NEB cDNA library preparation

Whitehead lab SOP: NEB Next Ultra RNA library preparation for Illumina (NEB#E7530S/L)-*adapted for ½ rxns*
Last updated: 10/17/14 (V4.0) by LK-JR

Preparer Name(s):_____
Date:_____
Project/Plate#/Samples Info:_____

Before Starting and General Notes:

- We are using ½ reaction volumes
- Protocol adapted from NEBNext Ultra RNA Library Prep Kit for Illumina (NEB #E7530)
- Extract RNA samples If starting from tissue, it is best to DNase-treat the RNA during the extraction protocol.
- Quantify RNA using Qubit or plate reader
- Check RNA quality on Bioanalyzer. For best results, use high quality RNA with a RIN>7
- Need 500ng Total RNA to start Starting total RNA concentration: we use either 250ng or 500ng. We have used 150ng a few times as well.
- This protocol targets a library size of 300-400bp; for different lengths, see NEB kit Appendix
- Store NEB Next Ultra RNA kit in -20°C
- Use spreadsheet with concentrations to determine amount sample + H_20 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
-****During steps using reservoirs, put the one(s) you are not using far away from you to avoid accidentally putting/picking up into/out of wrong reservoir (e.g. waste vs wash, etc.!!!)**

Reagents/Kits Needed:

NEB #E7530 NEBNext Ultra 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
80% Ethanol – **impt to be made fresh daily**

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
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Adaptor Ligation
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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 Berkeley QB3 Vincent J. Coates Genomics Sequencing Laboratory, which requires at least 10ul of 10nM of pooled library**

Day 1:

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

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 H_20	5ul		

Total Volume of MM: _____

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

mRNA Isolation, Fragmentation and Priming Starting with Total RNA

1. Dilute total RNA with nuclease-free H_20 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.*

2. Aliquot **NEBNext Oligo d(T)$_{25}$ beads** into a 2ml nuclease-free tube

*7.5ul beads *(____no.samples+2 samples)= _____ ul beads total*

Put on magnet until clear; then remove and discard supernatant (buffer that the beads come in)

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

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. 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.

12. 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.

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

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

15. 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.

16. Place the 24 well sample plate on the **magnetic plate at room temperature for 2 minutes**.

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

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

19a. Repeat steps 15-18.

19b. Impt 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 to achieve this.

20a. Add **25ul of Elution Buffer (Tris)** 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.

20b. Cap, quickly vortex and quick spin up to 1000rpm (STOP when gets to 1000rpm) on large centrifuge.

21. 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.

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

23. 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.*

24. Incubate the 24 well plate at **room temperature for 5 minutes**.

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

26. 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.

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

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

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

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

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

32. Wash the beads by adding **100ul of Elution Buffer (Tris)** to each well. Gently pipette mix entire volume to mix thoroughly (6x). *N.B. This is a wash because it only elutes with increased temperature incubation.*

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

34. Remove and discard all of the supernatant from the tube. 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.

35. Remove the plate from the magnetic rack.

36a. 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.**
Worked best to use 10-100ul electronic repeat pipetter to dispense into individual tubes, then pipette mix with manual p10 multichannel. Can gentle vortex if needed

36b. Quick spin up to 1000rpm (STOP when gets to 1000rpm) on large centrifuge and then incubate the samples at **94°C for 10 minutes (program: "*94-10MIN*").**
Note: This time will be different if your library size is not 300-400 bp (see NEB manual).

- During 10min, make the 1st strand cDNA synthesis mastermix.

36c. Immediately after place plate on magnetic rack.

37. Collect the purified mRNA by **transferring 5ul of the supernatant** to a clean nuclease-free 24 well plate.

38. Place the new 24 well plate with the purified mRNA on ice.

39a. **First Strand cDNA Synthesis:** Create mastermix and add to fragmented and primed mRNA.

Component:	***Volume/ Sample***	***No. samples(+2)***	***Total to add for MasterMix***
Murine RNase Inhibitor *(pink)*	0.25ul		
Protoscript II Reverse Transcriptase *(pink)*	0.5ul		
Nuclease-free H_20	4.25ul		

Total Volume of MM: _____

39b. **Add 5ul each well**. ***Samples on ice block while pipetting***

40a. Pipette mix, or light vortex/flick mix.

