NEBNext® Ultra™ II DNA Library Prep for Element Biosciences Sequencing

New England Biolabs

Library preparation methods need to be flexible as users begin to adopt newly emerging sequencing platforms. The Element AVITI system and Cloudbreak chemistry promise high quality sequencing data with an affordable, user-friendly benchtop system. The commercial availability of Element barcoded sequencing adaptors enables users to apply their preferred library prep method and still perform native sequencing on the Element instrument.

The NEBNext Ultra II DNA Library Prep Kit is an optimal choice for producing high-quality DNA sequencing libraries from a broad range of species, input amounts, and sample types including genomic DNA and cell-free DNA. The Element Elevate Adapter Kits can be easily incorporated into the NEBNext Ultra II DNA Library Kit workflow requiring no adaptor dilution from input amounts ranging from 0.5 ng to 1 µg of DNA. Sample purification bead cleanup recommendations are used to remove excess adaptor dimer and optimize the library size for efficient loading of the AVITI instrument.

PROTOCOL

Starting Material

Fragment input DNA (0.5 ng to 1 µg) using an instrument such as Covaris®. NEB recommends shearing DNA in 1X TE using a 350 bp program. If the DNA volume post-shearing is less than 50 µl, add 1X TE to a final volume of 50 µl.

1. End Prep

1.1 Mix the following components in a sterile, 0.2 ml nuclease-free tube (60 µl final volume):

COMPONENT	VOLUME
Sheared DNA (0.5 ng to 1 µg)	50 µl
● (green) NEBNext Ultra II End Prep Buffer	7 µl
● (green) NEBNext Ultra II End Prep Enzyme Mix	3 µl
Total Volume	60 µl

1.2 Set a 100 µl or 200 µl pipette to 50 µl and then pipette the entire volume up and down at least 10 times to mix thoroughly. Perform a quick spin to collect all liquid from the sides of the tube.

1.3 Place in a thermal cycler, with heated lid set to 75°C, and run the following program:

- 30 minutes at 20°C
- 30 minutes at 65°C
- Hold at 4°C

MATERIALS

- Input DNA of choice (0.5 ng to 1 µg)
- NEBNext Ultra II DNA Library Prep Kit (NEB #E7645)
- Element Elevate™ Long UDI Adapter Kit Set A (Element Biosciences #830-00010)* or Element Elevate Index and Adapter Kit (Element Biosciences #830-00005)
- User-supplied SP5 and SP27 outer PCR primer mix, 5 µM each. Required only for use with the Element Elevate Long UDI Adapter Kit set A (Element Biosciences #830-00010)

  Primer sequences:

  SP5 5' Phosphate-CATGTAATGCACGTACTTTCAGGGT

  SP27 5' GATCAGGTGAGGCTGCGACGACT"
- SPRIselect® Reagent Kit (Beckman Coulter, Inc. #B23317) or AMPure® XP Beads (Beckman Coulter, Inc. #A63881)
- 1X TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA)
- 0.1X TE (1 mM Tris-HCl, pH 8.0, 0.1 mM EDTA)
- 80% ethanol (freshly prepared)
- Pipettes with disposable tips
- Thin wall 200 µl PCR tubes (for example TempAssure® PCR flex-free 8-tube strips USA Scientific #1402-4708)
- Magnetic rack (NEB #S1515S), magnetic plate (Alpaqua® #A001322) or equivalent
- Thermal cycler
- Microcentrifuge
- Vortex Mixer
- Bioanalyzer®, TapeStation® (Agilent Technologies, Inc.) or similar fragment analyzer and consumables

2. Adaptor Ligation

2.1 Add the following components to the end prep reaction mixture:

COMPONENT	VOLUME
End-prepped DNA (from step 1.3)	60 µl
Element Adaptor* (no dilution required)	2.5 µl
● (red) NEBNext Ligation Enhancer	1 µl
● (red) NEBNext Ultra II Ligation Master Mix	30 µl
Total Volume	93.5 µl

* ***Note: This protocol is compatible with both Element Elevate Long UDI Adapter Kit Set A (Element Biosciences #830-00010) or Element Elevate Index and Adapter Kit (Element Biosciences #830-00005). Please ensure that the PCR primers used in step 5.1 are correct for the adaptor of choice.***

2.2 Set a 100 µl or 200 µl pipette to 80 µl and then pipette the entire volume up and down at least 10 times to mix thoroughly. Perform a quick spin to collect all liquid from the sides of the tube. ***(Caution: The NEBNext Ultra II Ligation Master Mix is very viscous. Care should be taken to ensure adequate mixing of the ligation reaction, as incomplete mixing will result in reduced ligation efficiency. The presence of a small amount of bubbles will not interfere with performance)***.

2.3 Incubate in a thermocycler with the heated lid off and run the following program.

- 15 minutes at 20°C
- Hold at 4°C

3. Cleanup of adaptor-ligated DNA

3.1 Vortex NEBNext Sample Purification Beads, SPRIselect or AMPure XP Beads to resuspend. If using AMPure XP Beads, remove from 4°C and keep at room temperature for 30 minutes prior to use.

