INSTRUCTION MANUAL

NEBNext® Multiplex Oligos for Illumina® (96 Index Primers)

NEB #E6609S/L

96/384 reactions

Version 5.0_6/24

Table of Contents

The NEBNext Multiplex Oligos for Illumina (96 Index Primers) Includes

The volumes provided are sufficient for preparation of up to 96 reactions (NEB #E6609S) and 384 reactions (NEB #E6609L). All reagents should be stored at –20°C.

NEBNext Adaptor for Illumina

USER® Enzyme

NEBNext Index/Universal Primer Mix Plate

- Each well contains the Universal PCR Primer plus one of the Index Primers (S size contains 1 plate, L size contains 4 identical plates)

For the list of additional materials required, please check the manual for your NEBNext Library Prep Kit.

Applications

The NEBNext Multiplex Oligos for Illumina (96 Index Primers) contains adaptors and primers that are ideally suited for multiplex sample preparation for next-generation sequencing on the Illumina platform (Illumina, Inc.).

Lot Control: The lots provided in the NEBNext Multiplex Oligos for Illumina (96 Index Primers) are managed separately and are qualified by additional functional validation. Individual reagents undergo standard enzyme activity and quality control assays, and also meet stringent criteria in the additional quality controls listed on each individual component page.

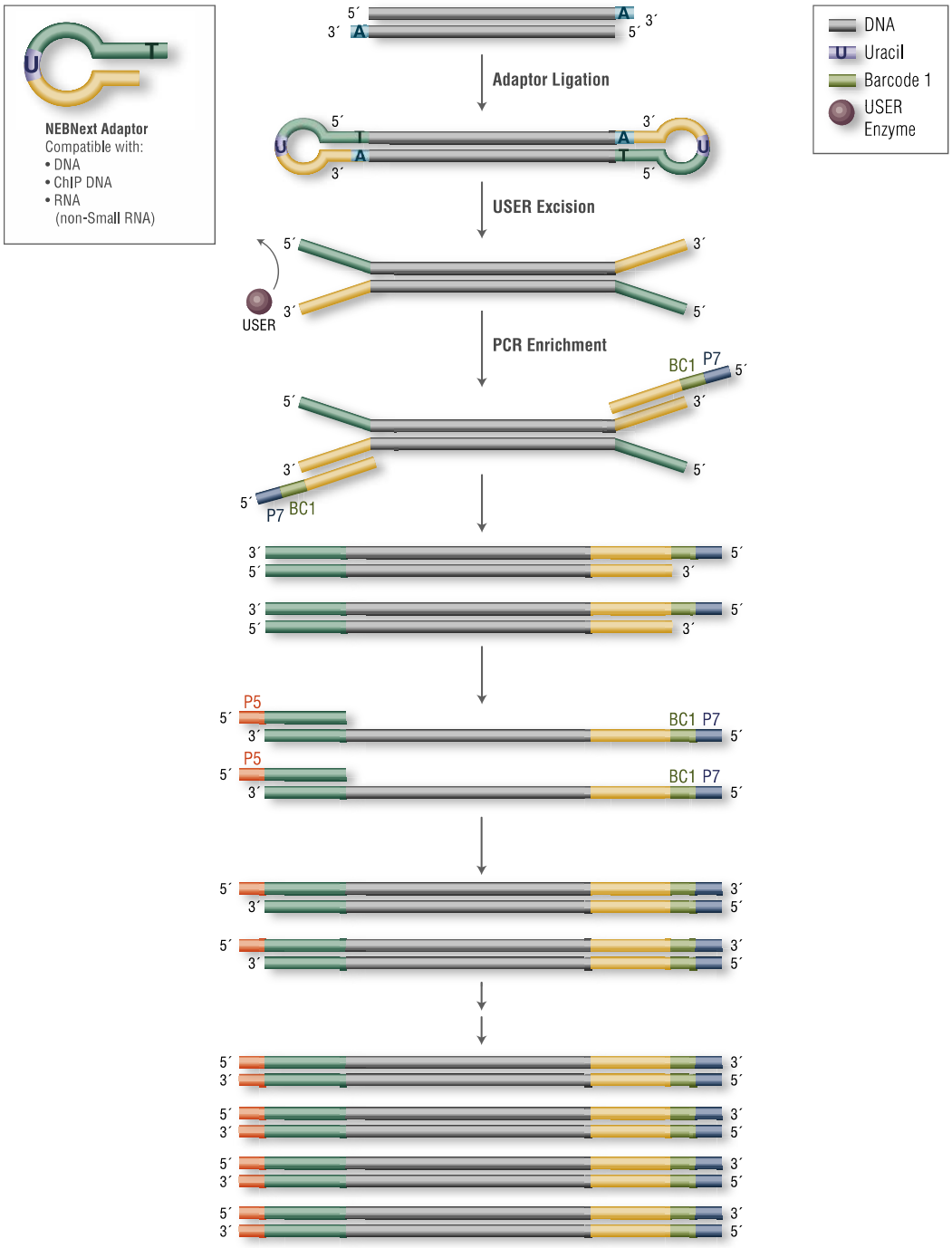
Functionally Validated: Each of the components is functionally validated through construction and sequencing of a genomic DNA libraries on the Illumina platform.

