**Whitehead lab SOP:**

**NEBNext Ultra Directional RNA library preparation for Illumina (NEB#E7420S/L)-*adapted for ½ rxns***

**Last updated:** 6/22/18 (V1.0) by SB

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

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

**Project/Plate#/Samples Info:\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_**

**Before Starting and General Notes:**

- We are using ½ reaction volumes

- Protocol adapted from NEBNext Ultra Directional RNA Library Prep Kit for Illumina (NEB #E7420)

- Extract RNA samples. If starting from tissue, it is best to DNase-treat the RNA during the extraction protocol. We are not DNase-treating at this time.

- Quantify RNA using Qubit or plate reader

- Check RNA quality on Bioanalyzer. For best results, use high quality RNA with a RIN>7

- Starting total RNA concentration: we use either 250ng or 500ng. We have used 60ng and150ng a few times as well.

- This protocol targets a library size of 200bp; for different lengths, see NEB kit Appendix

- Use spreadsheet with concentrations to determine amount sample + H20 to add for 500ng in dilutions in Step 1, and also assign unique index primer combinations

- See *NEB library spreadsheet example* spreadsheet for columns/calculations to include on printout

**Reagents/Kits Needed:**

NEB #E7420 NEBNext Ultra Directional RNA Library Prep Kit for Illumina

NEB #7490 NEBNext Poly(A) mRNA Magnetic Isolation Module

NEB #E7600 NEBNext Multiplex Oligos for Illumina (Dual Index Primers Set 1)

Agencourt #A63881 Agencourt AMPure XP beads

10 mM Tris-HCl, pH 7.5-8.0

0.1X TE, pH 8.0

Actinomycin D (Sigma #A1410, dissolved in DMSO to ug/ul)

DMSO (add 400 ul directly to 200mg bottle of Act D to make 5ug/ul stock solution, store in foil in freezer)

80% Ethanol – use fresh molecular quality 100% ethanol (within 1 month of being opened) and make 80% ethanol fresh daily

**Start with 8 samples (including a positive control) for the first set of libraries. If those look good, try 16 to 24 samples the next time before you move to 48 samples at a time.**

**General Workflow:** *(for additional info, see figure of steps on website)*

*go back and add compile estimated time for each step here*

**mRNA Isolation, Fragmentation and Priming Starting with Total RNA**

****

**First Strand cDNA Synthesis**

****

**Second Strand cDNA Synthesis**

****

**Purify Double-stranded cDNA using 1.8X Agencourt XP Beads**

****

**End Repair/dA-tail of cDNA library**

****

**Adaptor Ligation**

****

**Size Selection of Adaptor-ligated DNA**

****

**PCR Library Enrichment**

****

**Purify PCR Reaction using Agencourt AMPure XP Beads**

****

**QC using Bioanalyzer/Qubit/plate reader**

****

**Multiplex individual libraries to be run together in a single lane**

****

**Purify pooled libraries using AMPure XP beads**

**\*Our cDNA libraries are currently being sequenced at the UC Davis Genome Center on the Hi-Seq 4000, which requires at least 15ul of 10nM of pooled library per lane with no adapter dimer contamination.**

****

**Day 1:**

**Take NEBNext Poly(A) mRNA Magnetic Isolation Module (NEB #E4790) out of 4oC and let equilibrate to room temp for 30 min.**

**Get thermal cycler ready for 65-5min**

**1.1 Part I**

* Prepare First Strand Reaction Buffer and Random Primer Mix:

|  |  |  |  |
| --- | --- | --- | --- |
| ***Component:*** | ***Volume/Sample*** | ***No. samples*** *(no overage-see below)* | ***Total to add for MasterMix*** |
| First Strand Synthesis Rxn Buffer (5x)*-pink* | 4ul |  |  |
| NEB Next Random Primers -(pink) | 1ul |  |  |
| Nuclease-free H20 | 5ul |  |  |

***Total Volume of MM:***

* Keep the mix on ice during the mRNA isolation
* *Note: don’t need to calculate overage for this one because adding 7.5ul instead of 10ul (mastermix makes 10ul/sample)*

**1.2 mRNA Isolation, Fragmentation and Priming Starting with Total RNA**

1. Dilute total RNA with nuclease-free H20 to final volume of **25 ul** (according to spreadsheet calculations) in nuclease-free 24 well plate. Add water first, then put **on** **ice block to add RNA**. *Cap each row as you go to avoid mis-pipetting when working with many samples.* Note: total RNA can be from 50 ng-50 0ng. We have been using 250 to 300 ng of total RNA.

