RNA-Seq Protocol for *Daphnia*

This protocol is intended for RNA sequencing from either whole or dissected small arthropods, such as *Daphnia.* Sequencing is done on an Illumina platform. We use Illumina HiSeq 2500 paired ends, as our target libraries are ~500bp. Generally, store RNA at -80°C until cDNA synthesis is performed. Use aseptic technique. Follow steps in order unless otherwise noted. Ensure all buffers are fully thawed before use.

Materials required:

- Equipment:
  - -80°C Freezer
  - -20°C Freezer
  - 4°C Fridge
  - Clean ice bucket; also, ready access to crushed ice
  - Thermocycler
  - Vortexer
  - Centrifuge
  - Bioanalyzer
  - Qubit
  - Nanodrop (optional)
  - Micropipettors & tips (filter-tips ideal)
  - Dissection kit (if needed)
  - Dissection microscope (if needed)
  - Earth magnet rack for magnetic beads. Appropriate size for your project (96-well & 12-tube here)
  - Very clean working space
- Kits:
  - Zymo Research Tissue & Insect RNA MicroPrep (R2030)
  - NEBNext Poly(A) mRNA Magnetic Isolation Module (NEB #E7490)
  - NEBNext Ultra RNA Library Prep Kit for Illumina (NEB #E7530)
  - NEBNext Multiplex Oligos for Illumina (Dual Index Primers Set 1) (NEB #E7600)
  - Qubit High Sensitivity DNA Kit
  - Qubit RNA Kit
- Consumables:
  - Bioanalyzer chips (RNA nano or pico and DNA)
  - Qubit reagents and standards
  - Qubit tubes
  - RNaseZap
  - Gloves; Lab Coat
  - Ampure XP Beads, or equivalent (such as home-made Serapure)
  - 1.7 mL microcentrifuge tubes (DNAse/RNAse-free)
  - 96-well plates (0.2 mL wells) or 0.2 mL tubes
  - Plate sealers, if using plates; caps if using tubes
  - 100% Ethanol (Molecular-grade; room temperature)
  - 80% Ethanol (freshly prepared; room temperature)
  - 0.1X TE, pH 8.0 (room temperature)
  - DNase (-20°C)
  - DNase Buffer Solution (-20°C)
  - 10 mM Tris-HCl, pH 7.5-8.0 (room temperature)
  - 10 mM NaCl (optional; room temperature)
  - DNAse/RNAse-free double distilled water (ddH_2O; room temperature and 4°C)

Sample prep (~30 min):

What sample preparation entails will depend on your project. You will be isolating mRNA from whatever you bring to the next portion of the protocol. If you are targeting ovaries, for instance, you can increase the efficiency of your sequencing efforts by dissecting the ovary out of the animal. Dissections should be performed in the cleanest available water (cold, nuclease-free water is preferable) and using sterilized tools. A Bunsen burner can be useful for sterilizing tools before use. Take care to not allow samples to over-dry and use them immediately after isolation. Micropipettors are useful for transferring small samples with minimal contamination. RNases are always at work, so *work quickly*. Plan to do RNA isolation immediately after sample prep.

RNA Isolation (~30-45 min):

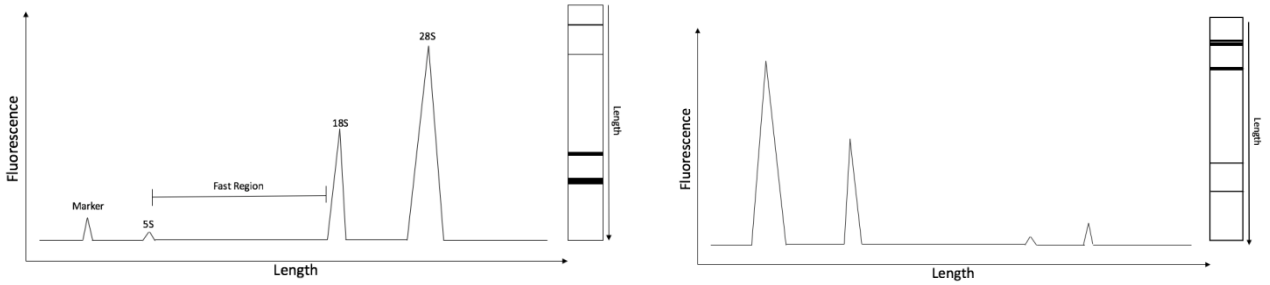
Always work in an RNase-free environment. Clean with RNase-Zap or equivalent. RNA can be isolated from a kit or using Trizol/Phenol-Chloroform Precipitation. This protocol refers to a Zymo RNA isolation kit. These kits provide a very clean RNA isolation in water. **NOTE**: The Zymo kit contains a one-time step to add 48 mL 100% ethanol to the 12 mL RNA Wash Buffer concentrate (R2030). Do this once before using the kit.

