**SOP:** NEBNext Ultra II DNA Library Prep Kit for Illumina (E7645)

**Last Updated:** 12/13/18 SMB

**Name(s):\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_**

**Date:\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_**

**Project/Sample Info:\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_**

- This SOP is modified from NEB Next Ultra II kit SOP for congruence with BestRAD library prep (Miller/O’Rourke protocol)

- Starting material is RAD library prep, 50ul

- See Full [NEB Instruction Manual](https://www.neb.com/-/media/catalog/datacards-or-manuals/manuale7645.pdf) for additional details/modifications for other applications, etc.

-good to have general estimate of input DNA amount (ng) of RAD product, if less than 50 ng need to skip size selection, if less than 100ng need to dilute adaptor. Can also use starting input to estimate appropriate number of cycles for test PCR

**1.1 NEBNext End Prep ~ 6 hours**

1. Mix the following in a sterile tube:

**Color Component Volume**

Green End Prep Enzyme Mix 3.0 ul

Green End Repair Reaction Buffer (10X) 7ul

na Fragmented DNA (RAD lib. prep) 50ul

Total volume: 60 ul

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

*- Use P100 or P200 set to 50ul to pipette mix entire volume at least 10X to thoroughly mix before spin*

3. Place in a thermocycler, with the heated lid on (≥75°C), and run the following program:

**Time Temp**

30 minutes 20°C

30 minutes 65°C

Hold 4°C

**1.2 Adaptor Ligation**

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

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

**Color Component Volume**

red NEBNext Adaptor for Illumina\* 2.5 ul

red Ligation Enhancer 1 ul

red Ultra II Ligation Master mix 30 ul

Total volume: 93.5 ul

*\*the NEBNext Adaptor for Illumina is in the Multiplex kit; we used NEB #E7335*

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

*- NEB recommends adding adaptor last and mix well immediately, or premix adaptor and sample and then add other ligation reagents*

*- Use P100 or P200 set to 80ul to pipette mix entire volume at least 10X to thoroughly mix before spin*

3. Incubate at 20°C for 15 minutes in a thermal cycler with heated lid off *(Protocol: “NEB\_DNA” ->“Adaptor”)*

4. Take out Ampure beads to warm to room temp

5. Add 3ul of USER enzyme to the ligation mixture from Step 3. (found in the multiplex kit)

6. Mix well and incubate at 37°C for 15 minutes (in thermocycler; heated lid ≥47°C)

*Samples can be stored at this point overnight at -20°C if needed*

**1.3A Size Selection of Adaptor-ligated DNA (RAD product input > 50 ng)**

The following size selection protocol is for RAD libraries with 250 bp inserts and 370bp library size. For libraries with different size fragment inserts, refer to Table 1.1 in the NEB instruction manual for the appropriate volume of beads to be added. The size selection protocol is based on a **starting volume of exactly 96.5ul**

- Put Q5 hot start at room temp to thaw while doing size selection step; once thawed gently mix by inverting tube several times to dissolve any precipitate

1. Vortex AMPure XP Beads to resuspend.

3. Add 30ul of resuspended AMPure XP Beads to the 96.5ul ligation reaction. Mix well by pipetting up and down at least 10 times.

- Can pour beads into reservoir and use multichannel to add if have multiple libraries

- Make sure to expel all the liquid out of the tips in the last step

4. Incubate for 5 minutes at room temperature.

5. Quickly spin the tube or plate and place the tube or plate on an appropriate magnetic stand to separate the beads from the supernatant. After the solution is clear (~5 minutes), carefully transfer the supernatant containing your DNA to a new tube or wells in plate **(Caution: do not discard the supernatant!)**. Discard the beads that contain the unwanted large fragments.

6. Add 15ul resuspended AMPure XP Beads to the supernatant, mix well as described in Step 3, 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 200ul 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. Do not disturb the beads.

9. Repeat Step 8 once, then go back with P10 to make sure remove all ethanol traces (keep on magnet).

10. Air dry the beads for 5 minutes while the tube/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.**

11. Elute the DNA target from the beads into 21.5ul of 10 mM Tris-HCI or 0.1X TE (low TE) and remove the tube/plate from the magnet. Pipette mix well (set pipette to 20ul, mix 10X) or cover and mix on a vortex. Incubate for at least 2 minutes at room temperature.

-Beads should suspend easily back into solution if are not overdried.

12. Quickly spin the tube and place it on a magnetic stand. After the solution is clear (~5 minutes), transfer Xul to a new PCR tube/wells for amplification.