2. Aliquot **10 ul** **NEBNext Oligo d(T)25 beads per sample** into a 2ml nuclease-free tube

*10ul beads \*( \_\_\_\_\_no.samples+2 samples)= \_\_\_\_\_\_ ul beads total*

3a. Add **RNA Binding Buffer** to wash beads into 2ml tube.

*50ul RNA Binding Buffer \* \_\_\_\_\_(no. samples +2 samples)= \_\_\_\_\_\_ ul total**RNA Binding Buffer*

*-if have large # samples, need to use 5ml tube, then split into two 2ml tubes to put on magnet*

3b.Pipette mix entire volume (6x) thoroughly. Solution will be very bubbly, pipette mix slowly to minimize bubbles, try not to retain bubbles in tip.

4. Place tubes on magnetic rack at room temperature for 2 minutes

5. Remove and discard all of the supernatant from the tube. Do not disturb the beads.

6. Remove the 2ml tube from the magnetic rack.

7. Repeat Steps 3-6.

8a. Resuspend the beads in **RNA Binding Buffer** and mix well

*25ul RNA Binding Buffer \* \_\_\_\_\_no. samples(\*1.1)= \_\_\_\_\_\_ ul total**RNA Binding Buffer*

*(if have in two 2 ml tubes, divide in half and add amount into each*

8b. Add **25ul** of **bead-RNA Binding Buffer** mix to each RNA sample on 24 well plate. *Seemed to work best to use 200ul repeat pipetter (not multichannel).*

8c. Pipette mix well, cap and quickly vortex and quick spin up to 1000rpm (STOP when gets to 1000rpm) on large centrifuge.

9. Place 24 well sample plate on thermal cycler and close lid. Heat sample at **65°C for 5 minutes** and hold at 4°C (program name: ***“65-5MIN”***) to denature RNA and facilitate binding of poly-A mRNA to the beads.

10. Remove the 24 well sample plate from the thermal cycler when the temperature reaches 4°C.

11. Resuspend the beads. Pipette up and down slowly 6 times to mix thoroughly.

12. Place 24 well sample plate on the bench and incubate at **room temperature for 5 minutes** to allow the mRNA to bind to the beads.

13. Resuspend the beads. Pipette up and down slowly 6 times to mix thoroughly.

14. Incubate for **5 minutes at room temperature** to allow the RNA to bind to the beads.

15. Place the 24 well sample plate on the **magnetic plate at room temperature for 2 minutes** to separate the poly-A mRNA bound to the beads from the solution.

16. Remove and discard all of the supernatant. Do not disturb the beads. *(Set 300 ul multichannel ~50ul for supernatant removal)*

17. Remove the 24 well sample plate from the magnetic plate.

18 . Wash the beads by adding **100ul** of **Wash Buffer** (can use disposable reservoir and 300 ul multichannel pipette) to each well to remove the unbound RNA. Pipette mix the entire volume of each well thoroughly **(6x)**, cap samples.

19. Place the 24 well sample plate on the **magnetic plate at room temperature for 2 minutes**. Get thermal cycler ready for 80-2min.

20. Remove and discard all of the supernatant from the tube. Do not disturb the beads.

21. Remove the 24 well sample plate from the magnetic plate.

22a. Repeat steps 18-21.

22b. Important to make sure get all the wash supernatant out before moving on to Elution buffer steps; can cap and do quick salad spin followed by going back through with p10 and then visually confirm that no supernatant remains.

23a. Add **25ul of Elution Buffer (Tris) from Poly A mRNA kit** to each well. Gently pipette mix entire volume to mix thoroughly (6x).

*Worked best to add 25ul with electronic 200ul repeat pipetter, followed by pipette mixing with manual repeat pipetter (300ul, set to 30ul) because vortex doesn’t work well when beads are out of solution already.*

23b. Cap, quickly vortex and quick spin up to 1000rpm. (STOP when gets to 1000rpm) on large centrifuge.

