LIBRARY PREPARATION

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

Instruction Manual

NEB #E6609S/L
96/384 reactions
Version 1.3 6/16

NEW ENGLAND BioLabs® Inc.

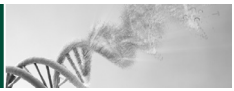


Table of Contents:

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

Required Materials Not Included:

Enzymes and buffers appropriate for DNA, ChIP or mRNA Library Preparation (NEB #E7645, E7595, E7420, E7530, E7370, E7445, E6040, E6110, E6240, E6056, E6000, E6200, E6100)

SPRIselect or AMPure® XP Beads (Beckman Coulter, Inc.)

DNA LoBind® Tubes (Eppendorf®)/PCR Plates

Magnetic Stand

Bioanalyzer® (Agilent Technologies, Inc.)

Freshly Prepared 80% Ethanol

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.). Each of these components must pass rigorous quality control standards and is lot controlled, both individually and as a set of reagents.

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.

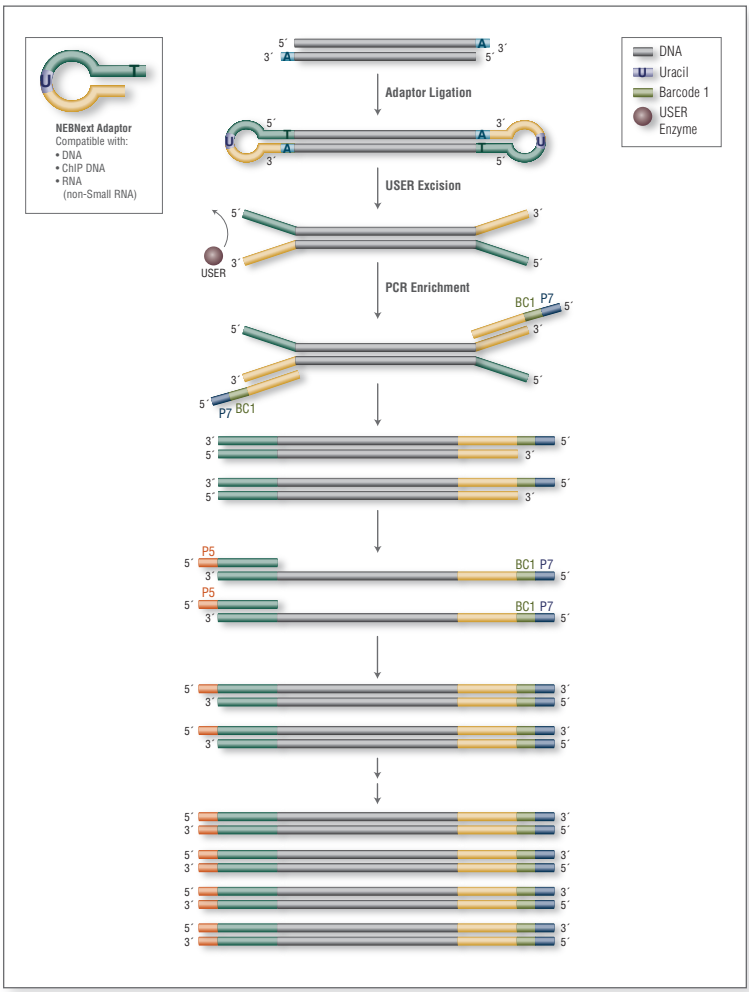
Functionally Validated: Each set of reagents is functionally validated together through construction and sequencing of genomic DNA libraries on the Illumina platform.

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 Overview:

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 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.

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



Please Refer to the Kit Specific Protocol for using the NEBNext Multiplex Oligos for Illumina:

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

- #E7645, NEBNext Ultra II DNA Library Prep Kit for Illumina
- #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
- #E6110, NEBNext mRNA Library Prep Master Mix Set for Illumina
- #E6240, NEBNext ChIP-Seq Library Prep Master Mix Set for Illumina
- #E6056, NEBNext Quick Ligation Module
- #E6000, NEBNext DNA Library Prep Reagent Set for Illumina
- #E6200, NEBNext ChIP-Seq Library Prep Reagent Set for Illumina
- #E6100, NEBNext mRNA Library Prep Reagent Set for Illumina

1

Setting up the PCR Reaction

NEBNext Multiplex Oligos for Illumina
(96 Index Primers)
Instruction Manual

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 Chapter 2.

1.1A.3. Thaw the NEBNext Index/Universal Primer Mix plate for 10-15 minutes at room temperature.

