NEBNext® Multiplex Oligos for Illumina® (Methylated Adaptor, Index Primers Set 1)

NEB #E7535S/L 24/96 reactions

Version 5.0_2/20

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The NEBNext Multiplex Oligos for Illumina (Methylated Adaptor, Index Primers Set 1) Includes:

The volumes provided are sufficient for preparation of up to 24 reactions (NEB #E7535S) and 96 reactions (NEB #E7535L). All reagents should be stored at –20°C. Colored bullets represent the color of the cap of the tube containing the reagent.

- (red) NEBNext Methylated Adaptor for Illumina
- (red) USER® Enzyme
- (blue) NEBNext Universal PCR Primer for Illumina
- (blue) NEBNext Index 1 Primer for Illumina
- (blue) NEBNext Index 2 Primer for Illumina
- (blue) NEBNext Index 3 Primer for Illumina
- (blue) NEBNext Index 4 Primer for Illumina
- (blue) NEBNext Index 5 Primer for Illumina
- (blue) NEBNext Index 6 Primer for Illumina
- (blue) NEBNext Index 7 Primer for Illumina
- (blue) NEBNext Index 8 Primer for Illumina
- (blue) NEBNext Index 9 Primer for Illumina
- (blue) NEBNext Index 10 Primer for Illumina
- (blue) NEBNext Index 11 Primer for Illumina
- (blue) NEBNext Index 12 Primer for Illumina

Required Materials Not Included

Compatible with the following library preparation kits:

- NEBNext Ultra™ DNA Library Prep Kit for Illumina (NEB #E7370)
- NEBNext DNA Library Prep Master Mix Set for Illumina (NEB #E6040)
- NEB EpiMark® Bisulfite Conversion Kit (NEB #E3318)

Required Materials Not Included (continued)

- NEB EpiMark Hot Start *Taq* DNA Polymerase (NEB #M0490)
- 10 mM dNTPs
- 80% Ethanol (freshly prepared)
- Magnetic rack or plate (e.g., NEBNext Magnetic Separation Rack (NEB #S1515), Alpaqua® 96S Super Magnet Plate (#A001322), or equivalent) for use with NEB#E7370, plus compatible tubes or plates
- DNA LoBind® Tubes (Eppendorf®)/PCR Plates for use with NEB#E6040, plus compatible magnet such as NEB# E1506
- Agencourt® AMPure® XP Beads (Beckman Coulter, Inc. #A63881)
- Bioanalyzer® (Agilent Technologies, Inc.)
- Nuclease-free Water
- 0.1X TE (or 10 mM Tris-HCl, pH 7.5-8.0)

Applications

The NEBNext Multiplex Oligos for Illumina (Methylated Adaptor, Index Primers Set 1) contains adaptors and primers that are ideally suited for multiplex sample preparation for whole genome bisulfite 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 (Methylated Adaptor, Index Primers Set 1) 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 a genomic DNA library on a Ion Torrent PGM.

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.

Section 1
Protocol for use with NEBNext Ultra DNA Library Prep Kit for Illumina (NEB #E7370)

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.

Stopping points in the protocol.

●

Colored bullets indicate the cap color or label stripe of the reagent to be added to a reaction.

Starting Material: 100 ng–1 µg of fragmented DNA.

Note: The starting material recommendations apply for use with bisulfite conversion.

1.1. NEBNext End Prep

1.1.1. Mix the following components in a sterile nuclease-free tube:

COMPONENT	VOLUME
● (green) End Prep Enzyme Mix	3.0 µl
● (green) End Repair Reaction Buffer (10X)	6.5 µl
Fragmented DNA	55.5 µl
Total Volume	65 µl

1.1.2. Mix by pipetting followed by a quick spin to collect all liquid from the sides of the tube.

1.1.3. Place in a thermocycler, with the heated lid on, and run the following program:

30 minutes @ 20°C

30 minutes @ 65°C

Hold at 4°C

1.2 Perform Adaptor Ligation

1.2.1. Add the following components directly to the End Prep reaction mixture and mix well:

COMPONENT	VOLUME
● (red) NEBNext Methylated Adaptor for Illumina (15 µM)	2.5 µl
● (red) Blunt/TA Ligase Master Mix	15 µl
● (red) Ligation enhancer	1 µl
Total Volume	83.5 µl