Where larger volumes, customized or bulk packaging are required, we encourage consultation with the Customized Solutions team at NEB. Please complete the NEB Custom Contact Form at www.neb.com/CustomContactForm to learn more.

Workflow Overview

Designed for use in library prep for DNA, ChIP DNA and RNA (but not Small RNA), the NEBNext non-indexed 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 primers included in this kit are pre-mixed with the universal primer 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, except for PCR free workflows.

Figure 1. Workflow demonstrating the use of NEBNext Multiplex Oligos for Illumina (96 Index Primers).



Library Preparation Kits for use with NEBNext Multiplex Oligos for Illumina (96 Index Primers)

Please refer to the kit specific **library preparation kit manual** for using the NEBNext Multiplex Oligos for Illumina **for additional required materials that are not included.**

Please refer to the NEBNext Multiplex Oligos Selection Chart at www.neb.com/oligos for a list of compatible applications.

Please note: for Illumina Sequencing instruments using patterned flow cells Unique Dual Indexing is recommended. Please see Illumina Index Hopping White Paper.

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

Index Sequence Files

For a link to download a sample sheet with the index sequences for use with the Illumina Experiment Manager (IEM) please go to our FAQs or Usage Guidelines tab on the relevant product page on www.neb.com: www.neb.com/E6609.

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 number of samples to be processed.

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 NEBNext Index/Universal Primer Mix plate for 10–15 minutes at room temperature.

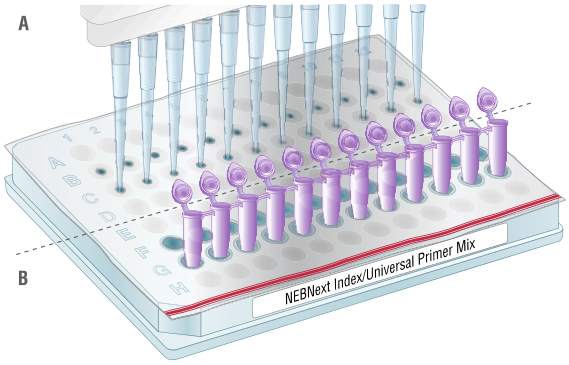
1.1A.4. Remove the hard plastic plate cover. Mix briefly by vortexing and then centrifuge the plate (280 × *g* for ~1 min) to collect all of the primer at the bottom of each well.

1.1A.5. Orient the NEBNext Index/Universal Primer Mix 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 the Universal Primer and the Index Primer. 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 Index/Universal Primer Mix Plate



1.1B Setting up the PCR reactions (96 samples)

1.1B.1. Thaw the NEBNext Index/Universal Primer Mix 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 NEBNext Index/Universal Primer 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 the Universal Primer and the Index Primer. 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

For sample sheets please see NEB.com, NEB #E6609 Product Page, "Protocols, Manuals and Usage Guidelines" Tab, Usage Guidelines.

Four Channel Chemistry Color Balancing

For all HiSeq®/MiSeq® sequencers:
Illumina uses four channel chemistry with 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. The following tables list some valid combinations (up to 8-plex) for each Set that can be sequenced together. For combinations > 8 choose any column and add any plex combinations as needed.

For the NovaSeq®6000/ NextSeq®/MiniSeq®:
Utilize red/ green or blue/ green 2 color chemistry, valid index combinations must include some indices that do not start with GG in the first two cycles. See Illumina document Document # 1000000041074 v12.

