAACGG	CCGTTGTC
F9	7-366	GCTTCACA	5-318	TAGCTTTA	TAAAGCTA
G9	7-367	CCGATCGT	5-319	AAACAGTC	GACTGTTT
H9	7-368	CCGTAAGC	5-320	ACCTCACT	AGTGAGGT
A10	7-369	AGTTGGAT	5-321	GACATTAA	TTAATGTC
B10	7-370	TAACACGC	5-322	ATGTACGT	ACGTACAT
C10	7-371	AGACTCAC	5-323	ATGACAAA	TTTGTCAT
D10	7-372	CAGAGTGT	5-324	CCACCTAC	GTAGGTGG
E10	7-373	ACATTACG	5-325	TGCTGTTG	CAACAGCA
F10	7-374	ATTACTAC	5-326	CATTTCAG	CTGAAATG
G10	7-375	TGGACCCT	5-327	CGTCCCTA	TAGGGACG
H10	7-376	CGCTTGCA	5-328	TACGATTA	TAATCGTA

WELL POSITION	EXPECTED i7 INDEX READ		EXPECTED i5 INDEX READ		
	i7 INDEX ID		i5 INDEX ID	FORWARD STRAND WORKFLOW	REVERSE STRAND WORKFLOW
A11	7-377	GTTGCCTT	5-329	AAAGATCG	CGATCTTT
B11	7-378	CCAATGAA	5-330	GTGAGCTA	TAGCTCAC
C11	7-379	ATCAGTTG	5-331	AACGCGGG	CCCGCGTT
D11	7-380	ACATACAC	5-332	CTTGTGCT	AGCACAAG
E11	7-381	CTCCATAT	5-333	GCTTACCC	GGGTAAGC
F11	7-382	GCTTAAGT	5-334	AGTAAACA	TGTTTACT
G11	7-383	ACACGTAT	5-335	CGATGTAA	TTACATCG
H11	7-384	CAAGAAGT	5-336	CCTAGTCG	CGACTAGG
A12	7-386	TATCAGTA	5-337	AGTAGTAA	TTACTACT
B12	7-387	CGAGTCAG	5-338	TACTAAGG	CCTTAGTA
C12	7-388	TTTCCATC	5-339	CATTCGGA	TCCGAATG
D12	7-389	CTTTAACT	5-340	AATCGTCA	TGACGATT
E12	7-390	GCCTCTAT	5-341	GCTGATTT	AAATCAGC
F12	7-391	CCTCCTTT	5-342	CGCGAAAG	CTTTCGCG
G12	7-392	CAGTTCCC	5-343	TTGCCACT	AGTGGCAA
H12	7-393	TACCTTGT	5-344	TTCGTGGA	TCCACGAA

Kit Components

The NEBNext Multiplex Oligos for Illumina (96 Unique Dual Index Primer Pairs Set 4) are functionally validated through library preparation using the NEBNext Library Prep Kits and sequencing on the Illumina platforms.

NEB #E6446S Table of Components

NEB #	CONCENTRATION	PRODUCT	VOLUME
E6612A	15 µM	NEBNext Adaptor for Illumina	0.96 ml
E6610A		USER Enzyme	0.288 ml
E6447A	5 µM each	NEBNext 96 Unique Dual Index Primer Pairs Plate (Set 4)	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
E6447A	5 µM each	NEBNext 96 Unique Dual Index Primer Pairs Plate (Set 4)	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	5/20
2.0	Update concentration of E6447A 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

New England Biolabs, Inc., 240 County Road, Ipswich, MA 01938-2723 Telephone: (978) 927-5054 Toll Free: (USA Orders) 1-800-632-5227 (USA Tech) 1-800-632-7799 Fax: (978) 921-1350 e-mail: info@neb.com