1.2.2. Mix by pipetting followed by a quick spin to collect all liquid from the sides of the tube.

1.2.3. Incubate for 15 minutes at 20°C in a thermal cycler.

1.2.4. Add 3 µl ● USER enzyme directly to the ligation reaction mixture from Step 1.2.3.

1.2.5. Mix well and incubate for 15 minutes at 37°C.

Note: USER treatment is required before bisulfite conversion.

1.3. Purify the Ligation Reaction Using Agencourt AMPure XP Beads

1.3.1. Vortex Agencourt AMPure XP Beads to resuspend.

1.3.2. Add 86.5 µl of resuspended Agencourt AMPure XP Beads to the ligation reaction (~ 86.5 µl). Mix well by pipetting up and down at least 10 times.

1.3.3. Incubate for 5 minutes at room temperature.

1.3.4. Put the tube on an appropriate magnetic stand to separate the beads from the supernatant. After the solution is clear (about 5 minutes), carefully remove and discard the supernatant. Be careful not to disturb the beads that contain DNA targets. **Caution: do not discard the beads.**

1.3.5. Add 200 µl of freshly prepared 80% ethanol to the tube while in the magnetic stand. Incubate at room temperature for 30 seconds, and then carefully remove and discard the supernatant.

1.3.6. Repeat Step 1.3.5 once.

1.3.7. Air dry the beads for 5 minutes while the tube is on the magnetic stand with the lid open. **Caution: Do not overdry the beads. This may result in lower recovery of DNA target.**

1.3.8. Elute DNA target from the beads into 13 µl nuclease free water. Mix well by pipetting up and down. Quickly spin the tube and place it on the appropriate magnetic stand to separate the beads from the supernatant. After the solution is clear, (about 5 minutes) carefully transfer 10 µl to a new tube for bisulfite conversion.

1.4. Perform bisulfite conversion using the EpiMark Bisulfite Conversion Kit (NEB #E3318) or other equivalent bisulfite conversion kit. Please follow the specific instructions from the corresponding kit.

1.5 Cleanup DNA Eluent of Bisulfite Conversion Kit

We strongly recommend cleanup of DNA eluent using Agencourt AMPure XP beads to remove any potential carryover inhibitors from the bisulfite conversion kit.

1.5.1. Vortex Agencourt AMPure XP Beads to resuspend.

1.5.2. Add resuspended Agencourt AMPure XP Beads to the DNA eluent at 5:1 ratio, i.e., 50 µl beads per 10 µl eluent. Mix well by pipetting up and down at least 10 times. Incubate for 5 minutes at room temperature.

1.5.3. Put the tube on an appropriate magnetic stand to separate the beads from the supernatant. After the solution is clear (about 5 minutes), carefully remove and discard the supernatant. Be careful not to disturb the beads that contain DNA targets.

1.5.4. Add 200 µl of freshly prepared 80% ethanol to the tube while in the magnetic stand. Incubate at room temperature for 30 seconds, and then carefully remove and discard the supernatant.

1.5.5. Repeat Step 1.5.4 once.

1.5.6. Air dry the beads for 5 minutes while the tube is on the magnetic stand with the lid open. **Caution: Do not overdry the beads. This may result in lower recovery of DNA target.**

1.5.7. Elute DNA target from the beads into 25 µl 0.1X TE or 10 mM Tris-HCl, pH 8.0. Mix well by pipetting up and down. Quickly spin the tube and incubate for 2 minutes at room temperature

1.5.8. Place the tube on an appropriate magnetic stand to separate the beads from the supernatant. After the solution is clear, (about 5 minutes) carefully transfer 20 µl to a sterile PCR tube for amplification.