For the NovaSeq®X and X Plus:
Utilize blue/ green 2 color chemistry, valid index combinations must include some indices that do not start with GG in the first two cycles For additional NovaSeq X and X Plus color balancing guidelines please contact NEB technical support at info@neb.com.

Low Plex pooling options shown here are only for Illumina four channel red/ green chemistry.

Please note: for Illumina Sequencing instruments using patterned flow cells (for example NovaSeq, NextSeq 1000/ 2000, Iseq) Unique Dual Indexing is recommended. Please see Illumina Index Hopping White Paper.

Table 2.1.

PLEX	WELL POSITION
2	P18-B6 and P22-B10 P37-D1 and P42-D6 P52-E4 and P76-G4 P68-F8 and P95-H11
3	P4-A4, P11-A11, and P12-A12 P15-B3, P22-B10, and P24-B12 P25-C1, P31-C7, and P33-C9 P37-D1, P42-D6, and P48-D12 P49-E1, P54-E6, and P55-E7 P64-F4, P69-F9, and P71-F11 P49-E1, P54-E6, and P55-E7 P64-F4, P69-F9, and P71-F11 P76-G4, P77-G5, and P83-G11 P87-H3, P93-H9, and P94-H10
4	P1-A1, P2-A2, P3-A3, and P4-A4 P5-A5, P6-A6, P8-A8, and P10-A10 P13-B1, P14-B2, P15-B3, and P16-B4 P17-B5, P18-B6, P19-B7, and P20-B8 P25-C1, P26-C2, P27-C3, and P30-C6 P28-C4, P29-C5, P32-C8, and P35-C11 P37-D1, P38-D2, P39-D3, and P40-D4 P45-D9, P46-D10, P47-D11, and P48-D12 P49-E1, P50-E2, P51-E3, and P52-E4 P56-E8, P58-E10, P59-E11, and P60-E12 P61-F1, P62-F2, P63-F3, and P69-F9 P64-F4, P65-F5, P66-F6, and P67-F7 P73-G1, P74-G2, P75-G3, and P76-G4 P80-G8, P82-G10, P83-G11, and P84-G12 P85-H1, P86-H2, P87-H3, and P89-H5 P91-H7, P94-H10, P95-H11, and P96-H12
5–7	Any 4 plex combination with any other primers
8	Any column

Table 2.32 lists each index sequence color coded to correspond based on four channel red/green guidelines. For combinations of valid indices, ensure that you will have signal in both the red and green channels in each cycle. See examples below:

Table 2.2

GOOD																
PRIMER	**INDEX SEQUENCE**									**PRIMER**	**INDEX SEQUENCE**							
P1-A1	T	T	A	C	C	G	A	C		P41-D5	G	A	C	G	T	C	A	T
P2-A2	A	G	T	G	A	C	C	T		P42-D6	C	T	T	A	C	A	G	C
P3-A3	T	C	G	G	A	T	T	C		P43-D7	T	C	C	A	T	T	G	C
P4-A4	C	A	A	G	G	T	A	C		P44-D8	A	G	C	G	A	G	A	T
	✓	✓	✓	✓	✓	✓	✓	✓			✓	✓	✓	✓	✓	✓	✓	✓

BAD																
PRIMER	**INDEX SEQUENCE**									**PRIMER**	**INDEX SEQUENCE**							
P9-A9	C	G	C	A	A	C	T	A		P56-E8	T	A	T	G	G	C	A	C
P10-A10	C	G	T	A	T	C	T	C		P57-E9	C	T	C	G	A	A	C	A
P11-A11	G	T	A	C	A	C	C	T		P58-E10	C	A	A	C	T	C	C	A
P12-A12	C	G	G	C	A	T	T	A		P59-E11	G	T	C	A	T	C	G	T
	✓	X	✓	X	✓	✓	✓	✓			✓	✓	✓	✓	✓	X	✓	✓

Table 2.3 Index Sequences (Color coded based on four channel red/ green guidelines)