3.2. Add **75µl (0.8X)** of resuspended beads to the Adaptor Ligation reaction (96.5 µl). Mix well by pipetting up and down at least 10 times. Be careful to expel all the liquid out of the tip during the last mix. Vortexing for 3-5 seconds on high can also be used. If centrifuging samples after mixing, be sure to stop the centrifugation before the beads start to settle out.

3.3. Incubate samples on the bench top for 5 minutes at room temperature.

3.4 Place the tube/plate on an appropriate magnetic rack to separate the beads from the supernatant. If necessary, quickly spin the sample to collect the liquid from the sides of the tube or plate wells before placing on the magnetic rack.

3.5 After 5 minutes (or when the solution is clear), carefully remove and discard the supernatant. Be careful not to disturb the beads that contain DNA targets ***(Caution: do not discard beads)***.

3.6 Add 200 µl of freshly prepared 80% ethanol to the tube/plate while on the magnetic rack. Incubate at room temperature for 30 seconds, and then carefully remove and discard the supernatant. Be careful not to disturb the beads that contain DNA targets.

3.7 Repeat Step 3.6 once for a total of two washes. Be sure to remove all visible liquid after the second wash. If necessary, briefly spin the tube/plate, place back on the magnet and remove traces of ethanol with a P10 pipette tip.

3.8 Air dry the beads for up to 5 minutes while the tube/plate is on the magnetic rack with the lid open.

(Caution: Do not over-dry the beads. This may result in lower recovery of the DNA target. Elute the samples when the beads are still dark brown and glossy looking, but when all visible liquid has evaporated. When the beads turn lighter brown and start to crack they are too dry.)

3.9 Remove the tube/plate from the magnetic rack. Elute the DNA target from the beads by adding **53 µl** of 0.1X TE.

3.10 Mix well by pipetting 10 times. Incubate for at least 2 minutes at room temperature. If necessary, quickly spin the sample to collect all liquid from the sides of the tube before placing back on the magnetic rack.

3.11 Place the tube/plate on the magnetic rack.. After 5 minutes (or when the solution is clear), transfer **50 µl** to a new PCR tube.

4. Size selection of adaptor-ligated DNA

4.1 Vortex NEBNext Sample Purification Beads, SPRIselect or AMPure XP Beads to resuspend.

4.2 Add **25 µl** of resuspended beads to the cleaned up DNA from step 3.11. Mix well by pipetting up and down at least 10 times. Be careful to expel all the liquid out of the tip during the last mix. Vortexing for 3-5 seconds on high can also be used. If centrifuging samples after mixing, be sure to stop the centrifugation before the beads start to settle out.

4.3 Incubate samples on bench top for 5 minutes at room temperature.

4.4 Place the tube/plate on an appropriate magnetic rack to separate the beads from the supernatant. If necessary, quickly spin the sample to collect the liquid from the sides of the tube or plate wells before placing on the magnetic rack.

4.5 After 5 minutes (or when the solution is clear), transfer the **supernatant** to a new tube. ***(Caution: do not discard supernatant)***.

4.6 Add **10 µl** of freshly resuspended beads to the supernatant. Mix well by pipetting up and down at least 10 times. Be careful to expel all the liquid out of the tip during the last mix. Vortexing for 3-5 seconds on high can also be used. If centrifuging samples after mixing, be sure to stop the centrifugation before the beads start to settle out.

4.7 Incubate samples on bench top for 5 minutes at room temperature.

4.8 Place the tube/plate on an appropriate magnetic rack to separate the beads from the supernatant. If necessary, quickly spin the sample to collect the liquid from the sides of the tube or plate wells before placing on the magnetic rack.

4.9 After 5 minutes (or when the solution is clear), remove the supernatant ***(Caution: do not discard beads).***

4.10 Add 200 µl of freshly prepared 80% ethanol to the tube/plate while on the magnetic rack. Incubate at room temperature for 30 seconds, and then carefully remove and discard the supernatant. Be careful not to disturb the beads that contain DNA targets.

4.11 Repeat Step 4.10 once for a total of two washes. Be sure to remove all visible liquid after the second wash. If necessary, briefly spin the tube/plate, place back on the magnet and remove traces of ethanol with a P10 pipette tip.

4.12 Air dry the beads for up to 5 minutes while the tube/plate is on the magnetic rack with the lid open.
(Caution: Do not over-dry the beads. This may result in lower recovery of the DNA target. Elute the samples when the beads are still dark brown and glossy looking, but when all visible liquid has evaporated. When the beads turn lighter brown and start to crack they are too dry.)

4.13 Remove the tube/plate from the magnetic rack. Elute the DNA target from the beads by adding **16 µl** of 0.1X TE. Mix well by pipetting up and down 10 times and incubate for 2 minutes at room temperature.

4.14 ***Without removing the beads,*** transfer **15 µl** to a new PCR tube.