-Will have 20ul elute total to use here

-See below quantities for test PCR followed by optimized PCR

13. Proceed to PCR Amplification in Section 1.4. *\*Can stop and store library at -20°C here if needed\**

**OR 1.3B Clean-up of Adaptor-ligated DNA (RAD product input < 50 ng)**

Size selection greatly reduces your library yield and should not be performed if you started with less than 50 ng. If your final library is not the size you desire, you can perform size selection on the final library product. See the Size selection for libraries in water or TE pdf in the protocol folder for directions.

1. Vortex AMPure XP Beads to resuspend.

- Should be at RT from above; if not, make sure to take out at least 30 min prior to use here.

2. Add 87ul (0.9X) of resuspended AMPure XP Beads to your ligation reaction (**exactly 96.5 ul**). Mix well by pipetting up and down at least 10X. Make are all liquid/beads expelled

3. Incubate for at least 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 (~5 minutes), carefully remove and discard the supernatant. Be careful not to disturb the beads that contain DNA targets **(Caution: do not discard beads).**

-Set multichannel pipette to 150ul

5. Add 200ul 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. After second wash, go back with P10 to remove all ethanol traces.

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.**

-Don’t take plate off magnet, static can cause beads to jump between wells. If need to check if dry, can gently lift plate partially up to see into well, so magnet will still pull down on beads.

8. Elute DNA target from beads into 22ul 0.1X TE and then remove the tube/plate from the magnet. Mix well by pipetting up and down at least 10 times. Cover, quickly spin the tube and incubate at room temperature for at least 2 minutes.

9. Place the sample on an appropriate magnetic stand to separate beads from supernatant. After the solution is clear (~5 min), carefully transfer 20ul supernatant to a new tube for PCR.

10. Proceed to PCR Amplification in Section 1.4. *\*Can stop and store library at -20°C here if needed\**

**1.4 Test PCR Enrichment of Adaptor Ligated DNA**

- First use 4ul DNA +11ul water with 10 cycles to conduct test PCR. Keep prePCR product in -20 freezer.

- Then check for DNA concentration with fragment analyzer (see corresponding spreadsheet), then use the other 16ul with the appropriate amount of cycles.

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

**Color Component Volume**

blue NEBNext Ultra II Q5Master Mix 25 ul

index plate Index Primer (i7 primer) 5 ul

index plate Universal PCR Primer1/i5 primer 5 ul

na nuclease free water 11ul

na Adaptor ligated DNA Fragments (=sample from above) 4 ul

1 Can use i5 primer instead of universal if want dual indices, see manual for valid barcode combinations, etc.

\*\*Remember to record Index Primer-Library/Well Key here or create spreadsheet\*\*- this index combo will not be sequenced

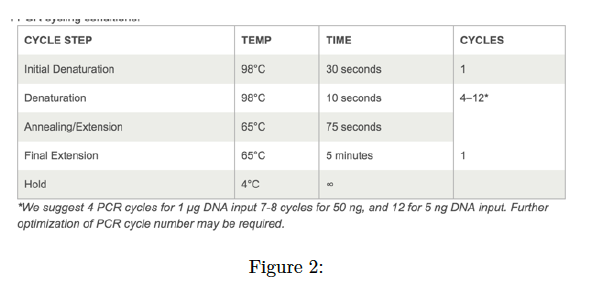
-Use P100 or P200 set to 40ul to pipette entire volume 10X, followed by quick spin.

-keep remainder of Adaptor ligated DNA (from step 1.3) in -20 freezer

2. PCR Cycling Conditions: 10 cycles for test

- can use starting input amount and table 4.1 in NEB manual to decide number of test cycles as well

-to make accurate calculations, need to number of cycles to be within linear phase of PCR reaction, so more than 3 cycles, but less than 12 cycles.



3. make sure Ampure beads are out (or take them out) and prep fragment analyzer while PCR is running

**1.5 Cleanup of test PCR Amplification**

1. Vortex AMPure XP Beads to resuspend.

- Should be at RT from above; if not, make sure to take out at least 30 min prior to use here.

2. Add 45ul (0.9X) of resuspended AMPure XP Beads to the PCR reactions (which are ~ 50ul). Mix well by pipetting up and down at least 10X. Make are all liquid/beads expelled

3. Incubate for at least 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 (~5 minutes), carefully remove and discard the supernatant. Be careful not to disturb the beads that contain DNA targets **(Caution: do not discard beads).**

-Set multichannel pipette to 150ul

5. Add 200ul 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. After second wash, go back with P10 to remove all ethanol traces.