1.6 PCR Amplification

Amplify bisulfite-converted libraries with a DNA polymerase that can read through uracils. Please refer to the manufacturer's instructions for cycling conditions.

Note: NEBNext Q5 Hot Start HiFi PCR Master Mix and NEBNext High-Fidelity 2X PCR Master Mix cannot be used for this application.

1.6. PCR Conditions using EpiMark Hot Start *Taq* DNA Polymerase (NEB #M0490)

1.6.1. Mix the following in a sterile PCR tube:

COMPONENT	VOLUME
Adaptor Ligated, bisulfite-converted DNA	20 µl
• (blue) NEBNext Universal PCR Primer	2.5 µl
• (blue) NEBNext Index (X) Primer	2.5 µl
5X EpiMark Hot Start *Taq* Reaction Buffer	10 µl
10 mM dNTPs (each)	1 µl
EpiMark Hot Start *Taq* (2 units/µl)	0.25 µl
Sterile water	13.75 µl
Total Volume	50 µl

1.6.2. PCR cycling conditions:

CYCLE STEP	TEMP	TIME	CYCLES
Denaturation	95°C	30 seconds	1
Cycling	95°C	15 seconds	13–15
	61°C	30 seconds	
	68°C	30 seconds	
Final Extension	68°C	5 minutes	1
Hold	4°C	∞	

Note: PCR conditions may vary if you are not using EpiMark Hot Start Taq DNA Polymerase.

1.6.3. Proceed to purify the PCR Reaction Using Agencourt AMPure XP Beads in Section 1.7.

1.7 Purify the PCR Reaction Using Agencourt AMPure XP Beads

1.7.1. Vortex Agencourt AMPure XP Beads to resuspend.

1.7.2. Add 45 µl (0.9X) of resuspended Agencourt AMPure XP Beads to the PCR reaction (~ 50 µl). Mix well by pipetting up and down at least 10 times.

1.7.3. Incubate for 5 minutes at room temperature.

1.7.4. Put the tube on an appropriate magnetic stand to separate the beads from the supernatant. After the solution is clear (about 5 minutes), carefully remove and discard the supernatant. Be careful not to disturb the beads that contain DNA targets.

1.7.5. Add 200 µl of freshly prepared 80% ethanol to the tube while in the magnetic stand. Incubate at room temperature for 30 seconds, and then carefully remove and discard the supernatant.

1.7.6. Repeat Step 1.7.5 once.

1.7.7. Air dry the beads for 5 minutes while the tube is on the magnetic stand with the lid open. **Caution: Do not overdry the beads. This may result in lower recovery of DNA target.**

1.7.8. Elute the DNA target from the beads into 28 µl 0.1X TE (or 10 mM Tris-HCl, pH 8.0). Mix well by pipetting up and down at least 10 times. Quickly spin the tube and incubate for 2 minutes at room temperature.

1.7.9. Place the tube on an appropriate magnetic stand to separate the beads from the supernatant. After the solution is clear (about 5 minutes), carefully transfer 25 µl of supernatant to a new tube.

1.7.10. Add 25 µl of resuspended Agencourt AMPure XP Beads to the supernatant (25 µl). Repeat Steps 1.7.3–1.7.7 once.

1.7.11. Elute DNA in 33 µl 0.1X TE. Place the tube on an appropriate magnetic stand to separate the beads from the supernatant. Mix well by pipetting up and down at least 10 times. Quickly spin the tube and incubate for 2 minutes at room temperature

1.7.12. Place the tube on an appropriate magnetic stand to separate the beads from the supernatant. After the solution is clear (about 5 minutes), carefully transfer 30 µl supernatant to a new tube. Store libraries at –20°C.

1.7.13. Check the size distribution on an Agilent Bioanalyzer high sensitivity chip.

Section 2
Protocol for use with NEBNext DNA Library Prep Master Mix Set for Illumina (NEB #E6040)

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.

Stopping points in the protocol.

●

Colored bullets indicate the cap color or label stripe of the reagent to be added to a reaction.

Starting Material: 1–5 µg of fragmented DNA.

