# Apostle MiniMax Methyl Library Preparation Kit (for Illumina®) User Manual

MiniMax Methyl Library Preparation Module
MiniMax Methyl Stubby Adapter (UDI) Module (for Illumina®)

Thoroughly Read This Manual Before Operation.
For Research Use Only.
Version2.1

# Statement

For research use only. Not for use in diagnostic procedures.

This instruction is intended for use with the MiniMax Methyl Library Preparation Kit (for Illumina®).

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# Announcement

## Environment

- Temperature: 20-25°C; Humidity: 40-60%.
- The personal protective equipment should be equipped during the experiment period.

## Temperature Control

- Avoid repeated freezing and thawing.
- Unless otherwise specified, the preparation of enzymatic reaction should be carried out on ice/cold metal bath.
- Make sure the thermal cycler reaches the designated temperature before loading any reaction tube in.

## Avoid Cross-contamination

- Briefly centrifuge all the tubes to collect the contents to the bottom before opening the lids.
- Physically separate the laboratory space, equipment and consumables among different steps.
- Clean the lab area using 0.5% sodium hypochlorite once finishing the experiment.

## Others

- Make sure the regents are fully thawed and thoroughly mixed prior to use.
- Unless otherwise specified, all the centrifuge steps are conducted at room temperature (20-25°C).
- Unless otherwise specified, all mixing steps listed as "mix thoroughly" should be performed by either vortexing for 10 sec or pipetting up and down for 10 times, and then briefly centrifuge to collect the contents.

# Introduction

MiniMax Methyl Library Preparation Kit (for Illumina®) is designed for preparation of high-quality methylation sequencing (Methyl-Seq) libraries from double-stranded DNA (dsDNA) on Illumina platforms. This A-T ligation-based kit offers a stable and efficient Methyl-Seq library preparation solution for whole genome sequencing, as well as hybridization capture based targeted sequencing powered by Apostle.

This kit is suitable for multiple DNA samples, including gDNA, cfDNA and FFPE DNA. Besides, it is compatible with series of commercial conversion solution, with DNA input ranging from10-1,000 ng. All the components have been verified by quality control with a stable and remarkable performance.

# Kit Content

The MiniMax Methyl Library Preparation Kit (for Illumina®) consists of Library Preparation Module and Adapter Module. Both modules are required for library construction.

### Apostle MiniMax Methyl Library Preparation Module

| Catalog# | Item | Package/Storage |
|---|---|---|
| 1002501 | MiniMax Methyl Library Preparation Module, 24 rxn | Box1 / -25 ~ -15°C<br>Box2 / 2 ~ 8°C |
| 1002502 | MiniMax Methyl Library Preparation Module, 96 rxn | Box1 / -25 ~ -15°C<br>Box2 / 2 ~ 8°C |

| Package# | Component | Volume 1002501 24 rxn | Volume 1002502 96 rxn | Storage |
|---|---|---|---|---|
| Box1 | MiniMax End Prep Buffer | 180 μL | 690 μL | -12 ~ -52°C |
| | MiniMax End Prep Enzyme | 120 μL | 460 μL | -12 ~ -52°C |
| | MiniMax Ligation Buffer | 750 μL | 2×1,500 μL | -12 ~ -52°C |
| | MiniMax DNA Ligase | 60 μL | 230 μL | -12 ~ -52°C |
| | 2X HiFi-Methyl PCR Master Mix | 720 μL | 2×1,600 μL | -12 ~ -52°C |
| | Nuclease Free Water | 2.5 mL | 8 mL | 2 ~ 8°C |
| | TE Buffer | 1 mL | 4 mL | 2 ~ 8°C |
| Box2 | MiniMax SP Beads | 4 mL | 15 mL | 2 ~ 8°C |