Online protocol: http://www.zymoresearch.com/downloads/dl/file/id/161/r2030i.pdf
*All centrifugation steps should be performed at 10,000-16,000 x g. Uses **two** Collection Tubes per extraction.*

1. Transfer your sample sample (up to 10 mg) into a ZR BashingBead™ Lysis Tube and add 800 µl RNA Lysis Buffer to this tube. **NOTE**: You can work directly with lysis if the sample should easily lyze (e.g., dissected gonads).
2. Cap and vortex for ~2-3 min. Doing this with the tube on its side seems to help.
3. **DNase I Digestion:** It is suggested you thaw the DNA Digestion Buffer at this point. Ice once thawed.
4. Centrifuge the ZR BashingBead™ Lysis Tube for 1 min.
5. Transfer 400 µL supernatant to a Zymo-Spin™ **IIIC** Column nested inside a Collection Tube and centrifuge for 30 s. Save the flow-through (what's in the Collection Tube)!
6. Add 400 µL ethanol (100%) to the flow-through in the Collection Tube (bottom tube) and mix well by pipetting up and down ~10 times.
7. Transfer the mixture to a Zymo-Spin™ **IC** Column nested inside a NEW Collection Tube and centrifuge for 30 s. Discard the flow-through by detaching the column from the Collection Tube, inverting the Collection Tube over a beaker, and reattaching the tubes.
8. **DNase I Digestion**: Add 400 µL RNA Wash Buffer to the column and centrifuge for 30 s. Discard the flow-through as before.
9. **DNase I Digestion**: Prepare a master mix containing 5 µL DNase I and 35 µL DNA Digestion Buffer for each sample in a 1.7 mL tube. Mix well by gentle inversion. Keep on ice until use.
10. **DNase I Digestion**: Add 40 µL of the DNaseI Reaction Mix to each column matrix. Incubate the column at room temperature (20-30°C) for 15 min. Continue to next step.
11. Add 400 µL RNA **Prep Buffer** to the column and centrifuge for 30 s. Discard the flow-through as before.
12. Add 700 µL RNA Wash Buffer to the column and centrifuge for 30 s. Discard the flow-through as before.
13. Add 400 µL RNA Wash Buffer to the column and centrifuge for 2 min to ensure complete removal of the wash buffer. Transfer the column carefully into a 1.7 mL tube.
14. Add 15 µL DNase/RNase-Free Water directly to the column matrix and centrifuge for 30 s. (Alternatively, for highly concentrated RNA use ≥ 6 µL elution).
15. Proceed immediately to quality control or store in a -80°C freezer (in B09).

Verify RNA Quality (~45 min):

It is advisable to confirm that RNA of appropriate quality and quantity was extracted from the sample. This step is not required, but highly recommended to avoid spending time and money on further steps. RNA quality can be assessed several ways, but a Bioanalyzer is the best option. This can tell you with high precision what RNA fragment sizes are present in your sample and whether more sampling is necessary. The following are hypothetical bioanalyzer electropherograms and gels.



Basically, you want to see high-weight peaks for the 18S and 28S rRNA subunits. If they are low molecular weight, or flattened, degredation occurred and this will affect all downstream processing. Quantify the amount of RNA using a Qubit RNA HS Assay Kit. Ensure all chemicals used are at room temperature. Keep sample on ice!

Online Qubit protocol here: https://tools.thermofisher.com/content/sfs/manuals/Qubit_RNA_Assay_QR.pdf

Purify mRNA from Total RNA (~2-3 hrs):

The extraction protocol will mostly yield ribosomal RNA (rRNA) which is generally not useful for transcript analysis. Therefore, we remove most of these rRNAs using a kit manufactured by NEB (E7490). This kit uses SPECIAL BEADS. **Do not use Ampure XP beads in this step**. Work in a clean environment free of RNase.

NOTE: For best results keep all the reagents used during the Poly(A) isolation except the NEBNext Oligo $d(T)_{25}$ beads, *on ice* when not in use. Starting material: 1–5 µg of DNAfree total RNA.