40b. Quick spin up to 1000rpm (STOP when gets to 1000rpm) on large centrifuge, then incubate for **50 minutes** at **42°C** *(Program: "**42-50MIN**").*

40c. Take the 2nd strand synthesis reaction buffer out of the freezer and thaw on ice.

41a. **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 H_20	24ul		
Second Strand Synthesis Reaction Buffer (10x-orange)	4ul		
Second Strand Synthesis Enzyme Mix (orange)	2ul		

Total Volume of MM: _____

41b. **Add 30ul to each well.** ***Samples on ice block while pipetting.***
42. Mix thoroughly by ***gentle pipette*** mixing, quick spin up to 1000rpm (STOP when gets to 1000rpm) on large centrifuge, then incubate on thermal cycler for **1 hour at 16°C, with heated lid set at ≤40°C** (program: "***NEB2NDST***")

43. Remove Ampure XP beads from 4°C and let equilibrate to room temp (usually 30 min)

Part II: Purify the Double-stranded cDNA using 1.8X Agencourt AMPure Beads
1. Vortex AMPure XP beads to Resuspend.

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).

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

4a. 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.

4b. After the solution is clear (~5 minutes), carefully remove and **discard the supernatant** *(~110 supernatant, so can use 300ul multichannel).* Do not disturb the beads that contain DNA targets.
While this is on rack, can prepare 80% ethanol if haven't done already during incubation steps.

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

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 (do these steps continuously).

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 **~5 minutes** while the 24 well plate is on the magnetic rack with the caps off. **Do not overdry!**

8a. Take samples off the magnet, and elute the DNA target from the beads **into 30ul nuclease-free H_20**. 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.

Day 2:

Part III: Perform End Repair/dA-tail of cDNA library

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. **Add 4.75ul to each well** of purified double stranded cDNA *(use electronic 10-100ul repeat pipette, but need to add 4.7ul bc only goes to one decimal)*
**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*

Take adaptor out at this time to thaw

2. Quick spin up to 1000rpm (STOP when gets to 1000rpm) on large centrifuge, then incubate samples in thermal cycler as follows (program: "***ENDREPAR"***)
30 minutes at 20℃
30 minutes at 65℃
Hold at 4℃

Remove AMPure from 4°C (near end of incubation cycle -needs ~30min at room temp)

3. Once temperature reaches 4℃, immediately proceed to Adaptor Ligation.

Part IV: Adaptor Ligation

-Dilute the NEBNext Adaptor for Illumina (15uM) to 1.5 uM with a 10-fold dilution (1:9) with nuclease-free water for immediate use.

Dilution calculation: (round up)
___ul diluted adaptor (calculation below)/10 =__ul stock adaptor
__ul stock adaptor (calculation last line)*9 =_____ul water

**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)*

1a. Make Ligation Mastermix:

Component:	***Volume/ Sample***	***No. samples(+1)****	***Total to add for MasterMix***
Blunt/TA Ligase MasterMix (red)	7.5 ul		
Diluted NEBNext Adaptor (red)	0.5 ul		
Nuclease-free water	1.25 ul		

don't need to do the 1.1 calculation for pipetting error* ***Total Volume of MM:* _____

1b. **Add 9.25ul to each well** *(use electronic 10-100ul repeat pipette, need to do 9.2ul, see above)*
N.B. that this is especially 'sticky'/viscous so need to be careful with pipetting to make sure right amount goes into each well.

2. Vortex and quick spin up to 1000rpm (STOP when gets to 1000rpm) on large centrifuge.

3. Incubate for **15 minutes at 20℃** on thermal cycler (program: "***NEBLIGAT***")

4. Add **1.5 ul of USER Enzyme** (red) to ligation mixture in each well (use single channel)

5. Mix well and incubate at **37℃ for 15 minutes** (program: "USERENZY")

Part V: Size Selection of Adaptor-ligated DNA

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

2. Add **6.75ul nuclease-free H_20 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.*

3. Add **22.5ul 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.*

4. Incubate at room temperature for 5 minutes.

5. 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), carefully transfer the supernatant containing your DNA to a new 24 well plate (*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 SUPERNATANT!!!
Discard the beads that have the unwanted large fragments

6. Add **10ul resuspended AMPure XP beads** to the supernatant, mix well (vortex) and incubate for 5 minutes at room temperature. *Use p10 multichannel.*

7a. Quickly spin the plate and place it on the magnetic stand to separate the beads from the supernatant.

