Protocol for use with NEBNext Ultra DNA Library Prep Kit for Illumina (E7370)

Starting material is RAD library prep, 55.5ul

1.1 NEBNext End Prep

1. Mix the following in a sterile tube:

Color	Component	Volume
Green	End Prep Enzyme Mix	3.0 µl
Green	End Repair Reaction Buffer (10X)	6.5 µl
na	Fragmented DNA	55.5 µl

   Total volume: 65 µl

2. Mix by pipetting followed by a quick spin to collect all liquid from the sides of the tube.
3. Place in a thermocycler, with the heated lid on, and run the follwing program:

Time	Temp
30 minutes	20°C
30 minutes	65°C
hold	4°C

1.2 Adaptor ligation

Because DNA input is < 100 ng, dilute the NEBNext Adaptor for Illumina (provided at 15 µM) 10-fold in 10 mM Tris-HCl to a final concentration of 1.5 µM, use immediately. ie, 1 ul adaptor into 9ul tris

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

Color	Component	Volume
red	Blunt-TA Ligase Master Mix	15 µl
red	NEBNext Adaptor for Illumina*	2.5 µl
red	ligation enhancer	1µl

   Total volume 83.5 µl
   *the NebNext Adaptor for Illumina is in the Multiplex kit

2. Mix by pipetting followed by a quick spin to collect all liquid from the sides of the tube.
3. Incubate at 20°C for 15 minutes in a thermal cycler.
   - Protocol: “NEB_DNA” ->“Adaptor”
4. Add 3 µl of USER enzyme to the ligation mixture from Step 3. (found in the multiplex kit)
5. Mix well and incubate at 37°C for 15 minutes.
   - in heat block

1.3 Size Selection of Adaptor-ligated DNA

The following size selection protocol is for libraries with 200 bp inserts only. For libraries with different size fragment inserts, refer to Table 1.1 for the appropriate volume of beads to be added. The size selection

protocol is based on a starting volume of 100 µl.

1. Vortex AMPure XP Beads to resuspend.
2. Add 13.5 µl dH2O to the ligation reaction for a 100 µl total volume.
3. Add 55 µl of resuspended AMPure XP Beads to the 100 µl ligation reaction. Mix well by pipetting up and down at least 10 times.
4. Incubate for 5 minutes at room temperature.
5. Quickly spin the tube and 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 the supernatant containing your DNA to a new tube **(Caution: do not discard the supernatant)**. Discard the beads that contain the unwanted large fragments.
6. Add 25 µl resuspended AMPure XP Beads to the supernatant, mix well and incubate for 5 minutes at room temperature.
7. Quickly spin the tube and place it 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 that contains unwanted DNA. Be careful not to disturb the beads that contain the desired DNA targets **(Caution: do not discard beads)**.
8. Add 200 µl of 80% freshly prepared ethanol to the tube while in the magnetic stand. Incubate at room temperature for 30 seconds, and then carefully remove and discard the supernatant.
9. Repeat Step 8 once.
10. 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.**
11. Remove the tube/plate from the magnet. Elute the DNA target from the beads into 17 µl of 10 mM Tris-HCl or 0.1 X TE. Mix well on a vortex mixer or by pipetting up and down. Incubate for 2 minutes at room temperature.
12. Quickly spin the tube and place it on a magnetic stand. After the solution is clear (about 5 minutes), transfer 15 µl to a new PCR tube for amplification.
13. Proceed to PCR Amplification in Section 1.4.

LIBRARY PARAMETERS	APPROXIMATE INSERT SIZE	150 bp	200 bp	250 bp	300-400 bp	400-500 bp	500-700 bp
	Total Library Size (insert + adaptor)	270 bp	320 bp	400 bp	400-500 bp	500-600 bp	600-800 bp
VOLUME TO BE ADDED (µl)	1st Bead Selection	65	55	45	40	35	30
	2nd Bead Selection	25	25	25	20	15	15

1.4 PCR Enrichment of Adaptor Ligated DNA

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

Color	Component	Volume
blue	NEBNext Q5 Hot Start HiFi PCR Master Mix	25 µl
blue	Index Primer	5 µl
blue	Universal PCR Primer	5 µl

2. PCR Cycling Conditions:

CYCLE STEP	TEMP	TIME	CYCLES
Initial Denaturation	98°C	30 seconds	1
Denaturation	98°C	10 seconds	4–12*
Annealing/Extension	65°C	75 seconds	
Final Extension	65°C	5 minutes	1
Hold	4°C	∞	

**We suggest 4 PCR cycles for 1 µg DNA input 7-8 cycles for 50 ng, and 12 for 5 ng DNA input. Further optimization of PCR cycle number may be required.*

3. Proceed to Cleanup of PCR Amplification Section 1.5.

1.5 Cleanup of PCR Amplification

1. Vortex AMPure XP Beads to resuspend.
2. Add 45 µl of resuspended AMPure XP Beads to the PCR reactions (~ 50 µl). Mix well by pipetting up and down at least 10 times.
3. Incubate for 5 minutes at room temperature.
4. Quickly spin the tube and place it 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 DNA targets(Caution do not discard beads).
5. Add 200 µl of 80% ethanol to the PCR plate while in the magnetic stand. Incubate at room temperature for 30 seconds, and then carefully remove and discard the supernatant.
6. Repeat Step 5 once.
7. Air dry the beads for 5 minutes while the 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.

8. Remove the tube/plate from the magnet. Elute DNA target from beads into 33 µl 0.1X TE. Mix well by pipetting up and down at least 10 times. Quickly spin the tube and incubate at room temperature for 2 minutes.
9. Place the sample on an appropriate magnetic stand to separate beads from supernatant. After the solution is clear (about 5 minutes), carefully transfer 28 µl supernatant to a new PCR tube. Libraries can be stored at –20°C.
10. Dilute 2-3 µl of the library 5 fold with 10 mM Tris-HCl or 0.1X TE and check the size distribution on an Agilent Bioanalyzer® (high sensitivity chip).