1. Dilute the total RNA with nuclease-free water to a final volume of 50 µL in a 0.2 mL PCR tube. Water quality is important. Store on ice until step 8.
2. In a second 0.2 mL PCR tube aliquot 20 µL of well resuspended NEBNext Magnetic Oligo $d(T)_{25}$ Beads.
3. Wash the beads by adding 100 µL of RNA binding buffer to the beads. Pipette the entire volume up and down 6 times to mix thoroughly.
4. Place this tube on the magnetic rack at room temperature for 2 min or until the solution is clear.
5. Remove and discard all of the supernatant from the tube. Take care not to disturb the beads.
6. Remove the tube from the magnetic rack.
7. Repeat steps 3–6 once for a total of 2 washes.
8. Resuspend the beads in 50 µL of RNA Binding Buffer and add the 50 µL of total RNA sample from step 1. Pipette the entire volume up and down to mix thoroughly.
9. Place the tubes on a thermal cycler and heat the sample at 65°C for 5 min and hold at 4°C to denature the RNA and facilitate binding of the polyA–RNA to the beads. Do not heat the lid.
10. Remove tubes 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 the tubes on the bench and incubate at room temperature for 5 min to allow the RNA to bind to the beads.
13. Resuspend the beads. Pipette up and down slowly 6 times to mix thoroughly.
14. Incubate for 5 more min on the bench at room temperature to allow the RNA to bind to the beads.
15. Place the tubes on the magnetic rack at room temperature for 2 min or until the solution is clear to separate the polyA–RNA bound to the beads from the solution.
16. Remove and discard all of the supernatant into a beaker. Take care not to disturb the beads.
17. Remove the tubes from the magnetic rack.
18. Wash the beads by adding 200 µL of Wash Buffer to remove unbound RNA. Pipette the entire volume up and down 6 times to mix thoroughly.
19. Place the tubes on the magnetic rack at room temperature for 2 min or until the solution is clear.
20. Remove and discard all the supernatant from each well of the plate. Take care not to disturb the beads.
21. Remove the tubes from the magnetic rack.
22. Repeat Steps 18–21.
23. Add 50 µL of Tris Buffer to each tube. Gently pipette the entire volume up and down 6 times to mix thoroughly. Tris is positively charged and may stick to the pipettor or tube. Be careful of this.
24. Place the tubes on a thermal cycler. Close the lid and heat the sample at 80°C for 2 min, then hold at 25°C to elute the polyA–RNA from the beads. Do not heat the lid.
25. Remove the tubes from the thermal cycler when the temperature reaches 25°C.
26. Add 50 µL of RNA Binding Buffer to each sample to allow the RNA to bind to the same beads. Gently pipette the entire volume up and down 6 times to mix thoroughly.
27. Incubate the tubes on the bench at room temperature for 5 min.
28. Resuspend the beads. Pipette up and down slowly 6 times to mix thoroughly.
29. Incubate the tubes on the bench at room temperature for 5 min to allow the RNA to bind to the beads.
30. Place the tubes on the magnetic stand at room temperature for 2 min or until the solution is clear.
31. Remove and discard all of the supernatant from each tube. Take care not to disturb the beads.
32. Remove the tubes from the magnetic rack.
33. Wash the beads once with 200 µL of Wash Buffer. Gently pipette the entire volume up and down 6 times to mix thoroughly.
34. Place the tubes on the magnetic rack at room temperature for 2 min or until the solution is clear.
35. Remove and discard all of the supernatant from each tube into a beaker. Take care not to disturb the beads.

    NOTE: It is important to remove **all** of the supernatant to successfully use the mRNA in downstream steps. Spin down the tube. Place the tube on the magnetic rack and with a 10 µL tip remove all wash buffer. (Caution: Do not disturb beads that contain the mRNA). !!!Do not overdry the beads!!!

36. Remove the tubes from the magnetic rack.

37. Elute the mRNA from the beads by adding 17 µL of the Tris Buffer, mix by pipetting 6 times and incubating the sample at +80°C for 2 min, then hold at 25°C to elute the polyA-RNA from the beads. Immediately, place the tubes on the magnetic rack for 2 min or until the solution is clear.
38. Collect the purified mRNA by transferring the supernatant to a new, labeled PCR Tube.
39. Place tube on ice. Return all reagents to freezer, fridge, etc.

NOTE: mRNA has not been reverse transcribed to cDNA yet. It is still fairly fragile, and should be stored on ice or in the -80C freezer until the next steps. However, avoid freeze-thaw cycles, as this degrades DNA and RNA. Ideally, continue until cDNA has been created to maximize yields.

Verify mRNA Quality (~45 min):

As before, it is advisable to confirm the purified mRNA quality using a Bioanalyzer. Now, we are interested in the yield and size distribution of the purified mRNA. 18S and 28S peaks should be diminished or gone entirely, as only poly(A) RNA should have been captured by the previous steps. Also quantify RNA using a Qubit.

mRNA Illumina Library Prep:

This protocol takes about two days. It can be done in one day if you are superman. Broadly speaking, we are reverse transcribing mRNA into cDNA and preparing it for Illumina sequencing. The cDNA synthesis should be finished before you stop working.