Note: The starting material recommendations apply for use with bisulfite conversion.

2.1. End Repair of Fragmented DNA

2.1.1. Mix the following components in a sterile microfuge tube:

COMPONENT	VOLUME
Fragmented DNA	1–85 µl
● (green) NEBNext End Repair Reaction Buffer (10X)	10 µl
● (green) NEBNext End Repair Enzyme Mix	5 µl
Sterile H_2O	variable
Total Volume	100 µl

2.1.2. Incubate in a thermal cycler for 30 minutes at 20°C.

2.2 Cleanup Using AMPure XP Beads

2.2.1. Vortex AMPure XP beads to resuspend.

2.2.2. Add 160 µl (1.6X) of resuspended AMPure XP beads to the end repair reaction. Mix thoroughly on a vortex mixer or by pipetting up and down at least 10 times.

2.2.3. Incubate for 5 minutes at room temperature.

2.2.4. Put the tube/PCR plate on an appropriate magnetic stand to separate beads from supernatant. After the solution is clear (about 5 minutes), carefully remove and discard the supernatant. Be careful not to disturb the beads that contain the DNA targets.

2.2.5. Add 200 µl of 80% freshly prepared ethanol to the tube/PCR plate while in the magnetic stand. Incubate at room temperature for 30 seconds, and then carefully remove and discard the supernatant.

2.2.6. Repeat Step 2.2.5 once.

2.2.7. Air dry beads for 5 minutes while the tube/PCR plate is on the magnetic stand with the lid open. **Caution: Do not overdry the beads. This may result in lower recovery of DNA target.**

2.2.8. Elute DNA target by adding 50 µl 0.1X TE or 10 mM Tris-HCl to the beads. Mix well on a vortex mixer or by pipetting up and down and incubate for 2 minutes at room temperature.

2.2.9. Put the tube/PCR plate in the magnetic stand until the solution is clear without disturbing the bead pellet, carefully transfer 42 µl of the supernatant to a fresh, sterile microfuge tube.

2.3. dA-Tailing of End Repaired DNA

2.3.1. Mix the following components in a sterile microfuge tube:

COMPONENT	VOLUME
End Repaired, Blunt DNA	42 µl
● (yellow) NEBNext dA-Tailing Reaction Buffer (10X)	5 µl
● (yellow) Klenow Fragment (3′→ 5′ exo⁻)	3 µl
Total Volume	50 µl

2.3.2. Incubate in a thermal cycler for 30 minutes at 37°C.

2.4 Cleanup using AMPure XP Beads

2.4.1. Vortex AMPure XP beads to resuspend.

2.4.2. Add 90 µl (1.8X) of resuspended AMPure XP beads to the dA-tailing reaction. Mix thoroughly on a vortex mixer or by pipetting up and down at least 10 times.

2.4.3. Incubate for 5 minutes at room temperature.

2.4.4. Put the tube/PCR plate on an appropriate magnetic stand to separate beads from supernatant. After the solution is clear (about 5 minutes), carefully remove and discard the supernatant. Be careful not to disturb the beads that contain the DNA targets.

2.4.5. Add 200 µl of 80% freshly prepared ethanol to the tube/PCR plate while in the magnetic stand. Incubate at room temperature for 30 seconds, and then carefully remove and discard the supernatant.

2.4.6. Repeat Step 2.4.5 once.

2.4.7. Air dry beads for 5 minutes while the tube/PCR plate is on the magnetic stand with the lid open. **Caution: Do not overdry the beads. This may result in lower recovery of DNA target.**

2.4.8. Elute DNA target by adding 30 µl 0.1X TE or 10 mM Tris-HCl to the beads. Mix well on a vortex mixer or by pipetting up and down and incubate for 2 minutes at room temperature.

2.4.9. Put the tube/PCR plate in the magnetic stand until the solution is clear without disturbing the bead pellet, carefully transfer 25 µl of the supernatant to a fresh, sterile microfuge tube.