INDEX PRIMER	EXPECTED INDEX SEQUENCE READ							
P1-A1	T	T	A	C	C	G	A	C
P2-A2	A	G	T	G	A	C	C	T
P3-A3	T	C	G	G	A	T	T	C
P4-A4	C	A	A	G	G	T	A	C
P5-A5	T	C	C	T	C	A	T	G
P6-A6	G	T	C	A	G	T	C	A
P7-A7	C	G	A	A	T	A	C	G
P8-A8	T	C	T	A	G	G	A	G
P9-A9	C	G	C	A	A	C	T	A
P10-A10	C	G	T	A	T	C	T	C
P11-A11	G	T	A	C	A	C	C	T
P12-A12	C	G	G	C	A	T	T	A
P13-B1	T	C	G	T	C	T	G	A
P14-B2	A	G	C	C	T	A	T	C
P15-B3	C	T	G	T	A	C	C	A
P16-B4	A	G	A	C	C	T	T	G
P17-B5	A	G	G	A	T	A	G	C
P18-B6	C	C	T	T	C	C	A	T

INDEX PRIMER	EXPECTED INDEX SEQUENCE READ							
P19-B7	G	T	C	C	T	T	G	A
P20-B8	T	G	C	G	T	A	A	C
P21-B9	C	A	C	A	G	A	C	T
P22-B10	T	T	A	C	G	T	G	C
P23-B11	C	C	A	A	G	G	T	T
P24-B12	C	A	C	G	C	A	A	T
P25-C1	T	T	C	C	A	G	G	T
P26-C2	T	C	A	T	C	T	C	C
P27-C3	G	A	G	A	G	T	A	C
P28-C4	G	T	C	G	T	T	A	C
P29-C5	G	G	A	G	G	A	A	T
P30-C6	A	G	G	A	A	C	A	C
P31-C7	C	A	G	T	G	C	T	T
P32-C8	C	T	T	G	C	T	A	G
P33-C9	T	G	G	A	A	G	C	A
P34-C10	A	G	C	T	A	A	G	C
P35-C11	G	A	A	C	G	G	T	T
P36-C12	G	G	A	A	T	G	T	C
P37-D1	T	A	C	G	G	T	C	T
P38-D2	C	C	A	G	T	A	T	C
P39-D3	T	C	T	A	C	G	C	A
P40-D4	G	T	A	A	C	C	G	A
P41-D5	G	A	C	G	T	C	A	T
P42-D6	C	T	T	A	C	A	G	C
P43-D7	T	C	C	A	T	T	G	C
P44-D8	A	G	C	G	A	G	A	T
P45-D9	C	A	A	T	A	G	C	C
P46-D10	A	A	G	A	C	A	C	C
P47-D11	C	C	A	G	T	T	G	A
P48-D12	T	G	G	T	G	A	A	G

INDEX PRIMER	EXPECTED INDEX SEQUENCE READ							
P49-E1	A	A	G	A	C	C	G	T
P50-E2	T	T	G	C	G	A	G	A
P51-E3	G	C	A	A	T	T	C	C
P52-E4	G	A	A	T	C	C	G	T
P53-E5	C	C	G	C	T	T	A	A
P54-E6	T	A	C	C	T	G	C	A
P55-E7	G	T	C	G	A	T	T	G
P56-E8	T	A	T	G	G	C	A	C
P57-E9	C	T	C	G	A	A	C	A
P58-E10	C	A	A	C	T	C	C	A
P59-E11	G	T	C	A	T	C	G	T
P60-E12	G	G	A	C	A	T	C	A
P61-F1	C	A	G	G	T	T	C	A
P62-F2	G	A	A	C	G	A	A	G
P63-F3	C	T	C	A	G	A	A	G
P64-F4	C	A	T	G	A	G	C	A
P65-F5	G	A	C	G	A	A	C	T
P66-F6	A	G	A	C	G	C	T	A
P67-F7	A	T	A	A	C	G	C	C
P68-F8	G	A	A	T	C	A	C	C
P69-F9	G	G	C	A	A	G	T	T
P70-F10	G	A	T	C	T	T	G	C
P71-F11	C	A	A	T	G	C	G	A
P72-F12	G	G	T	G	T	A	C	A
P73-G1	T	A	G	G	A	G	C	T
P74-G2	C	G	A	A	T	T	G	C
P75-G3	G	T	C	C	T	A	A	G
P76-G4	C	T	T	A	G	G	A	C
P77-G5	T	C	C	A	C	G	T	T
P78-G6	C	A	A	C	A	C	A	G
P79-G7	G	C	C	T	T	A	A	C