First Strand cDNA Synthesis (~60 min):

1. Make a master mix of the following (pink tube caps): 0.5 µL Murine RNase Inhibitor, 1 µL ProtoScript II Reverse Transcriptase, 3.5 µL Nuclease-free water.
2. Add 5 µL master mix to each sample for a final volume of 20 µL (assumes 2 µL used; 1 µL for Qubit and 1 µL for Bioanalyzer).

   NOTE: If you are following recommendations in Appendix A, for longer RNA fragments increase the incubation at 42°C from 15 min to 50 min in Step 2.

3. Incubate the sample in a preheated thermal cycler as follows:

   10 min at 25°C
   15 min at 42°C
   15 min at 70°C
   Hold at 4°C

4. **Immediately**, perform second strand cDNA synthesis reaction.

Perform Second Strand cDNA Synthesis (~70 min):

1. Make a master mix of the following (orange tube caps): 48 µL Nuclease-free water, 8 µL Second Strand Synthesis Reaction Buffer (10X), 4 µL Second Strand Synthesis Enzyme Mix.
2. Add 60 µL of this master mix to each First Strand Synthesis reaction (20 µL) from the last reaction.
3. Mix thoroughly by gentle pipetting.
4. Incubate in a thermal cycler for 1 hour at 16°C, with heated lid set at 40°C.

Purify the Double-stranded cDNA Using 1.8X Agencourt AMPure XP Beads (~45 min)

1. Thoroughly vortex AMPure XP Beads to resuspend and use at room temperature. Verify the opacity of the solution is not greater at the bottom of the bottle!
2. Add 144 µL (1.8X) of resuspended AMPure XP Beads to the second strand synthesis reaction (~80 µL). Mix well on a vortex mixer or by pipetting up and down at least 10 times. Do not pipet too fast. This can cause the beads to splash up into the pippetor.
3. Incubate for 5 min at room temperature.
4. Quickly spin the tube in a microcentrifuge to collect any sample on the sides of the tube. Place the tube on an appropriate magnetic rack to separate beads from supernatant. After the solution is clear (about 5 min), carefully remove and discard the supernatant. Be careful not to disturb the beads that contain DNA targets.
5. Add 200 µL of **freshly prepared** 80% ethanol to the tube while in the magnetic rack. Incubate at room temperature for 30 s, and then carefully remove and discard the supernatant into a beaker.
6. Repeat Step 5 once for a total of 2 washing steps.
7. Air dry the beads for 5 min while the tube is on the magnetic rack with lid open. !!!Do not overdry!!!

8. Remove the tube from the magnet. Elute the DNA target from the beads into 60 µL 0.1X TE Buffer or 10 mM Tris-HCl pH 8.0. Mix well on a vortex mixer or by pipetting up and down.
9. Quickly spin the tube and incubate for 2 min at room temperature. Place the tube in the magnetic rack until the solution is clear.
10. Remove 55.5 µL of the supernatant and transfer to a **new, labeled 0.2 mL PCR tube**.

>>>>>>>> **NOTE: If you need to stop at this point in the protocol samples can be stored at –20°C.** <<<<<<<<

Perform End Repair of cDNA Library (~70 min)

1. Make a master mix of the following (green tube caps): 6.5 µL NEBNext End Repair Reaction Buffer (10X), 8 µL NEBNext End Prep Enzyme Mix
2. Add 14.5 µL of this master mix to the 55.5 µL purified double stranded cDNA from the last step.
3. Incubate the sample in a thermal cycler (with the heated lid set at 75°C) as follows:

   30 min at 20°C
   30 min at 65°C
   Hold at 4°C

4. Proceed immediately to Adaptor Ligation using this End Repaired Reaction.

Perform Adaptor Ligation

Dilute the (red) NEBNext Adaptor for Illumina (15 µM) to 1.5 µM with a 10-fold dilution (1:9) with 10 mM TrisHCl and 10 mM NaCl for immediate use.*

1. Add the following components directly to the prep reaction mixture. (**Caution: do not premix the components to prevent adaptor-dimer formation; NO MASTER MIX**):

   End Repaired Reaction 65 µL
   (red) Blunt/TA Ligase Master Mix 15 µL
   Diluted **1.5 µM** NEBNext Adaptor* 1 µL
   Nuclease-free Water 2.5 µL

   Total volume 83.5 µL

   **The adaptor is provided in NEBNext Singleplex (NEB #E7350) or NEBNext Multiplex (NEB #E7335, #E7500, #E6609, #E7710, #E7730, or #E7600) Oligos for Illumina.*

2. Mix by pipetting followed by a quick spin to collect all liquid from the sides of the tube.
3. Incubate 15 min at 20°C in a thermal cycler. Turn off the heated lid on the thermal cycler.
4. Add 3 µL of (red) USER Enzyme to the ligation mixture from Step 3. Use new pipette tips for each reaction. Mix well by flicking the tube(s) and incubate at 37°C for 15 min.