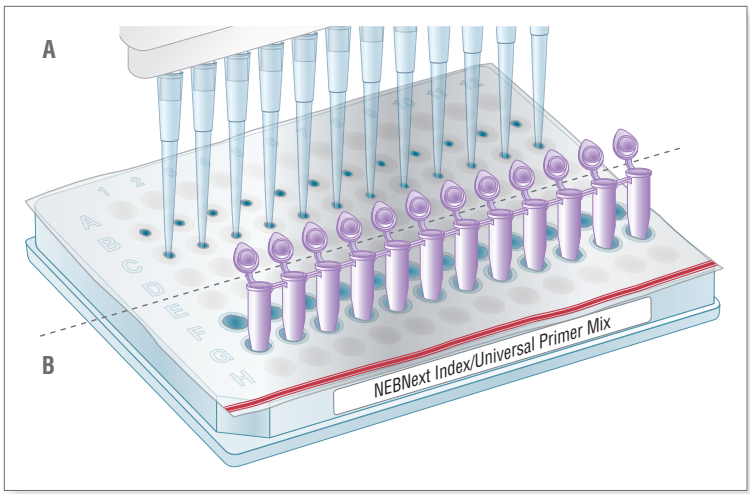
1.1A.4. Remove the hard plastic plate cover. Briefly centrifuge the plate (280 ×g for ~1 min) to collect all of the primer at the bottom of each well.

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. Briefly 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.

2

Index Pooling Guidelines

NEBNext Multiplex Oligos for Illumina
(96 Index Primers)
Instruction Manual

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/E6609 – NEBNext Multiplex Oligos for Illumina (96 Index Primers) (NEB #E6609).

For the HiSeq®/MiSeq®, 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 4-plex) that can be sequenced together. For combinations > 4 choose any 4-plex combination and add any other index as needed.

Table 2.1

PLEX	INDEX PRIMER
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
	P76-G4, P77-G5, and P83-G11
	P87-H3, P93-H9, and P94-H10

Table 2.1 (continued)

PLEX	INDEX PRIMER
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

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 examples below:

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
	✔	✖	✔	✖	✔	✔	✔	✔		✔	✔	✔	✔	✔	✖	✔	✔

Table 2.2 Index Sequences

INDEX PRIMER	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
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

INDEX PRIMER	INDEX SEQUENCE READ							
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
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

INDEX PRIMER	INDEX SEQUENCE READ							
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
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 Adaptor for Illumina

#E6612A: 0.96 ml Concentration: 15 µM

Note: Large kit contains 4 x 0.96 ml

5´-/5Phos/GAT CGG AAG AGC ACA CGT CTG AAC TCC AGT C/ideoxyU/A CAC TCT TTC CCT ACA CGA CGC TCT TCC GAT C-s-T-3´

Where -s- indicates phosphorothioate bond.

Store at –20°C

Quality Control Assays

16-Hour Incubation: 50 µl reactions containing this adaptor and 1 µg of HindIII digested Lambda DNA incubated for 16 hours at 37°C results in no detectable non-specific nuclease degradation as determined by agarose gel electrophoresis. 50 µl reactions containing this reaction buffer at 1X concentration and 1 µg T3 DNA incubated for 16 hours at 37°C also results in no detectable non-specific nuclease degradation as determined by agarose gel electrophoresis.

Endonuclease Activity: Incubation of a minimum of 5 µl of this adaptor with 1 µg of ϕX174 RF 1 DNA in assay buffer for 4 hours at 37°C in 50 µl reactions results in < 10% conversion to RF II as determined by agarose gel electrophoresis.

Phosphatase Activity: Incubation of a minimum of 10 µl of this adaptor in protein phosphatase assay buffer (1 M diethanolamine @ pH 9.8 and 0.5 mM $MgCl_2$) containing 2.5 mM *p*-nitrophenyl phosphate at 37°C for 4 hours yields no detectable *p*-nitrophenylene anion as determined by spectrophotometric analysis at 405 nm.

RNase Activity: Incubation of this adaptor with 40 ng of a FAM-labeled RNA transcript for 16 hours at 37°C results in no detectable RNase activity as determined by polyacrylamide gel electrophoresis.