INDEX PRIMER	EXPECTED INDEX SEQUENCE READ							
P80-G8	G	T	A	A	G	G	T	G
P81-G9	A	G	C	T	A	C	C	A
P82-G10	C	T	T	C	A	C	T	G
P83-G11	G	G	T	T	G	A	A	C
P84-G12	G	A	T	A	G	C	C	A
P85-H1	T	A	C	T	C	C	A	G
P86-H2	G	G	A	A	G	A	G	A
P87-H3	G	C	G	T	T	A	G	A
P88-H4	A	T	C	T	G	A	C	C
P89-H5	A	A	C	C	A	G	A	G
P90-H6	G	T	A	C	C	A	C	A
P91-H7	G	G	T	A	T	A	G	G
P92-H8	C	G	A	G	A	G	A	A
P93-H9	C	A	G	C	A	T	A	C
P94-H10	C	T	C	G	A	C	T	T
P95-H11	C	T	T	C	G	G	T	T
P96-H12	C	C	A	C	A	A	C	A

NEBNext Adaptors and Primers for Illumina

For sample sheets please see NEB.com, #E6609 Product Page, "Protocols, Manuals and Usage Guidelines" Tab, Usage Guidelines.

INDEX PRIMER	INDEX PRIMER SEQUENCE	EXPECTED INDEX SEQUENCE READ
P1-A1	5´-CAAGCAGAAGACGGCATACGAGAT**GTCGGTAA**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T-3´	TTACCGAC
P2-A2	5´-CAAGCAGAAGACGGCATACGAGAT**AGGTCACT**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T-3´	AGTGACCT
P3-A3	5´-CAAGCAGAAGACGGCATACGAGAT**GAATCCGA**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T-3´	TCGGATTC
P4-A4	5´-CAAGCAGAAGACGGCATACGAGAT**GTACCTTG**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T-3´	CAAGGTAC
P5-A5	5´-CAAGCAGAAGACGGCATACGAGAT**CATGAGGA**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T-3´	TCCTCATG
P6-A6	5´-CAAGCAGAAGACGGCATACGAGAT**TGACTGAC**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T-3´	GTCAGTCA
P7-A7	5´-CAAGCAGAAGACGGCATACGAGAT**CGTATTCG**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T-3´	CGAATACG
P8-A8	5´-CAAGCAGAAGACGGCATACGAGAT**CTCCTAGA**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T-3´	TCTAGGAG
P9-A9	5´-CAAGCAGAAGACGGCATACGAGAT**TAGTTGCG**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T-3´	CGCAACTA
P10-A10	5´-CAAGCAGAAGACGGCATACGAGAT**GAGATACG**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T-3´	CGTATCTC
P11-A11	5´-CAAGCAGAAGACGGCATACGAGAT**AGGTGTAC**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T-3´	GTACACCT
P12-A12	5´-CAAGCAGAAGACGGCATACGAGAT**TAATGCCG**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T-3´	CGGCATTA
P13-B1	5´-CAAGCAGAAGACGGCATACGAGAT**TCAGACGA**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T-3´	TCGTCTGA
P14-B2	5´-CAAGCAGAAGACGGCATACGAGAT**GATAGGCT**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T-3´	AGCCTATC
P15-B3	5´-CAAGCAGAAGACGGCATACGAGAT**TGGTACAG**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T-3´	CTGTACCA
P16-B4	5´-CAAGCAGAAGACGGCATACGAGAT**CAAGGTCT**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T-3´	AGACCTTG
P17-B5	5´-CAAGCAGAAGACGGCATACGAGAT**GCTATCCT**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T-3´	AGGATAGC
P18-B6	5´-CAAGCAGAAGACGGCATACGAGAT**ATGGAAGG**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T-3´	CCTTCCAT
P19-B7	5´-CAAGCAGAAGACGGCATACGAGAT**TCAAGGAC**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T-3´	GTCCTTGA
P20-B8	5´-CAAGCAGAAGACGGCATACGAGAT**GTTACGCA**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T-3´	TGCGTAAC
P21-B9	5´-CAAGCAGAAGACGGCATACGAGAT**AGTCTGTG**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T-3´	CACAGACT
P22-B10	5´-CAAGCAGAAGACGGCATACGAGAT**GCACGTAA**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T-3´	TTACGTGC
P23-B11	5´-CAAGCAGAAGACGGCATACGAGAT**AACCTTGG**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T-3´	CCAAGGTT
P24-B12	5´-CAAGCAGAAGACGGCATACGAGAT**ATTGCGTG**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T-3´	CACGCAAT
P25-C1	5´-CAAGCAGAAGACGGCATACGAGAT**ACCTGGAA**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T-3´	TTCCAGGT
P26-C2	5´-CAAGCAGAAGACGGCATACGAGAT**GGAGATGA**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T-3´	TCATCTCC
P27-C3	5´-CAAGCAGAAGACGGCATACGAGAT**GTACTCTC**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T-3´	GAGAGTAC
P28-C4	5´-CAAGCAGAAGACGGCATACGAGAT**GTAACGAC**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T-3´	GTCGTTAC
P29-C5	5´-CAAGCAGAAGACGGCATACGAGAT**ATTCCTCC**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T-3´	GGAGGAAT
P30-C6	5´-CAAGCAGAAGACGGCATACGAGAT**GTGTTCCT**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T-3´	AGGAACAC
P31-C7	5´-CAAGCAGAAGACGGCATACGAGAT**AAGCACTG**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T-3´	CAGTGCTT