24a. Place the 24 well sample plate on the thermal cycler. Close the lid and heat the samples at **80°C for 2 minutes,** then **hold at 25°C** (program name: **“*80-2MIN”***) to elute the Poly-A mRNA from the beads.

24b. Remove stock actinomycin D from the -20C to thaw. Keep away from light.

25. Remove the tube from the thermal cycler when the temperature reaches 25°C

26. Add **25ul of RNA Binding Buffer** to each well to allow the mRNA to rebind to the beads. Gently pipette mix entire volume to mix thoroughly (6x). *Worked best to add 25ul with electronic 200ul repeat pipetter, cap and gentle vortex, salad spin bc beads already in solution.*

27. Incubate the 24 well plate at **room temperature for 5 minutes**. *Get First Strand Synthesis rxn ready if you haven’t prepared it at the beginning already.*

28. Resuspend the beads. Pipette up and down slowly 6 times to mix thoroughly.

29. Incubate for **5 minutes at room temperature** to allow the RNA to bind to the beads.

30. Place the 24 well plate on the **magnetic rack at room temperature for 2 minutes**. *Get thermal cycler ready for 94-15 min.*

31. Remove and discard all of the supernatant from the tube *(volume in each well should be~50ul to set pipette).* Do not disturb the beads.

32. Remove the 24 well plate from the magnetic rack.

33. Wash the beads by adding **100ul of Wash Buffer**. Gently pipette mix entire volume to mix thoroughly (6x). Quickly spin down in *salad spinner*.

34. Place the 24 well plate on the **magnetic rack at room temperature for 2 minutes**.

35. Remove and discard all of the supernatant from the wells. Do not disturb the beads.

***Note: It is important to remove all of the supernatant to successfully fragment the mRNA in the subsequent steps. Spin down the 24 well plate, place the plate on the magnetic rack and with a 10ul tip remove all of the Elution Buffer (use the p10). Caution: Do not disturb beads that contain mRNA.***

36. Remove the 24 well plate from the magnetic rack.

37a. Elute mRNA from the beads by adding **7.5ul of the First Strand Synthesis Reaction Buffer and** **Random Primer mix (2x)** prepared at the start of the protocol to each well and pipette mix 6x. **Visually check that all beads go into solution.** *Used 20-200ul electronic repeat pipetter to dispense into individual tubes, then pipette mix with manual p10 multichannel. Multichannel can also be used if there is a large enough volume of MM. Can gentle vortex if neede.* Quick spin up to 1000rpm (STOP when gets to 1000rpm) on large centrifuge.

37b. Incubate the samples at **94°C for 15 minutes (program: “*94-15MIN*”).**

***Note: This time will be different if your library size is greater than 200 bp (see NEB manual).***

37c. During 15 min, make the 1st strand cDNA synthesis mastermix. **Note: leave enzymes in freezer until last minute.**

37d. Immediately after place plate on magnetic rack.

38. Collect the purified mRNA by **transferring 6.75ul of the supernatant** to a clean nuclease-free 24 well plate.

39. Place on ice and proceed directly to first strand cDNA synthesis. *Set thermal cycler to 1st strnd.*

**1.3 First Strand cDNA Synthesis:** Create mastermix and add to fragmented and primed mRNA.

Note: Dilute actinomycin D stock solution to 0.1 ug/ul in nuclease-free water for immediate use.

Note: Dilute solutions of Act D are very sensitive to light. In solution, Act D tends to adsorb to plastic and glass. For these reasons, unused dilute solution should be discarded and not stored for further use. However, frozen aliquots of a concentrated stock solution (5 ug/ul) in DMSO are expected to be stable for quite some time (6 months) at -20oC.

Note:

Vstock = ((0.1ug/ul)/(5ug/ul)) x Vdilution

Vwater = Vdilution - Vstock

Note: leave enzymes in freezer until last minute.