Lot Controlled

USER Enzyme

#E6610A: 0.288 ml
#E6610AA: 0.576 ml (2 vials)

Store at –20°C

Supplied in: 50 mM KCl, 5 mM NaCl, 10 mM Tris-HCl (pH 7.4 @ 25°C), 0.1 mM EDTA, 1 mM DTT, 175 µg/ml BSA and 50% Glycerol

Quality Control Assays

Non-Specific DNase Activity (16 Hour): A 50 µl reaction in NEBuffer 1 containing 1 µg of Lambda DNA and a minimum of 50 units of Uracil DNA Glycosylase incubated for 16 hours at 37°C results in a DNA pattern free of detectable nuclease degradation as determined by agarose gel electrophoresis. A 50 µl reaction in Endonuclease VIII Reaction Buffer containing 1 µg of Lambda-HindIII DNA and a minimum of 25 units of Endonuclease VIII incubated for 16 hours at 37°C results in a DNA pattern free of detectable nuclease degradation as determined by agarose gel electrophoresis.

Exonuclease Activity (Radioactivity Release): A 50 µl reaction in NEBuffer 1 containing 1 µg of a mixture of single and double-stranded [^{3}H] *E. coli* DNA and a minimum of 50 units of Uracil DNA Glycosylase incubated for 4 hours at 37°C releases < 0.1% of the total radioactivity. A 50 µl reaction in Endonuclease VIII Reaction Buffer containing 1 µg of a mixture of single and double-stranded [^{3}H] *E. coli* DNA and a minimum of 10 units of Endonuclease VIII incubated for 4 hours at 37°C releases < 0.5% of the total radioactivity.

Endonuclease Activity (Nicking): A 50 µl reaction in UDG Reaction Buffer containing 1 µg of supercoiled ϕX174 DNA and a minimum of 50 units of Uracil DNA Glycosylase incubated for 4 hours at 37°C results in < 10% conversion to the nicked form as determined by agarose gel electrophoresis.

Phosphatase Activity: Incubation of a minimum of 10 µl of USER at a 1X concentration in protein phosphatase assay buffer (1 M diethanolamine @ pH 9.8 and 0.5 mM $MgCl_2$) containing 2.5 mM *p*-nitrophenyl phosphate at 37°C for 4 hours yields no detectable *p*-nitrophenylene anion as determined by spectrophotometric analysis at 405 nm.

Lot Controlled

NEBNext Index/Universal Primer Mix

#E6611A: 1 plate (10 µl/well) Concentration: 5 µM each primer

Note: Large kit contains 4 plates (10 µl/well).

Description: 96 Index Primers & the Universal Primer are included for producing barcoded libraries.

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-s-T-3´

Store at –20°C

Where -s- indicates phosphorothioate bond.

INDEX PRIMER	INDEX PRIMER SEQUENCE	EXPECTED INDEX SEQUENCE READ
P1-A1	5´-CAAGCAGAAGACGGCATACGAGAT**GTCGGTAA**GTGACTGGAGTTCA GACGTGTGCTCTTCCGATC-s-T-3´	TTACCGAC
P2-A2	5´-CAAGCAGAAGACGGCATACGAGAT**AGGTCACT**GTGACTGGAGTTCA GACGTGTGCTCTTCCGATC-s-T-3´	AGTGACCT
P3-A3	5´-CAAGCAGAAGACGGCATACGAGAT**GAATCCGA**GTGACTGGAGTTCA GACGTGTGCTCTTCCGATC-s-T-3´	TCGGATTC
P4-A4	5´-CAAGCAGAAGACGGCATACGAGAT**GTACCTTG**GTGACTGGAGTTCAG ACGTGTGCTCTTCCGATC-s-T-3´	CAAGGTAC
P5-A5	5´-CAAGCAGAAGACGGCATACGAGAT**CATGAGGA**GTGACTGGAGTTCA GACGTGTGCTCTTCCGATC-s-T-3´	TCCTCATG
P6-A6	5´-CAAGCAGAAGACGGCATACGAGAT**TGACTGAC**GTGACTGGAGTTCA GACGTGTGCTCTTCCGATC-s-T-3´	GTCAGTCA
P7-A7	5´-CAAGCAGAAGACGGCATACGAGAT**CGTATTCG**GTGACTGGAGTTCAG ACGTGTGCTCTTCCGATC-s-T-3´	CGAATACG
P8-A8	5´-CAAGCAGAAGACGGCATACGAGAT**CTCCTAGA**GTGACTGGAGTTCAG ACGTGTGCTCTTCCGATC-s-T-3´	TCTAGGAG
P9-A9	5´-CAAGCAGAAGACGGCATACGAGAT**TAGTTGCG**GTGACTGGAGTTCAG ACGTGTGCTCTTCCGATC-s-T-3´	CGCAACTA
P10-A10	5´-CAAGCAGAAGACGGCATACGAGAT**GAGATACG**GTGACTGGAGTTCA GACGTGTGCTCTTCCGATC-s-T-3´	CGTATCTC
P11-A11	5´-CAAGCAGAAGACGGCATACGAGAT**AGGTGTAC**GTGACTGGAGTTCA GACGTGTGCTCTTCCGATC-s-T-3´	GTACACCT
P12-A12	5´-CAAGCAGAAGACGGCATACGAGAT**TAATGCCG**GTGACTGGAGTTCAG ACGTGTGCTCTTCCGATC-s-T-3´	CGGCATTA
P13-B1	5´-CAAGCAGAAGACGGCATACGAGAT**TCAGACGA**GTGACTGGAGTTCA GACGTGTGCTCTTCCGATC-s-T-3´	TCGTCTGA
P14-B2	5´-CAAGCAGAAGACGGCATACGAGAT**GATAGGCT**GTGACTGGAGTTCAG ACGTGTGCTCTTCCGATC-s-T-3´	AGCCTATC
P15-B3	5´-CAAGCAGAAGACGGCATACGAGAT**TGGTACAG**GTGACTGGAGTTCA GACGTGTGCTCTTCCGATC-s-T-3´	CTGTACCA
P16-B4	5´-CAAGCAGAAGACGGCATACGAGAT**CAAGGTCT**GTGACTGGAGTTCA GACGTGTGCTCTTCCGATC-s-T-3´	AGACCTTG
P17-B5	5´-CAAGCAGAAGACGGCATACGAGAT**GCTATCCT**GTGACTGGAGTTCAG ACGTGTGCTCTTCCGATC-s-T-3´	AGGATAGC
P18-B6	5´-CAAGCAGAAGACGGCATACGAGAT**ATGGAAGG**GTGACTGGAGTTCA GACGTGTGCTCTTCCGATC-s-T-3´	CCTTCCAT