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.**

-Don’t take plate off magnet, static can cause beads to jump between wells. If need to check if dry, can gently lift plate partially up to see into well, so magnet will still pull down on beads.

8. Elute DNA target from beads into 33ul 0.1X TE and then remove the tube/plate from the magnet. Mix well by pipetting up and down at least 10 times. Cover, quickly spin the tube and incubate at room temperature for at least 2 minutes.

9. Place the sample on an appropriate magnetic stand to separate beads from supernatant. After the solution is clear (~5 min), carefully transfer 30ul supernatant to a new PCR tube and label for inventory. Libraries can be stored at -20°C.

10A. Check the size distribution and concentration on Fragment Analyzer. Run test library undiluted, 1 in 5 dilution, and a 1:10 diliution to ensure an accurate read of concentration.

10B. You can also use qPCR (NEB Library Quant kit for Illumina E7630) to check the concentration. This gives you a less reliable concentration value but ensures that the adaptors necessary for sequencing are present in your library. You will need to arrange using the qPCR machine in the Pilsner lab ahead of time. See <https://www.neb.com/-/media/catalog/datacards-or-manuals/manuale7630.pdf> for details on use.

**1.6 Final PCR Enrichment of Adaptor Ligated DNA ~ 3 hours**

1. Calculate appropriate number of cycles for final PCR based on concentration of test PCR

-see spreadsheet in RAD test folder

-assume concentration doubles with each cycle (between 3-12 cycles)

-assume concentration of final PCR rxn will be 15/4 times the concentration of the test PCR at any cycle (higher input)

- back calculate minimum number of cycles that can be used to get a 10-30 nM library

-cannot use blown out (flat peaks) traces for accurate concentration

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

**Color Component Volume**

blue NEBNext Ultra II Q5Master Mix 25 ul

index plate Index Primer (i7 primer) 5 ul

index plate Universal PCR Primer1/i5 primer 5 ul

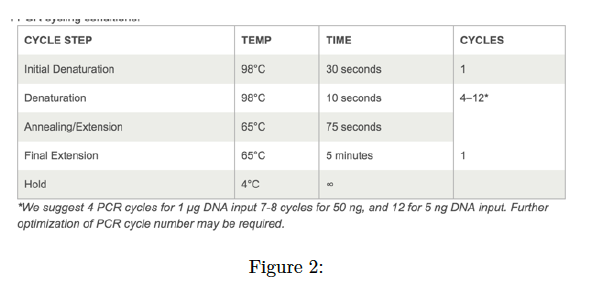
na Adaptor ligated DNA Fragments (=sample from step 1.3) 15 ul

1 Can use i5 primer instead of universal if want dual indices, see manual for valid barcode combinations, etc.

\*\*Remember to record Index Primer-Library/Well Key here or create spreadsheet\*\*

-Use P100 or P200 set to 40ul to pipette entire volume 10X, followed by quick spin.

2. PCR Cycling Conditions: use \_\_\_\_ cycles (calculated in step 1.6.1).



3. Take out Ampure beads to warm to room temperature and prep FA while PCR is running.

**1.7 Cleanup of final PCR Amplification**

1. Vortex AMPure XP Beads to resuspend.

- Should be at RT from above; if not, make sure to take out at least 30 min prior to use here.

2. Add 45ul (0.9X) of resuspended AMPure XP Beads to the PCR reactions (which are ~ 50ul). Mix well by pipetting up and down at least 10X. Make are all liquid/beads expelled

3. Incubate for at least 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 (~5 minutes), carefully remove and discard the supernatant. Be careful not to disturb the beads that contain DNA targets **(Caution: do not discard beads).**

-Set multichannel pipette to 150ul

5. Add 200ul 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. After second wash, go back with P10 to remove all ethanol traces.

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.**

-Don’t take plate off magnet, static can cause beads to jump between wells. If need to check if dry, can gently lift plate partially up to see into well, so magnet will still pull down on beads.

8. Elute DNA target from beads into 33ul 0.1X TE and then remove the tube/plate from the magnet. Mix well by pipetting up and down at least 10 times. Cover, quickly spin the tube and incubate at room temperature for at least 2 minutes.

9. Place the sample on an appropriate magnetic stand to separate beads from supernatant. After the solution is clear (~5 min), carefully transfer 30ul supernatant to a new PCR tube and label for inventory. Libraries can be stored at -20°C.

10. Check the size distribution and concentration on Fragment Analyzer (undiluted).