INDEX PRIMER	INDEX PRIMER SEQUENCE	EXPECTED INDEX SEQUENCE READ
P32-C8	5´-CAAGCAGAAGACGGCATACGAGAT**CTAGCAAG**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T-3´	CTTGCTAG
P33-C9	5´-CAAGCAGAAGACGGCATACGAGAT**TGCTTCCA**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T-3´	TGGAAGCA
P34-C10	5´-CAAGCAGAAGACGGCATACGAGAT**GCTTAGCT**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T-3´	AGCTAAGC
P35-C11	5´-CAAGCAGAAGACGGCATACGAGAT**AACCGTTC**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T-3´	GAACGGTT
P36-C12	5´-CAAGCAGAAGACGGCATACGAGAT**GACATTCC**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T-3´	GGAATGTC
P37-D1	5´-CAAGCAGAAGACGGCATACGAGAT**AGACCGTA**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T-3´	TACGGTCT
P38-D2	5´-CAAGCAGAAGACGGCATACGAGAT**GATACTGG**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T-3´	CCAGTATC
P39-D3	5´-CAAGCAGAAGACGGCATACGAGAT**TGCGTAGA**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T-3´	TCTACGCA
P40-D4	5´-CAAGCAGAAGACGGCATACGAGAT**TCGGTTAC**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T-3´	GTAACCGA
P41-D5	5´-CAAGCAGAAGACGGCATACGAGAT**ATGACGTC**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T-3´	GACGTCAT
P42-D6	5´-CAAGCAGAAGACGGCATACGAGAT**GCTGTAAG**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T-3´	CTTACAGC
P43-D7	5´-CAAGCAGAAGACGGCATACGAGAT**GCAATGGA**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T-3´	TCCATTGC
P44-D8	5´-CAAGCAGAAGACGGCATACGAGAT**ATCTCGCT**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T-3´	AGCGAGAT
P45-D9	5´-CAAGCAGAAGACGGCATACGAGAT**GGCTATTG**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T-3´	CAATAGCC
P46-D10	5´-CAAGCAGAAGACGGCATACGAGAT**GGTGTCTT**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T-3´	AAGACACC
P47-D11	5´-CAAGCAGAAGACGGCATACGAGAT**TCAACTGG**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T-3´	CCAGTTGA
P48-D12	5´-CAAGCAGAAGACGGCATACGAGAT**CTTCACCA**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T-3´	TGGTGAAG
P49-E1	5´-CAAGCAGAAGACGGCATACGAGAT**ACGGTCTT**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T-3´	AAGACCGT
P50-E2	5´-CAAGCAGAAGACGGCATACGAGAT**TCTCGCAA**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T-3´	TTGCGAGA
P51-E3	5´-CAAGCAGAAGACGGCATACGAGAT**GGAATTGC**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T-3´	GCAATTCC
P52-E4	5´-CAAGCAGAAGACGGCATACGAGAT**ACGGATTC**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T-3´	GAATCCGT
P53-E5	5´-CAAGCAGAAGACGGCATACGAGAT**TTAAGCGG**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T-3´	CCGCTTAA
P54-E6	5´-CAAGCAGAAGACGGCATACGAGAT**TGCAGGTA**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T-3´	TACCTGCA
P55-E7	5´-CAAGCAGAAGACGGCATACGAGAT**CAATCGAC**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T-3´	GTCGATTG
P56-E8	5´-CAAGCAGAAGACGGCATACGAGAT**GTGCCATA**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T-3´	TATGGCAC
P57-E9	5´-CAAGCAGAAGACGGCATACGAGAT**TGTTCGAG**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T-3´	CTCGAACA
P58-E10	5´-CAAGCAGAAGACGGCATACGAGAT**TGGAGTTG**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T-3´	CAACTCCA
P59-E11	5´-CAAGCAGAAGACGGCATACGAGAT**ACGATGAC**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T-3´	GTCATCGT
P60-E12	5´-CAAGCAGAAGACGGCATACGAGAT**TGATGTCC**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T-3´	GGACATCA
P61-F1	5´-CAAGCAGAAGACGGCATACGAGAT**TGAACCTG**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T-3´	CAGGTTCA
P62-F2	5´-CAAGCAGAAGACGGCATACGAGAT**CTTCGTTC**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T-3´	GAACGAAG
P63-F3	5´-CAAGCAGAAGACGGCATACGAGAT**CTTCTGAG**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T-3´	CTCAGAAG
P64-F4	5´-CAAGCAGAAGACGGCATACGAGAT**TGCTCATG**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T-3´	CATGAGCA