NEBNext Index/Universal Primer Mix (Cont.)

INDEX PRIMER	INDEX PRIMER SEQUENCE	EXPECTED INDEX SEQUENCE READ
P19-B7	5´-CAAGCAGAAGACGGCATACGAGAT**TCAAGGAC**GTGACTGGAGTTCA GACGTGTGCTCTTCCGATC-s-T-3´	GTCCTTGA
P20-B8	5´-CAAGCAGAAGACGGCATACGAGAT**GTTACGCA**GTGACTGGAGTTCAG ACGTGTGCTCTTCCGATC-s-T-3´	TGCGTAAC
P21-B9	5´-CAAGCAGAAGACGGCATACGAGAT**AGTCTGTG**GTGACTGGAGTTCA GACGTGTGCTCTTCCGATC-s-T-3´	CACAGACT
P22-B10	5´-CAAGCAGAAGACGGCATACGAGAT**GCACGTAA**GTGACTGGAGTTCA GACGTGTGCTCTTCCGATC-s-T-3´	TTACGTGC
P23-B11	5´-CAAGCAGAAGACGGCATACGAGAT**AACCTTGG**GTGACTGGAGTTCA GACGTGTGCTCTTCCGATC-s-T-3´	CCAAGGTT
P24-B12	5´-CAAGCAGAAGACGGCATACGAGAT**ATTGCGTG**GTGACTGGAGTTCAG ACGTGTGCTCTTCCGATC-s-T-3´	CACGCAAT
P25-C1	5´-CAAGCAGAAGACGGCATACGAGAT**ACCTGGAA**GTGACTGGAGTTCA GACGTGTGCTCTTCCGATC-s-T-3´	TTCCAGGT
P26-C2	5´-CAAGCAGAAGACGGCATACGAGAT**GGAGATGA**GTGACTGGAGTTCA GACGTGTGCTCTTCCGATC-s-T-3´	TCATCTCC
P27-C3	5´-CAAGCAGAAGACGGCATACGAGAT**GTACTCTC**GTGACTGGAGTTCAG ACGTGTGCTCTTCCGATC-s-T-3´	GAGAGTAC
P28-C4	5´-CAAGCAGAAGACGGCATACGAGAT**GTAACGAC**GTGACTGGAGTTCA GACGTGTGCTCTTCCGATC-s-T-3´	GTCGTTAC
P29-C5	5´-CAAGCAGAAGACGGCATACGAGAT**ATTCCTCC**GTGACTGGAGTTCAG ACGTGTGCTCTTCCGATC-s-T-3´	GGAGGAAT
P30-C6	5´-CAAGCAGAAGACGGCATACGAGAT**GTGTTCCT**GTGACTGGAGTTCAG ACGTGTGCTCTTCCGATC-s-T-3´	AGGAACAC
P31-C7	5´-CAAGCAGAAGACGGCATACGAGAT**AAGCACTG**GTGACTGGAGTTCA GACGTGTGCTCTTCCGATC-s-T-3´	CAGTGCTT
P32-C8	5´-CAAGCAGAAGACGGCATACGAGAT**CTAGCAAG**GTGACTGGAGTTCA GACGTGTGCTCTTCCGATC-s-T-3´	CTTGCTAG
P33-C9	5´-CAAGCAGAAGACGGCATACGAGAT**TGCTTCCA**GTGACTGGAGTTCAG ACGTGTGCTCTTCCGATC-s-T-3´	TGGAAGCA
P34-C10	5´-CAAGCAGAAGACGGCATACGAGAT**GCTTAGCT**GTGACTGGAGTTCAG ACGTGTGCTCTTCCGATC-s-T-3´	AGCTAAGC
P35-C11	5´-CAAGCAGAAGACGGCATACGAGAT**AACCGTTC**GTGACTGGAGTTCAG ACGTGTGCTCTTCCGATC-s-T-3´	GAACGGTT
P36-C12	5´-CAAGCAGAAGACGGCATACGAGAT**GACATTCC**GTGACTGGAGTTCAG ACGTGTGCTCTTCCGATC-s-T-3´	GGAATGTC
P37-D1	5´-CAAGCAGAAGACGGCATACGAGAT**AGACCGTA**GTGACTGGAGTTCA GACGTGTGCTCTTCCGATC-s-T-3´	TACGGTCT
P38-D2	5´-CAAGCAGAAGACGGCATACGAGAT**GATACTGG**GTGACTGGAGTTCAG ACGTGTGCTCTTCCGATC-s-T-3´	CCAGTATC