### Apostle MiniMax Methyl Stubby Adapter (UDI) Module (for Illumina®)

| Catalog# | Item | Methyl Stubby Adapter Volume | UDI-Index Primer Mix # & Volume | Amplification Primer Mix II Volume | Storage |
|---|---|---|---|---|---|
| 1003331 | MiniMax Methyl Stubby Adapter (UDI) Module Set A1 (for Illumina®), 24 rxn | 60 µL | 1-12 & 12×15 µL (Tube) | 20 µL | -60 ~ -10°C |
| 1003341 | MiniMax Methyl Stubby Adapter (UDI) Module Set B1 (for Illumina®), 96 rxn | 230 µL | 1-24 & 24×25 µL (Tube) | 75 µL | -60 ~ -10°C |
| 1003342 | MiniMax Methyl Stubby Adapter (UDI) Module Set B2 (for Illumina®), 96 rxn | 230 µL | 25-48 & 24×25 µL (Tube) | 75 µL | -60 ~ -10°C |

# Equipment and Consumable

## Equipment

| Item | Description |
| --- | --- |
| Ultrasonicator | Covaris™ M220 Focused-ultrasonicator<br>or equivalent |
| Digital electrophoresis | Agilent 2100 Electrophoresis Bioanalyzer® system (Cat # G2939AA)<br>Agilent 2200 TapeStation system (Cat # G2965AA)<br>Bioptic Qsep100 capillary gel electrophoresis system<br>or equivalent |
| Pipettor | General laboratory supplier |
| Thermal cycler | General laboratory supplier |
| Benchtop centrifuge | General laboratory supplier |
| Microcentrifuge | General laboratory supplier |
| Vortex mixer | General laboratory supplier |
| Magnet stand | Thermo Fisher DynaMag™ -2 Magnet (Cat # 12321D)<br>Thermo Fisher DynaMag™ -96 Side Magnet(Cat # 12331D)<br>BORTHEE-96 Side Magnet (Cat # MAG-96-11)<br>or equivalent |
| Qubit fluorometer | Thermo Fisher Qubit™ 3.0 Fluorometer (Cat # Q33216)<br>or equivalent |
| Timer | General laboratory supplier |

## Consumable

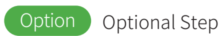
| Item | Description |
| --- | --- |
| Enzymatic conversion module | NEBNext® Enzymatic Methyl-seq Conversion Module (Cat #E7125S/L) |
| Bisulfite conversion module | QIAGEN EpiTect® Fast DNA Bisulfite Kit (Cat #59824/59826)<br>or equivalent |
| Absolute Ethanol | General laboratory supplier, analytical grade |
| 0.2 mL PCR Tube | Axygen MAXYMum Recovery™ PCR Tubes, 0.2 mL flat cap (Cat # PCR-02-L-C)<br>or equivalent |

# Workflow

| Workflow | gDNA/FFPE DNA | cfDNA | Duration |
|---|---|---|---|
| | Fragmentation | N/A | 30 min |
| STOP | | | |
| | MiniMax End Prep | | 60 min |
| | Methylated Adapter Ligation | | 15 min |
| | Post-ligation Cleanup | | 15 min |
| | Enzymatic Conversion<br>or<br>Option Bisulfite Conversion | | 6 hr<br>or<br>Option 2 hr |
| STOP | | | |
| | Library Amplification | | 20 min |
| | Post-amplification Cleanup | | 30 min |
| STOP | | | |

MiniMax Methyl Stubby Adapter

Safe Stopping Point


Optional Step

# Protocol

## Sample Requirement

**Sample Quality:** gDNA without serious degradation (tested by gel electrophoresis analysis); cfDNA without large fragment contamination; FFPE gDNA with average size > 500 bp and peak size at 15 Kb.

**Input Amount:** 10-1000 ng; The Qubit fluorometer or a similar fluorometric method is recommended for determining the dsDNA concentration.

**Sample Purity:** High quality DNA input, with $OD_{260}/OD_{280}$=1.8-2.0 and $OD_{260}/OD_{230}$=2.0-2.5.

### Step 1: Fragmentation

Please use the optimized protocol according to your fragmentation equipment and reagents. This kit has been validated on the DNA fragmented by the Covaris™ ultrasonicator.

**Note: Enzymatic fragmentation is forbidden in Methyl-Seq library preparation. For cfDNA, this step can be skipped.**

**Important notes:**

- Please adjust the size distribution according to the Illumina sequencing mode including SE75, SE150, PE150, PE300, etc.
- The DNA library with 200-250 bp on average is recommended for targeted sequencing.