2.5 Adaptor Ligation of dA-Tailed DNA

2.5.1. Mix the following components in a sterile microfuge tube:

COMPONENT	VOLUME
dA-Tailed DNA	25 µl
● (red) Quick Ligation Reaction Buffer (5X)	10 µl
● (red) Methylated Adaptor for Illumina	10 µl
● (red) Quick T4 DNA Ligase	5 µl
Total Volume	50 µl

2.5.2. Incubate in a thermal cycler for 15 minutes at 20°C.

2.5.3. Add 3 µl ● USER enzyme directly to the ligation reaction mixture from Step 2.5.2.

2.5.4. Mix well and incubate for 15 minutes at 37°C.

Note: USER treatment is required before bisulfite conversion.

2.6 Purify the Ligation Reaction Using Agencourt AMPure XP Beads

2.6.1. Vortex Agencourt AMPure XP Beads to resuspend.

2.6.2. Add 53 µl of the resuspended Agencourt AMPure XP Beads to the ligation reaction (~ 53 µl). Mix well by pipetting up and down at least 10 times.

2.6.3. Incubate for 5 minutes at room temperature.

2.6.4. Put the tube on an appropriate magnetic stand to separate the beads from the supernatant. After the solution is clear (about 5 minutes), carefully remove and discard the supernatant. Be careful not to disturb the beads that contain DNA targets.

2.6.5. Add 200 µl of freshly prepared 80% ethanol to the tube while in the magnetic stand. Incubate at room temperature for 30 seconds, and then carefully remove and discard the supernatant.

2.6.6. Repeat Step 2.6.5 once.

2.6.7. Air dry the beads for 5 minutes while the tube is on the magnetic stand with the lid open. **Caution: Do not overdry the beads. This may result in lower recovery of DNA target.**

2.6.8. Elute the DNA target from the beads into 13 µl nuclease free water. Mix well by pipetting up and down 10 times. Quickly spin the tube and incubate for 2 minutes at room temperature.

2.6.9. Place the tube on an appropriate magnetic stand to separate the beads from the supernatant. After the solution is clear (about 5 minutes) carefully transfer 10 µl to a new LoBind DNA tube for bisulfite conversion.

2.7 Perform bisulfite conversion using the EpiMark Bisulfite Conversion Kit (NEB #E3318) or other equivalent bisulfite conversion kit. Please follow the specific instructions from the corresponding kit.

2.8 Cleanup DNA Eluent of Bisulfite Conversion Kit

We strongly recommend cleanup of DNA eluent using Agencourt AMPure XP beads to remove any potential carryover inhibitors from the bisulfite conversion kit.

2.8.1. Vortex Agencourt AMPure XP Beads to resuspend.

2.8.2. Add the resuspended Agencourt AMPure XP Beads to the DNA eluent at 5:1 ratio, i.e., 50 µl beads per 10 µl eluent. Mix well by pipetting up and down at least 10 times. Incubate for 5 minutes at room temperature.

2.8.3. Put the tube on an appropriate magnetic stand to separate the beads from the supernatant. After the solution is clear (about 5 minutes), carefully remove and discard the supernatant. Be careful not to disturb the beads that contain DNA targets.

2.8.4. Add 200 µl of freshly prepared 80% ethanol to the tube while in the magnetic stand. Incubate at room temperature for 30 seconds, and then carefully remove and discard the supernatant.

2.8.5. Repeat Step 2.8.4 once.

2.8.6. Air dry the beads for 5 minutes while the tube is on the magnetic stand with the lid open. Caution: Do not overdry the beads. This may result in lower recovery of DNA target.

2.8.7. Elute DNA target from the beads into 25 µl 0.1X TE or (10 mM Tris-HCl). Mix well by pipetting up and down 10 times. Quickly spin the tube and incubate for 2 minutes at room temperature.

2.8.8. Place the tube on an appropriate magnetic stand to separate the beads from the supernatant. After the solution is clear (about 5 minutes) carefully transfer 20 µl to a sterile PCR tube for amplification.