INDEX PRIMER	INDEX PRIMER SEQUENCE	EXPECTED INDEX SEQUENCE READ
P65-F5	5´-CAAGCAGAAGACGGCATACGAGAT**AGTTCGTC**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T-3´	GACGAACT
P66-F6	5´-CAAGCAGAAGACGGCATACGAGAT**TAGCGTCT**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T-3´	AGACGCTA
P67-F7	5´-CAAGCAGAAGACGGCATACGAGAT**GGCGTTAT**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T-3´	ATAACGCC
P68-F8	5´-CAAGCAGAAGACGGCATACGAGAT**GGTGATTC**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T-3´	GAATCACC
P69-F9	5´-CAAGCAGAAGACGGCATACGAGAT**AACTTGCC**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T-3´	GGCAAGTT
P70-F10	5´-CAAGCAGAAGACGGCATACGAGAT**GCAAGATC**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T-3´	GATCTTGC
P71-F11	5´-CAAGCAGAAGACGGCATACGAGAT**TCGCATTG**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T-3´	CAATGCGA
P72-F12	5´-CAAGCAGAAGACGGCATACGAGAT**TGTACACC**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T-3´	GGTGTACA
P73-G1	5´-CAAGCAGAAGACGGCATACGAGAT**AGCTCCTA**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T-3´	TAGGAGCT
P74-G2	5´-CAAGCAGAAGACGGCATACGAGAT**GCAATTCG**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T-3´	CGAATTGC
P75-G3	5´-CAAGCAGAAGACGGCATACGAGAT**CTTAGGAC**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T-3´	GTCCTAAG
P76-G4	5´-CAAGCAGAAGACGGCATACGAGAT**GTCCTAAG**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T-3´	CTTAGGAC
P77-G5	5´-CAAGCAGAAGACGGCATACGAGAT**AACGTGGA**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T-3´	TCCACGTT
P78-G6	5´-CAAGCAGAAGACGGCATACGAGAT**CTGTGTTG**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T-3´	CAACACAG
P79-G7	5´-CAAGCAGAAGACGGCATACGAGAT**GTTAAGGC**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T-3´	GCCTTAAC
P80-G8	5´-CAAGCAGAAGACGGCATACGAGAT**CACCTTAC**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T-3´	GTAAGGTG
P81-G9	5´-CAAGCAGAAGACGGCATACGAGAT**TGGTAGCT**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T-3´	AGCTACCA
P82-G10	5´-CAAGCAGAAGACGGCATACGAGAT**CAGTGAAG**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T-3´	CTTCACTG
P83-G11	5´-CAAGCAGAAGACGGCATACGAGAT**GTTCAACC**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T-3´	GGTTGAAC
P84-G12	5´-CAAGCAGAAGACGGCATACGAGAT**TGGCTATC**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T-3´	GATAGCCA
P85-H1	5´-CAAGCAGAAGACGGCATACGAGAT**CTGGAGTA**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T-3´	TACTCCAG
P86-H2	5´-CAAGCAGAAGACGGCATACGAGAT**TCTCTTCC**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T-3´	GGAAGAGA
P87-H3	5´-CAAGCAGAAGACGGCATACGAGAT**TCTAACGC**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T-3´	GCGTTAGA
P88-H4	5´-CAAGCAGAAGACGGCATACGAGAT**GGTCAGAT**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T-3´	ATCTGACC
P89-H5	5´-CAAGCAGAAGACGGCATACGAGAT**CTCTGGTT**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T-3´	AACCAGAG
P90-H6	5´-CAAGCAGAAGACGGCATACGAGAT**TGTGGTAC**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T-3´	GTACCACA
P91-H7	5´-CAAGCAGAAGACGGCATACGAGAT**CCTATACC**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T-3´	GGTATAGG
P92-H8	5´-CAAGCAGAAGACGGCATACGAGAT**TTCTCTCG**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T-3´	CGAGAGAA
P93-H9	5´-CAAGCAGAAGACGGCATACGAGAT**GTATGCTG**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T-3´	CAGCATAC
P94-H10	5´-CAAGCAGAAGACGGCATACGAGAT**AAGTCGAG**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T-3´	CTCGACTT
P95-H11	5´-CAAGCAGAAGACGGCATACGAGAT**AACCGAAG**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T-3´	CTTCGGTT
P96-H12	5´-CAAGCAGAAGACGGCATACGAGAT**TGTTGTGG**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T-3´	CCACAACA