**Safe Stopping Point:** The fragmented DNA can be stored at 2 ~ 8°C overnight or -25 ~ -15°C for longer.

### Step 2: End Repair and A-Tailing

1. Thaw MiniMax End Prep Buffer at room temperature. Mix thoroughly and centrifuge to collect the contents. Keep the tube on ice.
2. Thaw MiniMax End Prep Enzyme on ice. Briefly vortex and centrifuge to collect the contents.
3. Set up each reaction in a 0.2 mL PCR tube on ice as follows:

| | |
|---|---|
| Fragmented DNA/cfDNA | 40 µL |
| MiniMax End Prep Buffer | 6 µL |
| MiniMax End Prep Enzyme | 4 µL |
| **Total** | **50 µL** |

**Note: If the input DNA is less than 40 µL, add Nuclease Free Water to a total volume of 40 µL.**

4. Mix thoroughly and centrifuge to briefly collect the contents.
5. Incubate the tube in the thermal cycler programmed as follows, with the heated lid set at 75°C. Make sure the thermal cycler is stabilized at 20°C before loading.

| | |
|---|---|
| 20°C | 30 min |
| 65°C | 30 min |
| 10°C | Hold |

**Note: The heated lid should be left open during the incubation at 20°C.**

## Step 3: Adapter Ligation

1. Thaw MiniMax Ligation Buffer at room temperature, mix thoroughly and centrifuge to collect the contents. Keep the tube on ice.

**Note: The MiniMax Ligation Buffer is viscous. Please pipette slowly and carefully to ensure the expected volume.**

2. Thaw MiniMax DNA Ligase on ice. Briefly vortex and centrifuge to collect the contents.
3. Remove the tube in **Step 2** from the thermal cycler and set up each reaction on ice as follows:

| | |
|---|---|
| End Repair & A-Tailing Product | 50 µL |
| MiniMax Methyl Stubby Adapter (as appropriate) [1] | 2 µL |
| MiniMax Ligation Buffer [2] | 26 µL |
| MiniMax DNA Ligase [2] | 2 µL |
| **Total** | **80 µL** |

**Note [1]: Make sure to add MiniMax Methyl Stubby Adapter before other reagents to avoid the adapter self-ligation.**

**Note [2]: If working on multiple samples, prepare Ligation Master Mix in advance with MiniMax Ligation Buffer and MiniMax DNA Ligase.**

**Important note:**

- The dilution ratio of MiniMax Methyl Stubby Adapter according to the amount of input DNA is recommended as follows:

| Input DNA (Fragmented DNA/cfDNA) | Adapter Stock Concentration | Dilution Ratio (250 bp*) | Dilution Ratio (160 bp*) | Adapter Insert Molar Ratio |
|---|---|---|---|---|
| 1000 ng | 15 µM | * | * | 5:1–3:1 |
| 500 ng | 15 µM | * | * | 10:1–6:1 |
| 250 ng | 15 µM | * | * | 25:1–15:1 |
| 100 ng | 15 µM | * | * | 50:1–30:1 |
| 50 ng | 15 µM | * | * | 100:1–60:1 |
| 25 ng | 15 µM | 2 | 2 | 100:1–60:1 |
| 10 ng | 15 µM | 5 | 4 | 100:1–75:1 |

* 250 bp is commonly used for gDNA fragmentation. 160 bp is the peak size observed for cfDNA in plasma.

**Note: When the input DNA ⩽ 25 ng, the adapter should be diluted prior to use.**

4. Mix thoroughly and centrifuge to collect the contents.
5. Incubate the tube in the thermal cycler programmed as follows . Make sure the thermal cycler is stabilized at 20°C before loading.

| | |
|---|---|
| 20°C | 15 min |
| 10°C | Hold |

**Note: The heated lid should be left open.**

## Step 4: Post-ligation Cleanup

1. Equilibrate the MiniMax SP Beads to room temperature for a minimum of 30 min. Thoroughly vortex the beads prior to use.