2.9 PCR Amplification

Amplify bisulfite-converted libraries with a DNA polymerase that can read through uracils. Please refer to the manufacturer's instructions for cycling conditions.

Note: NEBNext Q5 Hot Start HiFi PCR Master Mix and NEBNext High-Fidelity 2X PCR Master Mix cannot be used for this application.

2.9. PCR Conditions using EpiMark Hot Start *Taq* DNA Polymerase (NEB #M0490)

2.9.1. Mix the following in a sterile PCR tube:

COMPONENT	VOLUME
Adaptor Ligated, bisulfite-converted DNA	20 µl
• (blue) NEBNext Universal PCR Primer	2.5 µl
• (blue) NEBNext Index (X) Primer	2.5 µl
5X EpiMark Hot Start *Taq* Reaction Buffer	10 µl
10 mM dNTPs (each)	1 µl
EpiMark Hot Start *Taq* (2 units/µl)	0.25 µl
Sterile water	13.75 µl
Total Volume	50 µl

2.9.2. PCR cycling conditions:

CYCLE STEP	TEMP	TIME	CYCLES
Denaturation	95°C	30 seconds	1
Cycling	95°C	15 seconds	13–15
	61°C	30 seconds	
	68°C	30 seconds	
Final Extension	68°C	5 minutes	1
Hold	4°C	∞	

Note: PCR conditions may vary if you are not using EpiMark Hot Start Taq DNA Polymerase.

2.9.3. Proceed to purify the PCR Reaction Using Agencourt AMPure XP Beads in Section 2.10.

2.10 Cleanup Using Agencourt AMPure XP Beads

2.10.1. Vortex Agencourt AMPure XP Beads to resuspend.

2.10.2. Add 45 µl (0.9X) of resuspended Agencourt AMPure XP Beads to the PCR reaction (~ 50 µl). Mix well by pipetting up and down at least 10 times.

2.10.3. Incubate for 5 minutes at room temperature.

2.10.4. Put the tube on an appropriate magnetic stand to separate the beads from the supernatant. After the solution is clear (about 5 minutes), carefully remove and discard the supernatant. Be careful not to disturb the beads that contain DNA targets.

2.10.5. Add 200 µl of freshly prepared 80% ethanol to the tube while in the magnetic stand. Incubate at room temperature for 30 seconds, and then carefully remove and discard the supernatant.

2.10.6. Repeat Step 2.10.5 once.

2.10.7. Air dry the beads for 5 minutes while the tube is on the magnetic stand with the lid open. **Caution: Do not overdry the beads. This may result in lower recovery of DNA target.**

2.10.8. Elute DNA target from the beads into 28 µl 0.1X TE (or 10 mM Tris-HCl). Mix well by pipetting up and down at least 10 times. Quickly spin the tube.

2.10.9. Incubate for 2 minutes at room temperature. Place the tube on an appropriate magnetic stand to separate the beads from the supernatant. After the solution is clear (about 5 minutes) carefully transfer 25 µl of supernatant to a new tube.

2.10.10. Add 25 µl resuspended Agencourt AMPure XP Beads to the supernatant (25 µl). Mix well by pipetting up and down at least 10 times. Repeat Step 2.10.3–2.10.7 once.

2.10.11. Elute DNA in 33 µl in 0.1X TE (or 10 mM Tris-HCl, pH 8.0). Place the tube on an appropriate magnetic stand to separate the beads from the supernatant. Mix well by pipetting up and down 10 times. Quickly spin the tube and incubate for 2 minutes at room temperature.

2.10.12. Place the tube on an appropriate magnetic stand to separate the beads from the supernatant. After the solution is clear (about 5 minutes), carefully transfer 30 µl supernatant to a new tube. Store libraries at –20°C.