OLIGO	OLIGO SEQUENCE	EXPECTED INDEX SEQUENCE READ
NEBNext Adaptor for Illumina	5´-/5Phos/GAT CGG AAG AGC ACA CGT CTG AAC TCC AGT CdUA CAC TCT TTC CCT ACA CGA CGC TCT TCC GAT C*T-3´	N/A
NEBNext Universal PCR Primer for Illumina	5´-AAT GAT ACG GCG ACC ACC GAG ATC TAC ACT CTT TCC CTA CAC GAC GCT CTT CCG ATC*T-3´	N/A

Where -*- indicates phosphorothioate bond.

Kit Components

NEB #E6609S Table of Components

NEB #	CONCENTRATION	PRODUCT	VOLUME
E6612A	15 µM	NEBNext Adaptor for Illumina	0.96 ml
E6610A		USER Enzyme	0.288 ml
E6611A	5 µM each	NEBNext Index/Universal Primer Mix	1 plate (10 µl/well)

NEB #E6609L Table of Components

NEB #	CONCENTRATION	PRODUCT	VOLUME
E6612AA	15 µM	NEBNext Adaptor for Illumina	4 x 0.96 ml
E6610AA		USER Enzyme	2 x 0.576 ml
E6611AA	5 µM each	NEBNext Index/Universal Primer Mix	4 plates (10 µl/well)

Revision History

REVISION #	DESCRIPTION	DATE
1.0	N/A	3/16
1.1	Corrected note on page 6 and 7 from "Do not use reuse primer if the seal has been previously pierced to avoid contamination with other indexed primers." to "Do not reuse primer if the seal has been previously pierced to avoid contamination with other indexed primers."	4/16
1.2	Updated Figure 1.1 page 7.	5/16
1.3	Clarified primer concentration. Corrected primer sequences 5´ end.	6/16
2.0	Delete "Required Materials Not Included" and replace with guidance note. Change Index Primer sequences table to include adaptor, and NEBNext Universal Primer and rename it as NEBNext Adaptors and Primers for Illumina. Update the list of kits using the NEBNext Multiplex Oligos for Illumina. Delete Materials Create "Kit Component – Table of Components" for small and large size kits. Delete individual component information pages.	4/18
3.0	Edited Applications text. Added a description for Table 2.1. Corrected NEBNext Adaptors and Primers Table, changed -s- to a *. Added concentration to Table of Components.	8/18
4.0	Update to new manual format.	1/20
5.0	Updated Adaptors and Primers for Illumina.	5/22
6.0	Updated adaptor and index sequencing sections. Also added new logo to header and footer and updated legal footnote.	6/24