7b. 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!!!**

- *Remove index primers and PCR Master from freezer now*

*-Make **fresh** 80% ethanol needed in falcon tube: 200 ul x 5 washes x _____samples =_____ml 80% EtOH*

-Then Back-calculate: _____ml 80% EtOH=_____ml pure EtOH + ____ml water

8a. Add **200ul of 80% ethanol** to each well while in the magnetic stand *(Use disposable reservoir for ethanol and 300ul multichannel).*

8b. Incubate at room temperature for 30 seconds, and then carefully remove and discard the supernatant *(Use another disposable reservoir for **waste** and multichannel).*

9. Repeat step 8 two more times (total of 3 washes). On third wash step, pull off ethanol then quick spin in salad spinner, put back on magnet and use p10 multichannel/single as needed to remove all ethanol.

10. Air dry the beads for **5-6 minutes** while the plate is on the magnetic stand with the caps off.

11a. Elute the DNA target from the beads into **14ul of 10mM Tris-HCl, pH 8.0**. *Seems easier to do this in two rounds pulling off 5.75ul each time with p10 multichannel vs. using the 100 multichannel bc p10 has narrower tips that avoid bead contamination, etc. If do this, have caps off of one row at a time and be very aware of which rows you have added 1 or 2x into to keep track.*

11b. Mix well on vortex mixer or via pipette mixing. Quickly spin down the plate on the *salad spinner* and place it on the magnetic stand. After the solution is clear (about 5 minutes), **transfer 11.5ul** to a new 24 well plate for amplification.

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 NEBNext High-Fidelity 2X PCR Master Mix in the subsequent PCR step.

Part VI: 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.)

1. Add **12.5ul of NEBNext High Fidelity PCR Master Mix,** 2X (blue) to each well *(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)*

2. Add **0.5ul of each F and R index primer** appropriate for each well (see spreadsheet assignments). *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.*

3. Quickly spin samples on large centrifuge up to 1000rpm (STOP when reaches 1000rpm). Put on thermocycler for PCR library enrichment program *(*program: "**NEBPCR**"; *program set for 12 cycles, can adjust if needed). Check to make sure program is correct/hasn't been altered-*

1. Initial Denaturation: 98℃, 30 seconds
2. Denaturation: 98℃, 10 seconds

3. Annealing: 65℃, 30 seconds
4. Extension: 72℃, 30 seconds *(repeat step 2-4 for 12 cycles)*
5. Final Extension: 72℃, 5 minutes
6. Hold: 4℃, forever

Take out bioanalyzer reagents needed to come to room temp and check to see if need to make more filtered gel, etc.

Part VII: Purify the PCR Reaction using Agencourt AMPure XP Beads
1. Vortex Agencourt AMPure XP Beads to resuspend.

2. Add **25 ul (1.0X) 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 **5 minutes** while the tube is on the magnetic rack with the caps off.

8. Elute the DNA target from the beads into **11.5ul nuclease-free water**. Mix well via vortex or by pipetting mixing. Quickly spin plate in *salad spinner* and place it on the magnetic stand until the solution is clear.

9. Transfer **10ul of the supernatant** to a new 24 well plate and store at -20℃.

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. Agilent Bioanalyzer
- *High sensitivity DNA kit-see SOP*
- *No sample dilution*
- *See Appendix A-add in info for thresholds/cutoffs of what traces should look like, etc*

2. Qubit

- *ds DNA HS assay kit*

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.
2. Qubit concentrations roughly correlate to FU on the bioanalyzer on the scale of roughly:

ug/ml in sample	Bioanalyzer FU
3.41	~150
0.888	~30
1.74	~60
4.37	~140
1.74	~60
1.44	~50

So, estimating if your library has a high enough yield for pooling for sequencing based on ~1ug/ml being the lowest acceptable, depending on various things-roughly 50-60FU is lowest cutoff to ballpark if a library is acceptable or should be redone. However, if there are moderate primer/adaptor dimer issues and loss of some yield during pooled cleanup, a more **conservative, practical cutoff may be 100FU.**

- If see peak 300-500 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).

- Randomly the Bioanalyzer seems to have trouble finding the upper marker, even though it's clearly there. This prevents it from converting time of run to BP. To fix this, click on the sample (one at time if needed), click on the peak tab->thenbox for the peak that corresponds to the marker (usually the last one)->select manually assign upper marker.