2. Add 44 µL of MiniMax SP Beads to the same tube from **Step 3**. Mix thoroughly and then incubate the tube at 25°C for 5-10 min.

3. Briefly centrifuge and then place the tube on the magnet stand until the solution is clear (~5 min). Carefully remove and discard the supernatant without disturbing the beads.

**Note: The incubation time should be adjusted according to the actual performance of various magnet stands to ensure that the solution is completely clarified.**

4. Keeping the tube on the magnet stand, add 150 µL of 80% freshly prepared ethanol without disturbing the beads. Incubate the tube on the magnet stand at room temperature for 30 sec. Carefully remove and discard the supernatant.

5. Repeat step 4 once.

6. Briefly centrifuge and then place the tube on the magnet stand. Carefully remove all residual ethanol without disturbing the beads by using a 10-µL pipette tip.

7. Open the lid, dry the beads on the magnet stand at 25°C for 2-3 min

**Note: Over-dried beads may be more difficult to suspend.**

8. Remove the tube off the magnet stand. Add 21 µL of Nuclease Free Water to into the tube and then incubate at 25°C for 2 min.

9. Briefly centrifuge and then place the tube on the magnet stand. Incubate for 2 min until the solution is clear. Transfer 20 µL of the clear supernatant to a new 0.2 mL PCR tube. Avoid transfer of MiniMax SP beads.

**Safe Stopping Point:** The library can be stored at 2 ~ 8°C overnight or -25 ~ -15°C for longer.

## Step 5: Library Conversion

This kit has been validated to be highly compatible with bisulfite conversion and enzymatic conversion methods. The recommendations of choice are listed as follows:

| Input DNA | NEBNext® Enzymatic Methyl-seq Conversion Module (E7125S/L) | QIAGEN EpiTect® Bisulfite kit (59824) |
|---|---|---|
| 10-200 ng | ★ ★ ★ | ★ |
| 200-500 ng | ★ | ★ ★ ★ |
| 500-1000 ng | N/A | ★ ★ |

**The enzymatic conversion method is recommended with input DNA ranging from 10-200 ng. For more than 200 ng of input DNA, the bisulfite conversion method is recommended.**

1. Please proceed the conversion workflow as requested with the purified DNA library from **step 4**.

2. Dissolve the converted products with 20 µL of TE Buffer.

**As the converted products are single-stranded phase, the subsequent workflow should be proceeded as quickly as possible.**

## Step 6: Library Amplification

1. Thaw 2X HiFi-Methyl PCR Master Mix and MiniMax UDI-Index Primer Mix on ice. Mix thoroughly and centrifuge to collect the contents.
2. Set up each library amplification reaction in a 0.2 mL PCR tube on ice as follows:

| | |
|---|---|
| The Converted Product of **Step 5** | 20 μL |
| 2X HiFi-Methyl PCR Master Mix | 25 μL |
| MiniMax UDI-Index Primer Mix | 5 μL |
| **Total** | **50 μL** |

3. Mix thoroughly and centrifuge to collect the contents.
4. Incubate the tube in the thermal cycler programmed as follows , with the heated lid set at 105°C.

| | | |
|---|---|---|
| 98°C | 2 min | |
| 98°C<br>60°C<br>72°C | 15 sec<br>30 sec<br>30 sec | Varies<br>(refer to the following table) |
| 72°C<br>4°C | 2 min<br>Hold | |

| Input DNA (Fragmented DNA/cfDNA) | Number of Cycles Recommended (Enzymatic Conversion) | |
|---|---|---|
| | Library Yield ⩾ 500 ng | Library Yield ⩾ 1,000 ng |
| 500 ng | 4–5 | 5–6 |
| 200 ng | 5–6 | 6–7 |
| 100 ng | 6–7 | 7–8 |
| 50 ng | 7–8 | 8–9 |
| 10 ng | 9–10 | 10-11 |

| Input DNA (Fragmented DNA/cfDNA) | Number of Cycles Recommended (Bisulfite Conversion) | |
|---|---|---|
| | Library Yield ⩾ 500 ng | Library Yield ⩾ 1,000 ng |
| 1000 ng | 4–5 | 5–6 |
| 500 ng | 5–6 | 6–7 |
| 200 ng | 6–7 | 7–8 |
| 100 ng | 7–8 | 8–9 |
| 50 ng | 8–9 | 9–10 |
| 10 ng | 10–11 | 11–12 |

**Important notes:**

- For targeted sequencing with MiniMax Hybrid Capture Reagents (Cat # 1005101), the library yield should reach 500 ng.
- If bisulfite conversion has been performed, 1 or 2 additional cycles are necessary because of the DNA damage caused by the bisulfite conversion.