INDEX PRIMER	INDEX PRIMER SEQUENCE	EXPECTED INDEX SEQUENCE READ
P39-D3	5´-CAAGCAGAAGACGGCATACGAGAT**TGCGTAGA**GTGACTGGAGTTCA GACGTGTGCTCTTCCGATC-s-T-3´	TCTACGCA
P40-D4	5´-CAAGCAGAAGACGGCATACGAGAT**TCGGTTAC**GTGACTGGAGTTCAG ACGTGTGCTCTTCCGATC-s-T-3´	GTAACCGA
P41-D5	5´-CAAGCAGAAGACGGCATACGAGAT**ATGACGTC**GTGACTGGAGTTCAG ACGTGTGCTCTTCCGATC-s-T-3´	GACGTCAT
P42-D6	5´-CAAGCAGAAGACGGCATACGAGAT**GCTGTAAG**GTGACTGGAGTTCA GACGTGTGCTCTTCCGATC-s-T-3´	CTTACAGC
P43-D7	5´-CAAGCAGAAGACGGCATACGAGAT**GCAATGGA**GTGACTGGAGTTCA GACGTGTGCTCTTCCGATC-s-T-3´	TCCATTGC
P44-D8	5´-CAAGCAGAAGACGGCATACGAGAT**ATCTCGCT**GTGACTGGAGTTCAG ACGTGTGCTCTTCCGATC-s-T-3´	AGCGAGAT
P45-D9	5´-CAAGCAGAAGACGGCATACGAGAT**GGCTATTG**GTGACTGGAGTTCAG ACGTGTGCTCTTCCGATC-s-T-3´	CAATAGCC
P46-D10	5´-CAAGCAGAAGACGGCATACGAGAT**GGTGTCTT**GTGACTGGAGTTCAG ACGTGTGCTCTTCCGATC-s-T-3´	AAGACACC
P47-D11	5´-CAAGCAGAAGACGGCATACGAGAT**TCAACTGG**GTGACTGGAGTTCA GACGTGTGCTCTTCCGATC-s-T-3´	CCAGTTGA
P48-D12	5´-CAAGCAGAAGACGGCATACGAGAT**CTTCACCA**GTGACTGGAGTTCAG ACGTGTGCTCTTCCGATC-s-T-3´	TGGTGAAG
P49-E1	5´-CAAGCAGAAGACGGCATACGAGAT**ACGGTCTT**GTGACTGGAGTTCAG ACGTGTGCTCTTCCGATC-s-T-3´	AAGACCGT
P50-E2	5´-CAAGCAGAAGACGGCATACGAGAT**TCTCGCAA**GTGACTGGAGTTCAG ACGTGTGCTCTTCCGATC-s-T-3´	TTGCGAGA
P51-E3	5´-CAAGCAGAAGACGGCATACGAGAT**GGAATTGC**GTGACTGGAGTTCA GACGTGTGCTCTTCCGATC-s-T-3´	GCAATTCC
P52-E4	5´-CAAGCAGAAGACGGCATACGAGAT**ACGGATTC**GTGACTGGAGTTCAG ACGTGTGCTCTTCCGATC-s-T-3´	GAATCCGT
P53-E5	5´-CAAGCAGAAGACGGCATACGAGAT**TTAAGCGG**GTGACTGGAGTTCA GACGTGTGCTCTTCCGATC-s-T-3´	CCGCTTAA
P54-E6	5´-CAAGCAGAAGACGGCATACGAGAT**TGCAGGTA**GTGACTGGAGTTCA GACGTGTGCTCTTCCGATC-s-T-3´	TACCTGCA
P55-E7	5´-CAAGCAGAAGACGGCATACGAGAT**CAATCGAC**GTGACTGGAGTTCAG ACGTGTGCTCTTCCGATC-s-T-3´	GTCGATTG
P56-E8	5´-CAAGCAGAAGACGGCATACGAGAT**GTGCCATA**GTGACTGGAGTTCAG ACGTGTGCTCTTCCGATC-s-T-3´	TATGGCAC
P57-E9	5´-CAAGCAGAAGACGGCATACGAGAT**TGTTCGAG**GTGACTGGAGTTCA GACGTGTGCTCTTCCGATC-s-T-3´	CTCGAACA
P58-E10	5´-CAAGCAGAAGACGGCATACGAGAT**TGGAGTTG**GTGACTGGAGTTCA GACGTGTGCTCTTCCGATC-s-T-3´	CAACTCCA