**1.**

|  |  |  |  |
| --- | --- | --- | --- |
| ***Component:*** | ***Volume/Sample*** | ***No. samples(+2)*** | ***Total to add for MasterMix*** |
| Murine RNase Inhibitor *(pink)* | 0.25ul |  |  |
| Protoscript II Reverse Transcriptase *(pink)* | 0.5ul |  |  |
| Actinomycin D (0.1 ug/ul) | 2.5ul |  |  |

***Total Volume of MM:***

**1b**. **Add 3.25ul** each well. *Samples on ice block while pipetting*

1c. Pipette mix, or light vortex/flick mix. Quick spin up to 1000rpm (STOP when gets to 1000rpm) on large centrifuge.

2a. Incubate as follows (with heated lid set at 105oC): (Program**: “1STSTRND” under NEB-RNA**)

10 min at 25oC

15 min at 42oC

15 min at 70oC

Hold at 4oC

**Note: If you are making longer RNA fragments, follow recommendations in NEB Appendix A.**

2b. Take the 2nd strand synthesis reaction buffer out of the freezer and thaw on ice. **Leave the enzyme in the freezer.**

3. After incubation, immediately perform second strand cDNA synthesis.

**1.4 Perform Second Strand cDNA synthesis:**

1a. **Second Strand cDNA Synthesis:** Create mastermix and add the to the First Strand Synthesis Reaction

|  |  |  |  |
| --- | --- | --- | --- |
| ***Component:*** | ***Volume/Sample*** | ***No. samples(+2)*** | ***Total to add for MasterMix*** |
| Nuclease-free H20 | 24ul |  |  |
| Second Strand Synthesis Reaction Buffer with dUTP Mix (10x) -orange) | 4ul |  |  |
| Second Strand Synthesis Enzyme Mix (orange) *\*this is fragile – gentle pipette* | 2ul |  |  |

***Total Volume of MM:***

1b. **Add 30ul to each well. *Can use repeater. Samples on ice block while pipetting.***

2a. Mix thoroughly by ***gentle pipette*** mixing, quick spin up to 1000rpm (STOP when gets to 1000rpm) on large centrifuge.

3a. Incubate on thermal cycler for **1 hour at 16°C, with heated lid set at ≤40°C**(program: “***NEB2NDST***”)

3b. Remove Ampure XP beads from 4oC and let equilibrate to room temp (usually 30 min).

**1.5 Purify the Double-stranded cDNA using 1.8X Agencourt AMPure Beads:**

1. Vortex AMPure XP beads to Resuspend.

*Note: May need to invert tube, or even pipette mix to make sure beads not stuck to bottom of 1.5ml tube and fully in solution! When making new aliquots make sure bottom of stock bottle is not covered with beads by inverting bottle several times and visually confirming no beads stuck to bottom.*

2. **Add 72ul (1.8X) of resuspended AMPure XP beads** to each well of the second strand synthesis reaction. Mix well on a vortex mixer or by pipette mixing (10x).

3a. Incubate at room temperature for **5 minutes.**

3b. Prepare fresh 80% ethanol (full two-day protocol requires 1.6ml of 80% ethanol per sample)

*Total 80% ethanol needed: ­­­­­­­ 200 ul x 2 washes x \_\_\_\_\_\_samples =­­­­­­\_\_\_\_\_\_\_ml 80% EtOH*

*\*N.B. The ethanol wash steps for the AMPure remain at 200ul throughout the protocol (not halved like all the other reagents)*

*Can then Back-calculate: \_\_\_\_\_\_\_ml 80% EtOH=\_\_\_\_\_\_\_ml pure EtOH + \_\_\_\_ml water*

4. Quickly spin the plate *(in salad spinner)* to collect any sample on the sides of the wells. Place the plate on the magnetic rack 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 the DNA targets.**

5. Add **200ul of freshly prepared 80% ethanol** to each well while in the magnetic rack. Incubate at room temperature for 30 seconds, and then carefully remove and discard the supernatant.

6a. Repeat Step 5 once for a total of two washes.

6b. Spin down plate(s) in *salad spinner*, and then ***replace on magnet*** and remove all of the remaining supernatant with the p10 multichannel/singles as needed to ensure all ethanol is removed.

7. Air dry the beads for **~3 minutes** while the 24 well plate is on the magnetic rack with the caps off. **Do not overdry the beads. This may result in lower recovery of DNA target!**

8a. Take samples off the magnet, and elute the DNA target from the beads **into 30ul 10mM Tris-HCL or 0.1X TE buffer**. Mix well on a vortex mixer or by pipetting up and down.