## Step 7: Post-amplification Cleanup

1. Add 50 µL of MiniMax SP Beads into the Library Amplification tube. Mix thoroughly and then incubate the tube at 25°C for 5-10 min.
2. Briefly centrifuge and then place the tube on a magnet stand until the solution is clear (~5 min). Carefully remove and discard the supernatant without disturbing the beads.

**Note: The incubation time should be adjusted according to the actual performance of various magnet stands to ensure that the solution is completely clarified.**

3. Keeping the tube on the magnet stand, add 150 µL of freshly prepared 80% ethanol without disturbing the beads. Incubate the tube on the magnet stand at room temperature for 30 sec. Carefully remove and discard the ethanol.
4. Repeat step 3 once.
5. Briefly centrifuge and then place the tube on the magnet stand. Carefully remove all residual ethanol without disturbing the beads by using a 10-µL pipette tip.
6. Dry the beads on the magnet stand at room temperature for 5 min.

**Note: Over-dried beads may be more difficult to suspend.**

7. Remove the tube off the magnet stand. Thoroughly resuspend the beads in 30 µL of TE buffer. Incubate the tube at 25°C for 2 min.
8. Briefly centrifuge and place the tube on the magnet stand. Incubate for 2 min until the solution is clear. Transfer the clear supernatant to a new 0.2 mL PCR tube. Avoid transfer of MiniMax SP Beads.

STOP **Safe Stopping Point:** The library can be stored at 2 ~ 8°C overnight or -25 ~ -15°C for longer.

## Step 8: Library Evaluation

1. The Qubit™ fluorometer or qPCR is recommended for library quantification.
2. The Bioanalyzer® 2100 (Agilent) or Qsep 100 (Bioptic) is recommended for size distribution analysis.
3. The quality control standards include:
   a. No adapter dimers and free adapters.
   b. Relatively concentrated size distribution.
   c. Suitable for Illumina® platform in size.

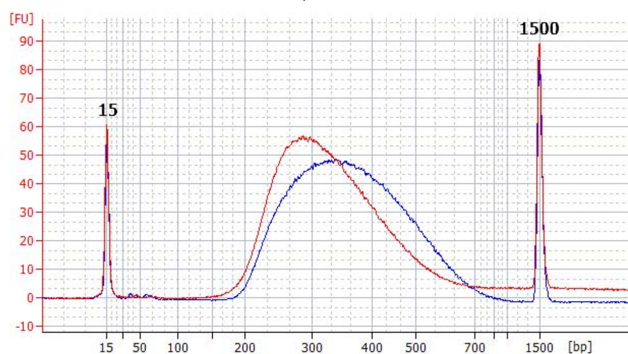



**Fig 1.** Size distribution of library prepared by using Apostle MiniMax Methyl Library Preparation Module coupled with Apostle MiniMax Methyl Stubby Adapter (UDI) Module (for Illumina®). The libraries were prepared from 50 ng of fragmented gDNA and were converted by using EM method with 7 PCR cycles (blue) and BS method with 9 PCR cycles (red), respectively.

## Step 9: Sequencing

The library is ready for sequencing on Illumina platforms.
In the period of hybridization & wash, the incubation temperature should be optimized from **65°C** to **63°C**. Please contact Nanodigmbio (support@njnad.com) for details.