   NOTE: This step is only required for use with NEBNext Adaptors. USER Enzyme can be found in the NEBNext SinglePlex (NEB#E7350) or Multiplex (NEB #E7335, #E7500, #E7710, #E7730, #E6609, #E7600) Oligos for Illumina.

Purify the Ligation Reaction Using AMPure XP Beads (~90 min)

NOTE: If you are selecting for larger size fragments (> 200 nt) follow the size selection recommendations in Appendix A, Chapter 4.

*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 (this step). Once thawed, gently mix by inverting the tube several times (no vortex).*

1. Add 12.5 µL of nuclease-free water to the ligation reaction to bring the reaction volume to 100 µL.
   NOTE: X refers to the original sample volume of 100 µL from the above step.
2. Add 100 µL (1.0X) resuspended AMPure XP Beads and mix well on a vortex mixer or by pipetting up and down at least 10 times.
3. Incubate for 5 min at room temperature.
4. *Quickly* spin the tube in a microcentrifuge and place the tube on an appropriate magnetic rack to separate beads from the supernatant. After the solution is clear (about 5 min), discard the supernatant that contain unwanted fragments into a beaker (Caution: do not discard the beads).
5. Add 200 µL of **freshly prepared** 80% ethanol to the tube while in the magnetic rack. Incubate at room temperature for 30 s, and then carefully remove and discard the supernatant into a beaker.

6. Repeat Step 5 once for a total of 2 washing steps.
7. Briefly spin the tube, and put the tube back in the magnetic rack.
8. **Completely** remove the residual ethanol, and air dry beads for 5 min while the tube is on the magnetic rack with the lid open. !!!Do not overdry!!! Watch for cracking/drying.
9. Caution: *Do not overdry the beads.* This may result in lower recovery of DNA target.
10. Remove the tube from the rack. Elute DNA target from the beads with 52 µL 0.1X TE or 10 mM Tris-HCl.
11. Mix well on a vortex mixer or by pipetting up and down, and incubate for 2 min at room temperature. Put the tube in the magnetic rack until the solution is clear.
11. Transfer 50 µL supernatant to a clean, labeled 0.2 mL tube. Discard beads and old tube.
12. To the 50 µL supernatant in the new tube, add **50 µL (1.0X)** of the resuspended AMPure XP Beads and mix well on a vortex mixer or by pipetting up and down at least 10 times.
13. Incubate for 5 min at room temperature.
14. Quickly spin the tube in a microcentrifuge and place the tube on an appropriate magnetic rack to separate beads from the supernatant. After the solution is clear (about 5 min), discard the supernatant that contains unwanted fragments into a beaker (Caution: do not discard the beads).
15. Add 200 µL of **freshly prepared** 80% ethanol to the tube while in the magnetic rack. Incubate at room temperature for 30 s, and then carefully remove and discard the supernatant into a beaker.
16. Repeat Step 14 once for a total of 2 washing steps.
17. Briefly spin the tube, and put the tube back in the magnetic rack.
18. **Completely** remove the residual ethanol, and air dry beads for 5 min while the tube is on the magnetic rack with the lid open. !!!Do not overdry!!! Watch for cracking/drying.
19. Remove the tube from the rack. Elute DNA target from the beads with 22 µL 0.1X TE or 10 mM TrisHCl.
12. Mix well on a vortex mixer or by pipetting up and down, and incubate for 2 min at room temperature. Put the tube in the magnetic rack until the solution is clear.
20. Without disturbing the bead pellet, transfer **20 µL** of the supernatant to a clean, labeled PCR tube and proceed to PCR enrichment. You can choose to retain the old tube, but ensure it is clearly labeled & store at 4°C or -20°C.

PCR Enrichment of Adaptor Ligated DNA (~50 min)

Follow **Procedure A** if you are using the following oligos (10 µM primer). At the time of writing, the Xu lab uses Procedure A with NEB #E7600:

NEBNext Singleplex Oligos for Illumina (NEB #E7350); NEBNext Multiplex Oligos for Illumina (Set 1, NEB #E7335; Set 2, NEB #E7500; Set 3, NEB #E7710; Set 4, NEB #E7730); NEBNext Multiplex Oligos for Illumina (Dual Index Primers, NEB #E7600)

Follow **Procedure B** if you are using NEBNext Multiplex Oligos for Illumina (96 Index Primers, NEB #E6609).

Procedure A PCR Library Enrichment (Skip if using Procedure B):

WARNING: Take care to to write exactly which samples have what primer combinations!
If you forget, your data are worthless!