8b. Quickly spin the plate in the *salad spinner* and then place it on the magnetic rack until the solution becomes clear (approx. 2min).

9. Remove **28.5ul of the supernatant** and transfer to a clean nuclease-free 24 well plate. *Cap each row as you go to ensure no mistakes/cross contamination of samples.*

***Stop Here (if doing protocol over 2 days) and place samples in -20°C freezer. While no one has done controlled experiments, NEB tech suggests that library is stable in freezer at this stage for months to years.***

**Day 2:**

**1.6: Perform End Repair of cDNA library:**

**\* take Day 1 out of freezer to thaw at RT (won’t thaw in time it takes to make MM on ice block)**

1a. Create End Repair mastermix:

|  |  |  |  |
| --- | --- | --- | --- |
| ***Component:*** | ***Volume/Sample*** | ***No. samples(\*1.1)*** | ***Total to add for MasterMix*** |
| NEBNext End Repair Reaction Buffer (10X) | 3.25ul |  |  |
| NEBNext End Prep Enzyme Mix (green) | 1.5ul |  |  |

***Total Volume of MM:***

1b. **Move library plate to ice block. Add 4.75ul to each well** of purified double stranded cDNA . Quick spin up to 1000rpm (STOP when gets to 1000rpm) on large centrifuge.

*\*can do this on ice block to keep samples cool, but need to thaw on ice first because when put directly onto ice block from freezer, samples may not thaw completely, preventing homogenous mixing*

***1c. Take adaptor out at this time to thaw and remove AMPure from 4°C (near end of incubation cycle. needs ~30min at room temp)***

2. Incubate samples in thermal cycler as follows (with the heated lid set at 750C) (program: “***ENDREPAR”***)

30 minutes at 20***°***C

30 minutes at 65***°***C

Hold at 4***°***C

3. Once temperature reaches 4***°***C, immediately proceed to Adaptor Ligation.

**1.7 Perform Adaptor Ligation*:***

*-Dilute the NEBNext Adaptor for Illumina (15uM) to 1.5 uM with a 10-fold dilution (1:9) with 10 mM Tris-HCl for immediate use.*

*Dilution calculation: (round up)*

\_\_\_\_ul diluted adaptor (calculation below)/10 =\_\_\_ul stock adaptor

\_\_\_ul stock adaptor (calculation last line)\*9 =\_\_\_\_\_ul 10 mM Tris-HCl

*\*Can round up amount of diluted adaptor needed to nearest 10 to simplify calculations (e.g. need 16.5ul diluted adaptor for 32 samples, make 20ul)*

1. Add the following components directly to the end prep reaction mixture. (**Caution: do not pre-mix the components to prevent adaptor-dimer formation**):

|  |  |  |  |
| --- | --- | --- | --- |
| ***Component:*** | ***Volume/Sample*** |  |  |
| Blunt/TA Ligase MasterMix (red) | 7.5 ul |  |  |
| **Diluted** NEBNext Adaptor (red) | 0.5 ul |  |  |
| Nuclease-free water | 1.25 ul |  |  |

2. Mix by pipetting followed by a quick spin up to 1000rpm (STOP when gets to 1000rpm) on large centrifuge.

3a. Incubate for **15 minutes at 20*°*C** **with the heated lid off** on thermal cycler (program: “***NEBLIGAT***”)

3b. A precipitate can form upon thawing of the **NEBNext Q5 Hot Start HiFi PCR Master Mix.** To ensure optimal performance, **place the master mix at room temperature** while purifying the ligation reaction. Once thawed, **gently mix by inverting the tube several times.**

3c. Remove the index primers from freezer.