NEBNext Index/Universal Primer Mix (Cont.)

INDEX PRIMER	INDEX PRIMER SEQUENCE	EXPECTED INDEX SEQUENCE READ
P59-E11	5´-CAAGCAGAAGACGGCATACGAGAT**ACGATGAC**GTGACTGGAGTTCA GACGTGTGCTCTTCCGATC-s-T-3´	GTCATCGT
P60-E12	5´-CAAGCAGAAGACGGCATACGAGAT**TGATGTCC**GTGACTGGAGTTCAG ACGTGTGCTCTTCCGATC-s-T-3´	GGACATCA
P61-F1	5´-CAAGCAGAAGACGGCATACGAGAT**TGAACCTG**GTGACTGGAGTTCA GACGTGTGCTCTTCCGATC-s-T-3´	CAGGTTCA
P62-F2	5´-CAAGCAGAAGACGGCATACGAGAT**CTTCGTTC**GTGACTGGAGTTCAG ACGTGTGCTCTTCCGATC-s-T-3´	GAACGAAG
P63-F3	5´-CAAGCAGAAGACGGCATACGAGAT**CTTCTGAG**GTGACTGGAGTTCAG ACGTGTGCTCTTCCGATC-s-T-3´	CTCAGAAG
P64-F4	5´-CAAGCAGAAGACGGCATACGAGAT**TGCTCATG**GTGACTGGAGTTCAG ACGTGTGCTCTTCCGATC-s-T-3´	CATGAGCA
P65-F5	5´-CAAGCAGAAGACGGCATACGAGAT**AGTTCGTC**GTGACTGGAGTTCAG ACGTGTGCTCTTCCGATC-s-T-3´	GACGAACT
P66-F6	5´-CAAGCAGAAGACGGCATACGAGAT**TAGCGTCT**GTGACTGGAGTTCAG ACGTGTGCTCTTCCGATC-s-T-3´	AGACGCTA
P67-F7	5´-CAAGCAGAAGACGGCATACGAGAT**GGCGTTAT**GTGACTGGAGTTCAG ACGTGTGCTCTTCCGATC-s-T-3´	ATAACGCC
P68-F8	5´-CAAGCAGAAGACGGCATACGAGAT**GGTGATTC**GTGACTGGAGTTCAG ACGTGTGCTCTTCCGATC-s-T-3´	GAATCACC
P69-F9	5´-CAAGCAGAAGACGGCATACGAGAT**AACTTGCC**GTGACTGGAGTTCAG ACGTGTGCTCTTCCGATC-s-T-3´	GGCAAGTT
P70-F10	5´-CAAGCAGAAGACGGCATACGAGAT**GCAAGATC**GTGACTGGAGTTCA GACGTGTGCTCTTCCGATC-s-T-3´	GATCTTGC
P71-F11	5´-CAAGCAGAAGACGGCATACGAGAT**TCGCATTG**GTGACTGGAGTTCAG ACGTGTGCTCTTCCGATC-s-T-3´	CAATGCGA
P72-F12	5´-CAAGCAGAAGACGGCATACGAGAT**TGTACACC**GTGACTGGAGTTCAG ACGTGTGCTCTTCCGATC-s-T-3´	GGTGTACA
P73-G1	5´-CAAGCAGAAGACGGCATACGAGAT**AGCTCCTA**GTGACTGGAGTTCAG ACGTGTGCTCTTCCGATC-s-T-3´	TAGGAGCT
P74-G2	5´-CAAGCAGAAGACGGCATACGAGAT**GCAATTCG**GTGACTGGAGTTCAG ACGTGTGCTCTTCCGATC-s-T-3´	CGAATTGC
P75-G3	5´-CAAGCAGAAGACGGCATACGAGAT**CTTAGGAC**GTGACTGGAGTTCAG ACGTGTGCTCTTCCGATC-s-T-3´	GTCCTAAG
P76-G4	5´-CAAGCAGAAGACGGCATACGAGAT**GTCCTAAG**GTGACTGGAGTTCAG ACGTGTGCTCTTCCGATC-s-T-3´	CTTAGGAC
P77-G5	5´-CAAGCAGAAGACGGCATACGAGAT**AACGTGGA**GTGACTGGAGTTCA GACGTGTGCTCTTCCGATC-s-T-3´	TCCACGTT
P78-G6	5´-CAAGCAGAAGACGGCATACGAGAT**CTGTGTTG**GTGACTGGAGTTCAG ACGTGTGCTCTTCCGATC-s-T-3´	CAACACAG