1. To the cDNA (20 µL) from Step 19 Section 1.8 add the following components and mix by gentle pipetting (do **not** make a master mix):

   (blue) NEBNext Q5 Hot Start HiFi PCR Master Mix 2X 25 µL
   (blue) Index (X) Primer/i7 Primer[1,2] 2.5 µL
   (blue) Universal PCR Primer/i5 Primer[1,3] 2.5 µL

   Total volume 50 µL

   [1] The primers are provided in NEBNext Singleplex (NEB #E7350) or Multiplex (NEB #E7335, #E7500, #E7710, #E7730, #E7600) Oligos for Illumina. **For use with Dual Index Primers (NEB #E7600), look at the NEB #E7600 manual for valid barcode combinations and tips for setting up PCR reactions**.
   [2] For use with NEBNext Multiplex Oligos (NEB #E7335, #E7500, #E7710, #E7730) use only one Index Primer per PCR reaction. **For use with Dual Index Primers (NEB #E7600) use only one i7 Primer per reaction**.
   [3] **For use with Dual Index Primers (NEB #E7600) use only one i5 Primer per reaction**.

Link to #E7600 manual: https://www.neb.com/products/e7600-nebnext-multiplex-oligos-for-illumina-dual-index-primers-set-1#tabselect2

2. PCR Cycling Conditions

Cycle step	Temperature	Time	Cycles
Initial denaturation	98°C	30 s	1
Denaturation Annealing/Extension	98°C 65°C	10 s 75 s	12-15[1,2]
Final Extension	65°C	5 min	1
Hold	4°C	∞	

[1] The number of PCR cycles should be adjusted based on RNA input. If 10 ng total RNA is the starting input, it is recommended to perform 15 cycles of PCR.

[2] It is important to limit the number of PCR cycles to avoid overamplification. If overamplification occurs, larger molecular weight products (> 500 bp) will appear on the Bioanalyzer trace.

3. Proceed to Purify the PCR Reaction.

Procedure B PCR Library Enrichment (skip if using Procedure A):

1. To the cDNA (20 µL) from Step 19 Section 1.8 add the following components and mix by gentle pipetting:

   (blue) NEBNext Q5 Hot Start HiFi PCR Master Mix 2X 25 µL
   (blue) Index/ Universal Primer Mix* 5 µL

   Total volume 50 µL

   * The primers are provided in NEBNext Multiplex Oligos for Illumina, NEB #E6609. Refer to NEB #E6609 manual for valid barcode combinations and tips for setting up PCR reactions.

2. PCR Cycling Conditions:

Cycle step	Temperature	Time	Cycles
Initial denaturation	98°C	30 s	1
Denaturation Annealing/Extension	98°C 65°C	10 s 75 s	12-15*,**
Final Extension	65°C	5 min	1
Hold	4°C	∞	

[1] The number of PCR cycles should be adjusted based on RNA input. If 10 ng total RNA is the starting input, it is recommended to perform 15 cycles of PCR.

[2] It is important to limit the number of PCR cycles to avoid overamplification. If overamplification occurs, larger molecular weight products (> 500 bp) will appear on the Bioanalyzer trace.

3. Proceed to Purifying the PCR Reaction.

Purify the PCR Reaction using Agencourt AMPure XP Beads (~50 min)

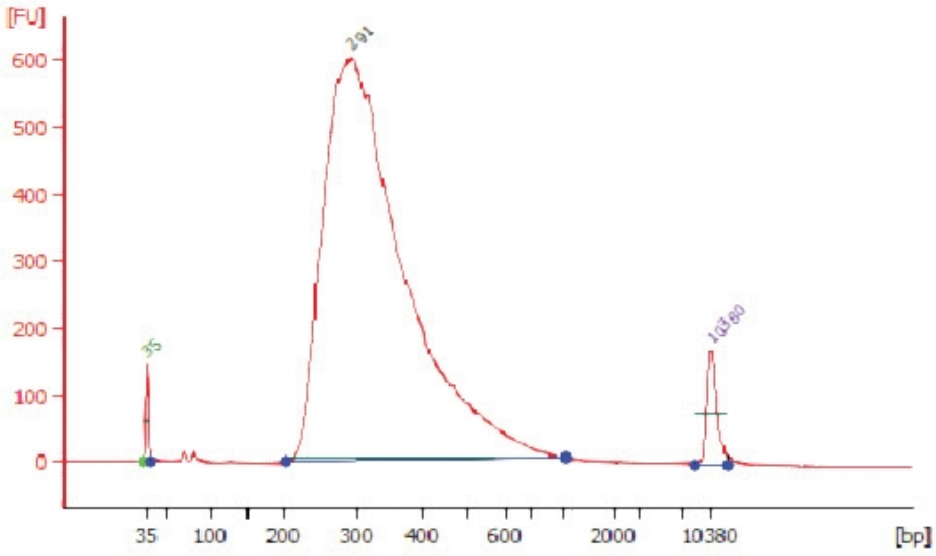
NOTE: X refers to the original sample volume from the above step.