**1.8 Purify the Ligation Reaction Using AMPure XP beads:**

**Note: if you are selection for larger size fragments (> 200 nt), follow the size selection recommendations in Appendix A, Chapter 4.**

1a. Vortex AMPure beads to Resuspend *(can leave out all day at RT after being removed from 4oC).*

1b. Add **8 ul nuclease-free H20 to each well** to adjust the final volume after ligation for a 50ul total volume (former volume was 43.25ul). *Use resevoir and p10 multichannel, or repeat electronic pipette. Can add 0.1ul water more to make up for volume omitted in end repair and adaptor ligation steps bc of electronic pipette if needed.*

2. Add **50 ul (1.0X) of resuspended AMPure XP beads** to the 50ul ligation reaction *(can reference chart on pg 13 in the appendix)*. Pipette mix well (at least 10X). *Use 200ul electronic multichannel-adding 22.4ul.*

3. Incubate at **room temperature for 5 minutes.**

4. Quickly spin down the plate in *salad spinner* and place the plate on the magnetic stand to separate the beads from the supernatant. After the solution is clear (about 5 minutes), discard the supernatant that contain unwanted fragments (*Use 300ul multichannel bc should have ~72.5ul supernatant, but was consistently a bit less, so can set to ~68-70ul).* **CAUTION: DO NOT DISCARD THE BEADS!!!**

5. Add 200 ul of freshly prepared 80% ethanol to the tubes while in the magnetic rack. Incubate at room temperature for 30 seconds, and then carefully remove and discard the supernatant.

6. Repeat Step 5 once for a total of 2 washes.

7. Quickly spin the plate and place it back on the magnetic .

8. Completely removed the residual ethanol with a p-10 and **air dry beads for 3 minutes** while the plate is on the magnetic rack with the lids off. **Do not overdry the beads**.

9. Remove the plate from the rack. Elute DNA target from the beads with **26 ul 10 mM Tris-HCl**. Mix well on a vortex mixer or by pipetting up and down, and **incubate for 2 minutes at room temperature**. Put the tube in the magnetic rack until the solution is clear.

10. **Transfer 25 ul of supernatant** to a clean PCR tube. Discard beads.

11. To the 25 ul supernatant, **add 25 ul (1.0X) of resuspended AMPure XP beads** and mix well by pipetting up and down 10 times.

12. Incubate for 5 minutes at room temperature.

13. Quickly spin the plate and place on magnetic rack to separate beads from supernatant. After the solution is clear (about 5 minutes), carefully remove the supernatant that contains unwanted DNA. Do not disturb the beads that contain the desired DNA targets *(~80ul-use 300ul multichannel pipette)*. **CAUTION: DO NOT DISCARD THE BEADS!!!**

14a. Add **200ul of 80% ethanol** to each well while in the magnetic stand. *(Use disposable reservoir for ethanol and 300ul multichannel).*

14b. Incubate at room temperature for 30 seconds, and then carefully remove and discard the supernatant.*(Use multichannel).*

15. Repeat step 14 once for a total of 2 wash steps.

16. Briefly spin the tube, and put the tube back in the magnetic rack.

17. Completely remove the residual ethanol with a p-10. Air dry the beads for **3 minutes** while the plate is on the magnetic stand with the caps off.

**Caution: Do not overdry the beads. This may result in lower recovery of the DNA target**.

18a. Remove the plate from the rack. Elute DNA target from the beads with **9.5 ul of 10mM Tris-HCl, pH 8.0**. Mix well on vortex mixer or by pipette mixing. Quickly spin down the plate on the *salad spinner. Make sure that the beads to not get pulled down to the bottom of the wells and stays in solution.*

18b. Incubate for **2 minutes at room temperature**. Plate plate on magnetic rack until the solution is clear (about 5 minutes).

19. Without disturbing the bead pellet, **transfer 8.5ul** to a new 24 well plate and proceed to PCR enrichment.

*Note: Be sure not to transfer any beads. Trace amounts of bead carry over may affect the optimal performance of the polymerase used in the subsequent PCR step.*

**1.9A PCR Library Enrichment:**

*(Assign unique indexed primer combinations to each sample on spreadsheet beforehand; Refer to manual for NEB E7600 NEBNext Multiplex Oligos for Illumina to determine how to assign unique indexed primer combinations. Indices need to be balanced for A,G,C,T.)*

1a. Add **1.5ul USER enzyme** and **12.5ul of NEBNext Q5 Hot Start HiFi PCR Master Mix** (blue) to each well. Mix by gently pipetting. *(use 10-100ul electronic repeat pipette- avoid contamination by ejecting MM on top side of tube without touching tip to tube and letting surface tension pull off/making sure full aliquot goes in). our repeater can only do 12.4 or 12.6 ul… looks like we need to do this step manually.*

1b. Add **1.25ul of F and 1.25ul of R index primer** appropriate for each well (see spreadsheet assignments). Mix by gentle pipetting. *Use p2 to individually add and mix directly into solution*

*\*\*Use aliquots of primers with individual lids and only keep one primer open at a time to avoid cross-contaminating primers and be very careful with tips, etc.*

1c. Quickly spin samples on large centrifuge up to 1000rpm (STOP when reaches 1000rpm).