2.10.13. Check the size distribution on an Agilent Bioanalyzer high sensitivity chip.

NEBNext Adaptors and Primers for Illumina

PRODUCT	INDEX PRIMER SEQUENCE	EXPECTED INDEX PRIMER SEQUENCE READ
NEBNext Index 1 Primer for Illumina	5´-CAAGCAGAAGACGGCATACGAGAT**CGTGAT**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC-s-T-3´	ATCACG
NEBNext Index 2 Primer for Illumina	5´-CAAGCAGAAGACGGCATACGAGAT**ACATCG**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC-s-T-3´	CGATGT
NEBNext Index 3 Primer for Illumina	5´-CAAGCAGAAGACGGCATACGAGAT**GCCTAA**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC-s-T-3´	TTAGGC
NEBNext Index 4 Primer for Illumina	5´-CAAGCAGAAGACGGCATACGAGAT**TGGTCA**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC-s-T-3´	TGACCA
NEBNext Index 5 Primer for Illumina	5´-CAAGCAGAAGACGGCATACGAGAT**CACTGT**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC-s-T-3´	ACAGTG
NEBNext Index 6 Primer for Illumina	5´-CAAGCAGAAGACGGCATACGAGAT**ATTGGC**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC-s-T-3´	GCCAAT
NEBNext Index 7 Primer for Illumina	5´-CAAGCAGAAGACGGCATACGAGAT**GATCTG**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC-s-T-3´	CAGATC
NEBNext Index 8 Primer for Illumina	5´-CAAGCAGAAGACGGCATACGAGAT**TCAAGT**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC-s-T-3´	ACTTGA
NEBNext Index 9 Primer for Illumina	5´-CAAGCAGAAGACGGCATACGAGAT**CTGATC**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC-s-T-3´	GATCAG
NEBNext Index 10 Primer for Illumina	5´-CAAGCAGAAGACGGCATACGAGAT**AAGCTA**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC-s-T-3´	TAGCTT
NEBNext Index 11 Primer for Illumina	5´-CAAGCAGAAGACGGCATACGAGAT**GTAGCC**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC-s-T-3´	GGCTAC
NEBNext Index 12 Primer for Illumina	5´-CAAGCAGAAGACGGCATACGAGAT**TACAAG**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC-s-T-3´	CTTGTA
NEBNext Methylated Adaptor for Illumina	5´-/PO_4/GAT /Me-dC/GG AAG AG/Me-dC/ A/Me-dC/A/Me-dC/ GT /Me-dC/TG AA/ Me-dC /T/Me-dC//Me-dC /AGT/Me-dC//dU/A /Me-dC/A/Me-dC /T/Me-dC/T TT/Me-dC/ /Me-dC/ /Me-dC/T A/Me-dC/A /Me-dC/ GA /Me-dC/G/Me-dC/ T/Me-dC/T T/Me-dC//Me-dC/ GAT /Me-dC/-s-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-s-T-3´	N/A

Where -s- indicates phosphorothioate bond.

Note: If fewer than 12 indexes are used in a lane for sequencing, it is recommended to use the following indexes:
Pool of 2 samples: Index #6 and 12
Pool of 3 samples: Index #4, 6 and 12
Pool of 6 samples: Index #2, 4, 5, 6, 7 and 12

Frequently Asked Questions (FAQs)

Q: *How is NEBNext Methlyated Adaptor (NEB #E7535) different from NEBNext adaptor for Illumina (NEB #E7335 & #E7350)?*

A. The sequences of NEBNext Methylated Adaptor and NEBNext Adaptor for Illumina are identical except that all cytosines in NEBNext adaptor are replaced with methylated cytosines. They both can be used to prepare libraries for Illumina sequencing, however, only the NEBNext Methylated Adaptor can be used to prepare bisulfite-converted libraries for Illumina sequencing.

Q: *What are the major applications of NEBNext Methylated Adaptor for Illumina?*

A. The major applications of NEBNext Methylated Adaptor for Illumina are to prepare libraries for whole-genome bisulfite sequencing or reduced representation bisulfite sequencing (RRBS) for methylation analysis on the Illumina platform.