INDEX PRIMER	INDEX PRIMER SEQUENCE	EXPECTED INDEX SEQUENCE READ
P79-G7	5´-CAAGCAGAAGACGGCATACGAGAT**GTTAAGGC**GTGACTGGAGTTCA GACGTGTGCTCTTCCGATC-s-T-3´	GCCTTAAC
P80-G8	5´-CAAGCAGAAGACGGCATACGAGAT**CACCTTAC**GTGACTGGAGTTCAG ACGTGTGCTCTTCCGATC-s-T-3´	GTAAGGTG
P81-G9	5´-CAAGCAGAAGACGGCATACGAGAT**TGGTAGCT**GTGACTGGAGTTCAG ACGTGTGCTCTTCCGATC-s-T-3´	AGCTACCA
P82-G10	5´-CAAGCAGAAGACGGCATACGAGAT**CAGTGAAG**GTGACTGGAGTTCA GACGTGTGCTCTTCCGATC-s-T-3´	CTTCACTG
P83-G11	5´-CAAGCAGAAGACGGCATACGAGAT**GTTCAACC**GTGACTGGAGTTCAG ACGTGTGCTCTTCCGATC-s-T-3´	GGTTGAAC
P84-G12	5´-CAAGCAGAAGACGGCATACGAGAT**TGGCTATC**GTGACTGGAGTTCAG ACGTGTGCTCTTCCGATC-s-T-3´	GATAGCCA
P85-H1	5´-CAAGCAGAAGACGGCATACGAGAT**CTGGAGTA**GTGACTGGAGTTCA GACGTGTGCTCTTCCGATC-s-T-3´	TACTCCAG
P86-H2	5´-CAAGCAGAAGACGGCATACGAGAT**TCTCTTCC**GTGACTGGAGTTCAG ACGTGTGCTCTTCCGATC-s-T-3´	GGAAGAGA
P87-H3	5´-CAAGCAGAAGACGGCATACGAGAT**TCTAACGC**GTGACTGGAGTTCAG ACGTGTGCTCTTCCGATC-s-T-3´	GCGTTAGA
P88-H4	5´-CAAGCAGAAGACGGCATACGAGAT**GGTCAGAT**GTGACTGGAGTTCA GACGTGTGCTCTTCCGATC-s-T-3´	ATCTGACC
P89-H5	5´-CAAGCAGAAGACGGCATACGAGAT**CTCTGGTT**GTGACTGGAGTTCAG ACGTGTGCTCTTCCGATC-s-T-3´	AACCAGAG
P90-H6	5´-CAAGCAGAAGACGGCATACGAGAT**TGTGGTAC**GTGACTGGAGTTCAG ACGTGTGCTCTTCCGATC-s-T-3´	GTACCACA
P91-H7	5´-CAAGCAGAAGACGGCATACGAGAT**CCTATACC**GTGACTGGAGTTCAG ACGTGTGCTCTTCCGATC-s-T-3´	GGTATAGG
P92-H8	5´-CAAGCAGAAGACGGCATACGAGAT**TTCTCTCG**GTGACTGGAGTTCAG ACGTGTGCTCTTCCGATC-s-T-3´	CGAGAGAA
P93-H9	5´-CAAGCAGAAGACGGCATACGAGAT**GTATGCTG**GTGACTGGAGTTCAG ACGTGTGCTCTTCCGATC-s-T-3´	CAGCATAC
P94-H10	5´-CAAGCAGAAGACGGCATACGAGAT**AAGTCGAG**GTGACTGGAGTTCA GACGTGTGCTCTTCCGATC-s-T-3´	CTCGACTT
P95-H11	5´-CAAGCAGAAGACGGCATACGAGAT**AACCGAAG**GTGACTGGAGTTCA GACGTGTGCTCTTCCGATC-s-T-3´	CTTCGGTT
P96-H12	5´-CAAGCAGAAGACGGCATACGAGAT**TGTTGTGG**GTGACTGGAGTTCA GACGTGTGCTCTTCCGATC-s-T-3´	CCACAACA