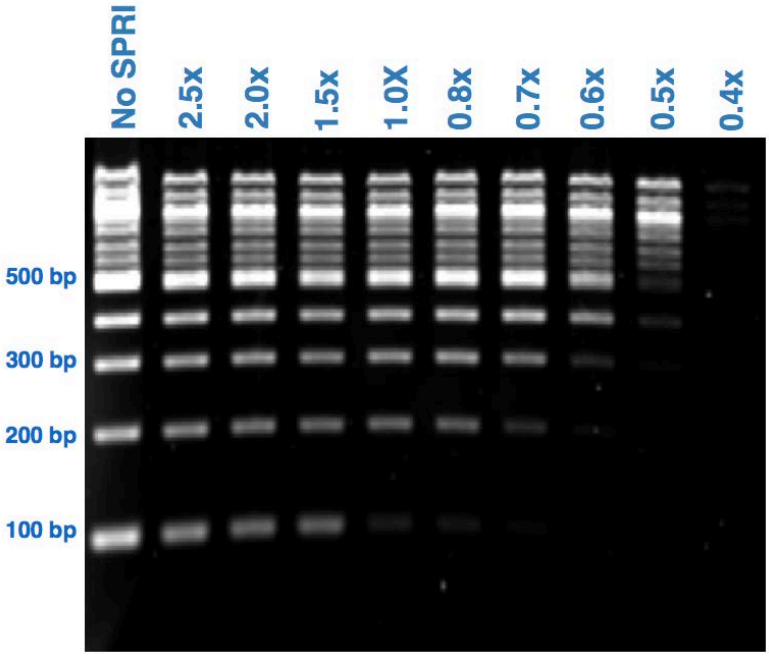
1. Vortex Agencourt AMPure XP Beads to resuspend. Make sure they are very well mixed. Don't let the beads sit around before using. Use at room temperature.
2. Add 45 µL (0.9X) of resuspended Agencourt AMPure XP Beads to the PCR reaction (~ 50 µL). Mix well on a vortex mixer or by pipetting up and down at least 10 times.
3. Incubate for 5 min at room temperature.
4. *Quickly* spin the tube in a microcentrifuge and place the tube on an appropriate magnetic rack to separate beads from the supernatant. After the solution is clear (about 5 min), carefully remove and discard the supernatant into a beaker. Be careful not to disturb the beads that contain DNA targets.

5. Add 200 µL of **freshly prepared** 80% ethanol to the tube while in the magnetic rack. Incubate at room temperature for 30 s, and then carefully remove and discard the supernatant into a beaker.
6. Repeat Step 5 once for a total of 2 washing steps.
7. Air dry the beads for 5 min while the tube is on the magnetic rack with the lid open. !!!Do not overdry!!!
8. Remove the tube from the rack. Elute the DNA target from the beads into 23 µL 0.1X TE. Mix well on a vortex mixer or by pipetting up and down, quickly spin the tube in a microcentrifuge and incubate for 2 min at room temperature. Place it in the magnetic rack until the solution is clear.
9. Transfer **20 µL** of the supernatant to a clean, well-labeled 0.2 mL tube, and store at –20°C.

Assess library quality on a Bioanalyzer® (Agilent High Sensitivity Chip or DNA Chip; ~45 mins).