# Appendix 1: MiniMax Methyl Stubby Adapter (UDI) (for Illumina®) Index Information

The index sequences of MiniMax UDI-Index Primer Mix in MiniMax Methyl Stubby Adapter (UDI) Module (for Illumina®) are listed as follows:

| UDI-Primer-Mix | i5 index (HiSeq® 2000/2500, MiSeq®, NovaSeq® v1.0 systems) | i5 index (HiSeq® 3000, 4000, HiSeq® X, NextSeq®, MiniSeq®, NovaSeq® v1.5 systems) | i7 index (all Illumina® systems) |
|---|---|---|---|
| UDI-Primer-Mix.01 | ATATGCGC | GCGCATAT | CTGATCGT |
| UDI-Primer-Mix.02 | TGGTACAG | CTGTACCA | ACTCTCGA |
| UDI-Primer-Mix.03 | AACCGTTC | GAACGGTT | TGAGCTAG |
| UDI-Primer-Mix.04 | TAACCGGT | ACCGGTTA | GAGACGAT |
| UDI-Primer-Mix.05 | GAACATCG | CGATGTTC | CTTGTCGA |
| UDI-Primer-Mix.06. | CCTTGTAG | CTACAAGG | TTCCAAGG |
| UDI-Primer-Mix.07 | TCAGGCTT | AAGCCTGA | CGCATGAT |
| UDI-Primer-Mix.08 | GTTCTCGT | ACGAGAAC | ACGGAACA |
| UDI-Primer-Mix.09 | AGAACGAG | CTCGTTCT | CGGCTAAT |
| UDI-Primer-Mix.10 | TGCTTCCA | TGGAAGCA | ATCGATCG |
| UDI-Primer-Mix.11 | CTTCGACT | AGTCGAAG | GCAAGATC |
| UDI-Primer-Mix.12 | CACCTGTT | AACAGGTG | GCTATCCT |
| UDI-Primer-Mix.13 | ATCACACG | CGTGTGAT | TACGCTAC |
| UDI-Primer-Mix.14 | CCGTAAGA | TCTTACGG | TGGACTCT |
| UDI-Primer-Mix.15 | TACGCCTT | AAGGCGTA | AGAGTAGC |
| UDI-Primer-Mix.16 | CGACGTTA | TAACGTCG | ATCCAGAG |
| UDI-Primer-Mix.17 | ATGCACGA | TCGTGCAT | GACGATCT |
| UDI-Primer-Mix.18 | CCTGATTG | CAATCAGG | AACTGAGC |
| UDI-Primer-Mix.19 | GTAGGAGT | ACTCCTAC | CTTAGGAC |
| UDI-Primer-Mix.20 | ACTAGGAG | CTCCTAGT | GTGCCATA |
| UDI-Primer-Mix.21 | CACTAGCT | AGCTAGTG | GAATCCGA |
| UDI-Primer-Mix.22 | ACGACTTG | CAAGTCGT | TCGCTGTT |
| UDI-Primer-Mix.23 | CGTGTGTA | TACACACG | TTCGTTGG |
| UDI-Primer-Mix.24 | GTTGACCT | AGGTCAAC | AAGCACTG |
| UDI-Primer-Mix.25 | ACTCCATC | GATGGAGT | CCTTGATC |
| UDI-Primer-Mix.26 | CAATGTGG | CCACATTG | GTCGAAGA |
| UDI-Primer-Mix.27 | TTGCAGAC | GTCTGCAA | ACCACGAT |
| UDI-Primer-Mix.28 | CAGTCCAA | TTGGACTG | GATTACCG |
| UDI-Primer-Mix.29 | ACGTTCAG | CTGAACGT | GCACAACT |
| UDI-Primer-Mix.30. | AACGTCTG | CAGACGTT | GCGTCATT |
| UDI-Primer-Mix.31 | TATCGGTC | GACCGATA | ATCCGGTA |