5. PCR Enrichment of size-selected DNA

5.1 Add the following components to the cleaned up, size-selected DNA from step 4.14

COMPONENT	VOLUME (µl) PER REACTION
Adaptor-Ligated DNA Fragments with beads (Step 4.14)	15 µl
PCR Primer Mix*	10 µl
● (blue) NEBNext Ultra II Q5 Master Mix	25 µl
Total Volume	50 µl

* User-supplied SP5 and SP27 outer PCR primer mix, 5 µM each (if using Element Elevate Long UDI Adapter Kit) or Element Unique Index Pair (if using Element Elevate Index and Adapter Kit)

5.2 Set a 100 µl or 200 µl pipette to 40 µl and then pipette the entire volume up and down at least 10 times to mix thoroughly. Perform a quick spin to collect all liquid from the sides of the tube.

5.3 Place the tube on a thermal cycler and perform PCR amplification using the following PCR cycling conditions:

CYCLE STEP	TEMP	TIME	CYCLES
Initial Denaturation	98°C	30 seconds	1
Denaturation	98°C	10 seconds	5-14*
Annealing/Extension	65°C	75 seconds	
Final Extension	65°C	5 minutes	1
Hold	4°C	∞	

* The number of PCR cycles should be chosen based on the input amount and sample type. Thus, samples prepared with a different method prior to library prep may require re-optimization of the number of PCR cycles. The number of cycles should be high enough to provide sufficient library fragments for a successful sequencing run, but low enough to avoid PCR artifacts and over-cycling (high molecular weight fragments on Bioanalyzer). The number of PCR cycles recommended in Table 5.3 should be viewed as a starting point to determine the number of PCR cycles best for standard library prep samples.

Table 5.3: Recommended Number of PCR Cycles

INPUT AMOUNT	PCR CYCLES
1000 ng	5
500 ng	5
100 ng	5-6
50 ng	7-8
10 ng	9-10
5 ng	10-11
1 ng	12-13
0.5 ng	13-14

6. Cleanup of PCR Reaction

6.1 Vortex NEBNext Sample Purification Beads, SPRIselect or AMPure XP Beads to resuspend.

6.2 Add **40µl (0.8X)** of resuspended beads to the PCR reaction (50 µl). Mix well by pipetting up and down at least 10 times. Be careful to expel all the liquid out of the tip during the last mix. Vortexing for 3-5 seconds on high can also be used. If centrifuging samples after mixing, be sure to stop the centrifugation before the beads start to settle out.

6.3 Incubate samples on bench top for 5 minutes at room temperature.

6.4 Place the tube/plate on an appropriate magnetic rack to separate the beads from the supernatant. If necessary, quickly spin the sample to collect the liquid from the sides of the tube or plate wells before placing on the magnetic rack.

6.5 After 5 minutes (or when the solution is clear), carefully remove and discard the supernatant. Be careful not to disturb the beads that contain DNA targets ***(Caution: do not discard beads)***.

6.6 Add 200 µl of freshly prepared 80% ethanol to the tube/plate while on the magnetic rack. Incubate at room temperature for 30 seconds, and then carefully remove and discard the supernatant. Be careful not to disturb the beads that contain DNA targets.

6.7 Repeat Step 6.6 once for a total of two washes. Be sure to remove all visible liquid after the second wash. If necessary, briefly spin the tube/plate, place back on the magnet and remove traces of ethanol with a P10 pipette tip.

6.8 Air dry the beads for up to 5 minutes while the tube/plate is on the magnetic rack with the lid open.
(Caution: Do not over-dry the beads. This may result in lower recovery of the DNA target. Elute the samples when the beads are still dark brown and glossy looking, but when all visible liquid has evaporated. When the beads turn lighter brown and start to crack they are too dry.)

6.9 Remove the tube/plate from the magnetic rack. Elute the DNA target from the beads by adding **33 µl** of 0.1X TE.

6.10 Mix well by pipetting 10 times. Incubate for at least 2 minutes at room temperature. If necessary, quickly spin the sample to collect all liquid from the sides of the tube before placing back on the magnetic rack.

6.11 Place the tube/plate on the magnetic rack. After 5 minutes (or when the solution is clear), and without disturbing the bead pellet, transfer **30 µl** to a new PCR tube and store at -20°C . This is the final library.

7. Assessment of Library Yield and Size

7.1 Check the yield and size distribution on Agilent High Sensitivity D1000 Tapestation ScreenTape® Agilent High Sensitivity Bioanalyzer chip.

Note: If a peak at ~ 85 bp (primers) or ~156 bp (adaptor-dimer) is visible in the TapeStation traces, bring up the sample volume (from Step 6.11) to 50 µl with 0.1X TE buffer and repeat the SPRIselect Bead or NEBNext Sample Purification Bead Cleanup Step (Section 6). Adaptor dimer peak range is derived from values observed on TapeStation. Peaks may appear shifted on other library analysis instruments such as Bioanalyzer or Fragment Analyzer. Optionally, this additional cleanup can be performed after creating an equimolar pool of multiple libraries.

FIGURE 1: **Example of libraries prepared from 100 ng input of human NA12878 DNA using either the short (blue) or long (orange) Element Elevate adaptors run on the Agilent HSD1000 ScreenTape. (Libraries were diluted 5-fold in 0.1X TE prior to loading on the Tapestation).**