Q: *Is library prep workflow different when NEBNext Methylated Adaptor is used?*

A. If the libraries are for whole genome sequencing or targeted resequencing, there is no change in library prep workflow when NEBNext Methylated Adaptor is used. However, if the libraries are for bisulfite sequencing, there are two important changes to the workflow: 1) A bisulfite treatment step is required after adaptor ligation/USER cleavage and before PCR amplification. There are many bisulfite conversion kits commercially available, including EpiMark Bisulfite Conversion Kit from New England Biolabs (NEB #E3318); and 2) NEBNext High-Fidelity 2X PCR Master Mix and other high fidelity DNA polymerases including Phusion DNA polymerase should not be used to amplify bisulfite converted, uracil-containing DNA libraries. Instead, the bisulfite converted DNA should be PCR-amplified using a DNA polymerase that can bypass uracil, for example, EpiMark Hot Start Taq DNA Polymerase from New England Biolabs (NEB #M0490).

Kit Components

NEB #E7535S Table of Components

NEB #	PRODUCT	VOLUME
E7536A	NEBNext Methylated Adaptor for Illumina	0.24 ml
E7338A	USER Enzyme	0.072 ml
E6861A	NEBNext Universal PCR Primer for Illumina	0.120 ml
E7311A	NEBNext Index 1 Primer for Illumina	0.010 ml
E7312A	NEBNext Index 2 Primer for Illumina	0.010 ml
E7313A	NEBNext Index 3 Primer for Illumina	0.010 ml
E7314A	NEBNext Index 4 Primer for Illumina	0.010 ml
E7315A	NEBNext Index 5 Primer for Illumina	0.010 ml
E7316A	NEBNext Index 6 Primer for Illumina	0.010 ml
E7317A	NEBNext Index 7 Primer for Illumina	0.010 ml
E7318A	NEBNext Index 8 Primer for Illumina	0.010 ml
E7319A	NEBNext Index 9 Primer for Illumina	0.010 ml
E7320A	NEBNext Index 10 Primer for Illumina	0.010 ml
E7321A	NEBNext Index 11 Primer for Illumina	0.010 ml
E7322A	NEBNext Index 12 Primer for Illumina	0.010 ml

NEB #E7535L Table of Components

NEB #	PRODUCT	VOLUME
E7536AA	NEBNext Methylated Adaptor for Illumina	0.96 ml
E7338AA	USER Enzyme	0.288 ml
E6861AA	NEBNext Universal PCR Primer for Illumina	0.480 ml
E7311AA	NEBNext Index 1 Primer for Illumina	0.040 ml
E7312AA	NEBNext Index 2 Primer for Illumina	0.040 ml
E7313AA	NEBNext Index 3 Primer for Illumina	0.040 ml
E7314AA	NEBNext Index 4 Primer for Illumina	0.040 ml
E7315AA	NEBNext Index 5 Primer for Illumina	0.040 ml
E7316AA	NEBNext Index 6 Primer for Illumina	0.040 ml
E7317AA	NEBNext Index 7 Primer for Illumina	0.040 ml
E7318AA	NEBNext Index 8 Primer for Illumina	0.040 ml
E7319AA	NEBNext Index 9 Primer for Illumina	0.040 ml
E7320AA	NEBNext Index 10 Primer for Illumina	0.040 ml
E7321AA	NEBNext Index 11 Primer for Illumina	0.040 ml
E7322AA	NEBNext Index 12 Primer for Illumina	0.040 ml

Revision History

REVISION #	DESCRIPTION	DATE
1.0	New Document	
2.0	Include protocol for changes in concentration of NEBNext SinglePlex and Multiplex Oligos for Illumina. Changed all AMPure Bead elutions to 0.1X TE or 10 mM Tris-HCl. Changed ratio of AMPure Beads to 0.9X in final clenup after PCR reaction. Added 2 minute incubation after eluting DNA from AMPure Beads. Change Index Primer and Universal Primer concentration from 25 µM to 10 µM. All primer part #'s have changed.	1/15
3.0	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
4.0	Add new list for “Required Materials not Included”. Delete Kit Specific Protocol list. Apply new format to this manual. Delete part B of Section 1.6 and 2.9.	5/19
5.0	Updated to new manual format.	2/20

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