| UDI-Primer-Mix | i5 index (HiSeq® 2000/2500, MiSeq®, NovaSeq® v1.0 systems) | i5 index (HiSeq® 3000, 4000, HiSeq® X, NextSeq®, MiniSeq®, NovaSeq® v1.5 systems) | i7 index (all Illumina® systems) |
|---|---|---|---|
| UDI-Primer-Mix.32 | CGCTCTAT | ATAGAGCG | CGTTGCAA |
| UDI-Primer-Mix.33 | GATTGCTC | GAGCAATC | GTGAAGTG |
| UDI-Primer-Mix.34 | GATGTGTG | CACACATC | CATGGCTA |
| UDI-Primer-Mix.35 | CGCAATCT | AGATTGCG | ATGCCTGT |
| UDI-Primer-Mix.36 | TGGTAGCT | AGCTACCA | CAACACCT |
| UDI-Primer-Mix.37 | GATAGGCT | AGCCTATC | TGTGACTG |
| UDI-Primer-Mix.38 | AGTGGATC | GATCCACT | GTCATCGA |
| UDI-Primer-Mix.39 | TTGGACGT | ACGTCCAA | AGCACTTC |
| UDI-Primer-Mix.40 | ATGACGTC | GACGTCAT | GAAGGAAG |
| UDI-Primer-Mix.41 | GAAGTTGG | CCAACTTC | GTTGTTCG |
| UDI-Primer-Mix.42 | CATACCAC | GTGGTATG | CGGTTGTT |
| UDI-Primer-Mix.43 | CTGTTGAC | GTCAACAG | ACTGAGGT |
| UDI-Primer-Mix.44 | TGGCATGT | ACATGCCA | TGAAGACG |
| UDI-Primer-Mix.45 | ATCGCCAT | ATGGCGAT | GTTACGCA |
| UDI-Primer-Mix.46 | TTGCGAAG | CTTCGCAA | AGCGTGTT |
| UDI-Primer-Mix.47 | AGTTCGTC | GACGAACT | GATCGAGT |
| UDI-Primer-Mix.48 | GAGCAGTA | TACTGCTC | ACAGCTCA |
| UDI-Primer-Mix.49 | ACAGCTCA | TGAGCTGT | GAGCAGTA |
| UDI-Primer-Mix.50 | GATCGAGT | ACTCGATC | AGTTCGTC |
| UDI-Primer-Mix.51 | AGCGTGTT | AACACGCT | TTGCGAAG |
| UDI-Primer-Mix.52 | GTTACGCA | TGCGTAAC | ATCGCCAT |
| UDI-Primer-Mix.53 | TGAAGACG | CGTCTTCA | TGGCATGT |
| UDI-Primer-Mix.54 | ACTGAGGT | ACCTCAGT | CTGTTGAC |
| UDI-Primer-Mix.55 | CGGTTGTT | AACAACCG | CATACCAC |
| UDI-Primer-Mix.56 | GTTGTTCG | CGAACAAC | GAAGTTGG |
| UDI-Primer-Mix.57 | GAAGGAAG | CTTCCTTC | ATGACGTC |
| UDI-Primer-Mix.58. | AGCACTTC | GAAGTGCT | TTGGACGT |
| UDI-Primer-Mix.59. | GTCATCGA | TCGATGAC | AGTGGATC |
| UDI-Primer-Mix.60 | TGTGACTG | CAGTCACA | GATAGGCT |
| UDI-Primer-Mix.61 | CAACACCT | AGGTGTTG | TGGTAGCT |
| UDI-Primer-Mix.62 | ATGCCTGT | ACAGGCAT | CGCAATCT |
| UDI-Primer-Mix.63 | CATGGCTA | TAGCCATG | GATGTGTG |
| UDI-Primer-Mix.64 | GTGAAGTG | CACTTCAC | GATTGCTC |
| UDI-Primer-Mix.65 | CGTTGCAA | TTGCAACG | CGCTCTAT |