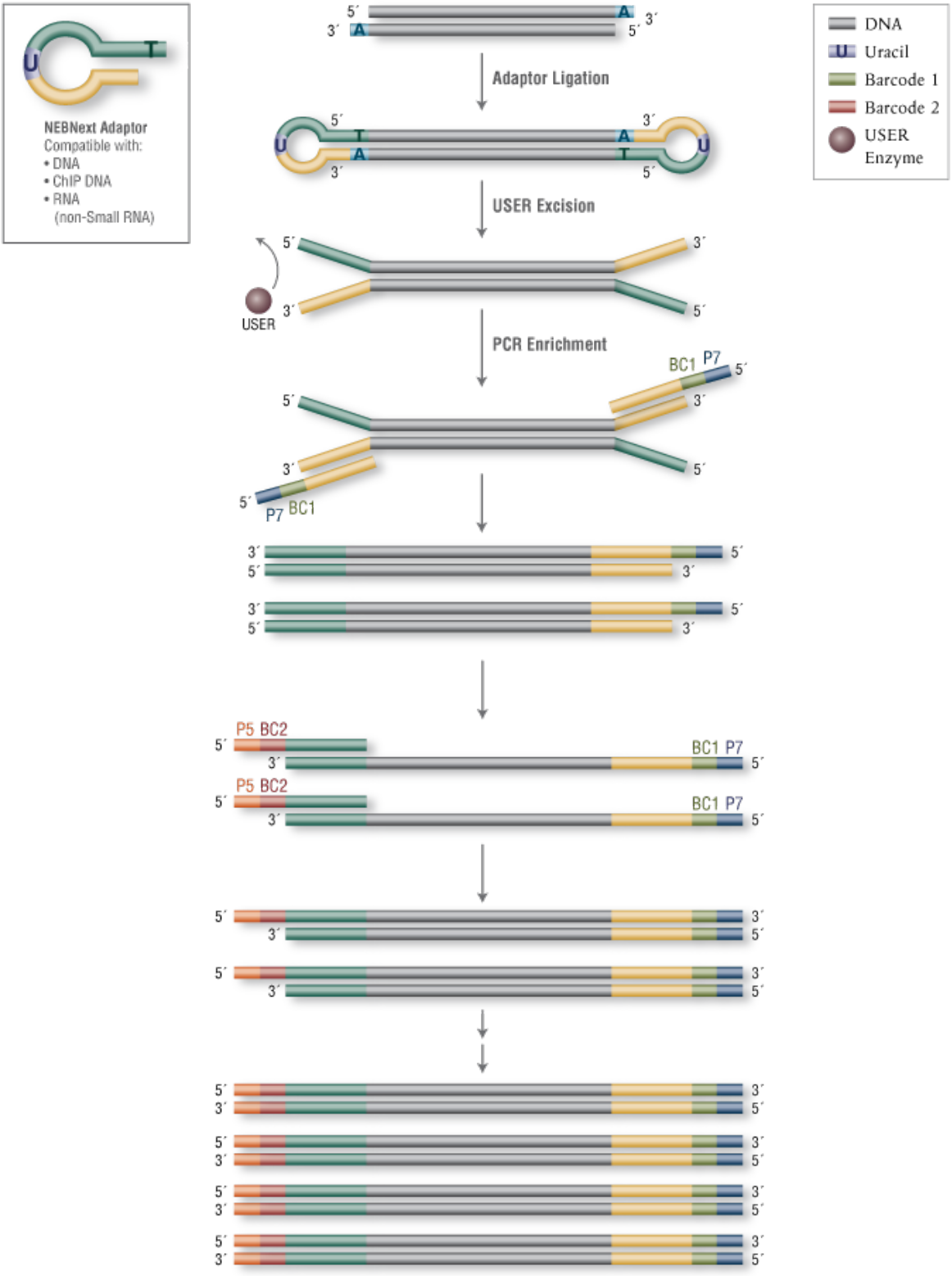
1. Dilute 2–3 µL of the library in 10 mM Tris or 0.1X TE.
2. Run 1 µL in a DNA High Sensitivity Chip.
3. Check that the electropherogram shows a narrow distribution with a peak size approximately 300 bp. Note: If a peak at ~ 80 bp (primers) or 128 bp (adaptor-dimer) is shown in the Bioanalyzer traces; Bring up the sample volume (Step 9, Section 1.10) to 50 µL exactly with nuclease-free water and repeat the AMPure XP Bead clean up step (Section 1.10).





Ampure XP, NEBNext Oligo d(T)$_{25}$, and Seramag Speedbeads all work the same way in regards to magnets. Generally, nucleotides are weakly negatively charged and the beads are weakly positively charged. When combined, nucleotides bind to the beads with an affinity proportional to the relative concentration of beads used (see gel above). In the presence of a magnetic field, the beads will physically move and bring the nucleotides with them leaving behind the fluid (and other chemicals) they were suspended in. Adding positively charged Tris-HCl or TE will remove nucleotides from the beads and allow you to remove a solution containing either the beads+nucleotides (no-magnet) or just the nucleotides (after placing on a magnet).

Important precautions: Avoid disturbing the bead pellet when beads are drawn to a magnet. Do not allow a bead+nucleotide clump to dry to the point of cracking. Remove all ethanol after wash steps. Mix beads thoroughly with a vortex before using, and use at room temperature. Use aliquots of beads so that the majority of the stock can remain at 4°C.


NEBNext Adaptor
Compatible with:
• DNA
• ChIP DNA
• RNA
(non-Small RNA)
DNA
Uracil
Barcode 1
Barcode 2
USER Enzyme
Adaptor Ligation
USER Excision
PCR Enrichment
USER
P5 BC2
BC1 P7