2. **PCR Cycling Conditions**: *program set for 12 cycles, can adjust if needed). Check to make sure program is correct/hasn’t been altered. You can use 12-15 cycles but the fewer the cycles, the better.*

1. USER digestion: 37oC, 15 minutes

2. Initial Denaturation: 98***°***C, 30 seconds

3. Denaturation: 98***°***C, 10 seconds

4. Annealing/Extension: 65***°***C, 75 seconds

***Repeat step 3-4 for a total of 12 cycles***

5. Final Extension: 65***°***C, 5 minutes

6. Hold: 4***°***C, forever

3. While PCR cycles are running

a. label final cDNA 1.5 ml tubes with turtle ID, rna subsample, and “cDNA made: MM/DD/YY”

b. Prep Fragment analyzer (conditioning solution, gel, inlet buffer, rinse buffer) for QC of libraries

**1.10 Purifying PCR Library Enrichment**

*Note: X referes to the original sample volume from the above step.*

1. Vortex Agencourt AMPure XP Beads to resuspend.

2. Add **22.5 ul (0.9X) of resuspended Agencourt AMPure XP Beads** to the PCR reaction in each well. Mix well via vortex or pipette mixing (at least 10X).

3. Incubate for **5 minutes at room temperature**

4. Quickly spin the plate and place the tube on the magnetic stand to separate the beads from the supernatant. After the solution is clear (about 5 minutes), carefully remove and **discard the supernatant. Do not disturb the beads that contain the DNA targets.** (~50ul supernatant)

5. Add **200ul of 80% ethanol** to each well while in the magnetic rack. Incubate at room temperature for **30 seconds**, and then carefully remove and discard the supernatant.

6. Repeat step 5 (total of 2 washing steps). Then spin down plate(s) in *salad spinner*, and then ***replace on magnet*** and remove all of the remaining supernatant with the p10 multichannel/singles as needed to ensure all ethanol is removed.

7. Air dry the beads for **3 minutes** while the tube is on the magnetic rack with the caps off.

8a. Elute the DNA target from the beads into **11.5ul 0.1X TE or 10mM Tris-HCl**. Mix well via vortex or by pipetting mixing. Quickly spin plate in salad spinner.

8b. Incubate for **2 minutes at room temperature**. Place it on the magnetic stand until the solution is clear.

9. Transfer **10ul of the supernatant** to 1.5ml tubes and store at -20***°***C.

***Place samples on ice and proceed to library QC, OR put samples in -80°C if doing on different day.***

***Location and label of samples in -80°C freezer:*** *\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_*

***Library QC:***

***1. AATI Fragment Analyzer***

* *High sensitivity large fragment DNA kit (DNF-464)-see quick start protocol*
* *No sample dilution (but this may vary).*

**Tips and other Relevant/Random Notes**

* We did a qubit clean up test on libraries made from individual samples and found:

1. Doing this can ‘lose’ a large portion of the library yield, but does effectively get rid of adaptor and primer dimer issues.

* If see peak 200-400 on bioanalyzer (see Appendix A), should be good. But also sometimes will see split peaks in this range (either just two humps, or two completely split peaks), along with trailing humps into slightly larger sizes. Talking with Minyong at Q3B, these are most likely PCR artifacts that will be removed downstream and are not a problem (i.e., your libraries are still good).
* We are looking for a 300-500bp peak on the fragment analyzer. The Lower marker usually run between 15 and 20 minutes and if the pro size software doesn’t recognize the lower marker you can right click the peak and set as lower marker manually.

**SB’s Notes about 75bp spike:**

From a brief discussion with Jane 7/5/18

* Discussed ampure bead concentration problem 🡪 retry failed libraries with new bead aliquot
* Can pool together and clean up with beads at end
* Extra clean up only necessary for libraries that are about same concentration as spikes