| UDI-Primer-Mix | i5 index (HiSeq® 2000/2500, MiSeq®, NovaSeq® v1.0 systems) | i5 index (HiSeq® 3000, 4000, HiSeq® X, NextSeq®, MiniSeq®, NovaSeq® v1.5 systems) | i7 index (all Illumina® systems) |
|---|---|---|---|
| UDI-Primer-Mix.66 | ATCCGGTA | TACCGGAT | TATCGGTC |
| UDI-Primer-Mix.67 | GCGTCATT | AATGACGC | AACGTCTG |
| UDI-Primer-Mix.68 | GCACAACT | AGTTGTGC | ACGTTCAG |
| UDI-Primer-Mix.69 | GATTACCG | CGGTAATC | CAGTCCAA |
| UDI-Primer-Mix.70 | ACCACGAT | ATCGTGGT | TTGCAGAC |
| UDI-Primer-Mix.71 | GTCGAAGA | TCTTCGAC | CAATGTGG |
| UDI-Primer-Mix.72 | CCTTGATC | GATCAAGG | ACTCCATC |
| UDI-Primer-Mix.73 | AAGCACTG | CAGTGCTT | GTTGACCT |
| UDI-Primer-Mix.74 | TTCGTTGG | CCAACGAA | CGTGTGTA |
| UDI-Primer-Mix.75 | TCGCTGTT | AACAGCGA | ACGACTTG |
| UDI-Primer-Mix.76 | GAATCCGA | TCGGATTC | CACTAGCT |
| UDI-Primer-Mix.77 | GTGCCATA | TATGGCAC | ACTAGGAG |
| UDI-Primer-Mix.78 | CTTAGGAC | GTCCTAAG | GTAGGAGT |
| UDI-Primer-Mix.79 | AACTGAGC | GCTCAGTT | CCTGATTG |
| UDI-Primer-Mix.80 | GACGATCT | AGATCGTC | ATGCACGA |
| UDI-Primer-Mix.81 | ATCCAGAG | CTCTGGAT | CGACGTTA |
| UDI-Primer-Mix.82 | AGAGTAGC | GCTACTCT | TACGCCTT |
| UDI-Primer-Mix.83 | TGGACTCT | AGAGTCCA | CCGTAAGA |
| UDI-Primer-Mix.84 | TACGCTAC | GTAGCGTA | ATCACACG |
| UDI-Primer-Mix.85 | GCTATCCT | AGGATAGC | CACCTGTT |
| UDI-Primer-Mix.86 | GCAAGATC | GATCTTGC | CTTCGACT |
| UDI-Primer-Mix.87 | ATCGATCG | CGATCGAT | TGCTTCCA |
| UDI-Primer-Mix.88 | CGGCTAAT | ATTAGCCG | AGAACGAG |
| UDI-Primer-Mix.89 | ACGGAACA | TGTTCCGT | GTTCTCGT |
| UDI-Primer-Mix.90 | CGCATGAT | ATCATGCG | TCAGGCTT |
| UDI-Primer-Mix.91 | TTCCAAGG | CCTTGGAA | CCTTGTAG |
| UDI-Primer-Mix.92 | CTTGTCGA | TCGACAAG | GAACATCG |
| UDI-Primer-Mix.93 | GAGACGAT | ATCGTCTC | TAACCGGT |
| UDI-Primer-Mix.94 | TGAGCTAG | CTAGCTCA | AACCGTTC |
| UDI-Primer-Mix.95 | ACTCTCGA | TCGAGAGT | TGGTACAG |
| UDI-Primer-Mix.96 | CTGATCGT | ACGATCAG | ATATGCGC |

**Note: As the reading mode of the dual index differs in various Illumina® platforms, please select the corresponding sequence information according to the Illumina systems.**

## Appendix 2: Frequently Asked Question

| Problems | Possible Reason | Solution |
|---|---|---|
| Low library yield | Inaccurate quantification of input DNA | The Qubit fluorometer is recommended for the quantification of fragmented DNA. |
| | a. Low input DNA with BS module.<br>b. Compared with the EM module, the BS module needs 1 or 2 additional PCR cycles to reach the similar recovery efficiency. | Increase PCR cycles; Convert low input DNA with EM module. |
| Low conversion efficiency (＜99%) of unmethylated cytosine | Contamination of post-converted library | a. Use filter tips.<br>b. Replace the gloves and change experiment table after the conversion process. |
| | a. The BS module is out of date.<br>b. The oxidation-caused crystallization of bisulfite may significantly inhibit the conversion efficiency. | a. Use the BS module within the period of validity.<br>b. Minimize the exposure to air. |
| | Input DNA is too much. | For EM module, the input DNA should be under 200 ng. |

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