Quality Control Assays

16-Hour Incubation: 50 µl reactions containing 1 µl NEBNext Index/Universal Primer Mix and 1 µg of HindIII digested Lambda DNA incubated for 16 hours at 37°C results in no detectable non-specific nuclease degradation as determined by agarose gel electrophoresis. 50 µl reactions containing NEBNext Index/Universal Primer Mix for Illumina and 1 µg of T3 DNA incubated for 16 hours at 37°C results in no detectable non-specific nuclease degradation as determined by agarose gel electrophoresis.

Endonuclease Activity: Incubation of a 50 µl reaction containing 1 µl NEBNext Index/Universal Primer Mix with 1 µg of ϕX174 RF I supercoiled DNA for 4 hours at 37°C results in less than 10% conversion to RF II (nicked molecules) as determined by agarose gel electrophoresis.

RNase Activity: Incubation of a 10 µl reaction containing 1 µl NEBNext Index/Universal Primer Mix with 40 ng of RNA transcript for 16 hours at 37°C resulted in no detectable degradation of RNA as determined by gel electrophoresis.

Phosphatase Activity: Incubation of NEBNext Index/Universal Primer Mix in protein phosphatase assay buffer (1 M diethanolamine @ pH 9.8 and 0.5 mM $MgCl_2$) containing 2.5 mM *p*-nitrophenyl phosphate at 37°C for 4 hours yields no detectable *p*-nitrophenylene anion as determined by spectrophotometric analysis at 405 nm.

Lot Controlled

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

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

CANADA
New England Biolabs, Ltd.
Telephone: (905) 665-4632
Toll Free: 1-800-387-1095
Fax: (905) 665-4635
Fax Toll Free: 1-800-563-3789
e-mail: info.ca@neb.com
www.neb.ca

CHINA, PEOPLE'S REPUBLIC
New England Biolabs (Beijing), Ltd.
Telephone: 010-82378265/82378266
Fax: 010-82378262
e-mail: info@neb-china.com
www.neb-china.com

FRANCE
New England Biolabs France
Free Call: 0800-100-632
Free Fax: 0800-100-610
e-mail: info.fr@neb.com
www.neb-online.fr

GERMANY & AUSTRIA
New England Biolabs GmbH
Telephone: +49/(0)69/305 23140
Free Call: 0800/246 5227 (Germany)
Free Call: 00800/246 52277 (Austria)
Fax: +49/(0)69/305 23149
Free Fax: 0800/246 5229 (Germany)
e-mail: info.de@neb.com
www.neb-online.de

JAPAN
New England Biolabs Japan, Inc.
Telephone: +81 (0)3 5669 6191
Fax: +81 (0)3 5669 6192
e-mail: info.jp@neb.com
www.nebj.jp

SINGAPORE
New England Biolabs Pte. Ltd.
Telephone: +65 63859623
Fax: +65 63859617
e-mail: sales.sg@neb.com
www.neb.sg

UNITED KINGDOM
New England Biolabs (UK) Ltd.
Telephone: (01462) 420616
Call Free: 0800 318486
Fax: (01462) 421057
Fax Free: 0800 435682
e-mail: info.uk@neb.com
www.neb.uk.com

NEW ENGLAND BioLabs® Inc.