Protocol for Enrichment of mRNAs, excluding Globin mRNA, from Whole Blood Total RNA

Overview

This protocol describes the enrichment of poly(A) mRNA followed by globin mRNA & rRNA depletion (Section 1 and 2). The enriched RNA contains only mRNA (excluding globin) and not non-coding RNA. The enriched RNA is then used as input for directional RNA library preparation for sequencing on an Illumina instrument (Section 3).

This protocol requires the following NEBNext products:

- NEBNext Poly(A) mRNA Magnetic Isolation Module (NEB #E7490)
- NEBNext Globin and rRNA Depletion Kit (Human/Mouse/Rat) with RNA Sample Purification Beads (NEB #E7755)
- NEBNext Ultra II Directional RNA Library Prep for Illumina with Sample Purification Beads (NEB #E7765)

Symbols

	This is a point where you can safely stop the protocol and store the samples prior to proceeding to the next step in the protocol.
⚠	*This caution sign signifies a step in the protocol that has two paths leading to the same end point.*
●	*Colored bullets indicate the cap color of the reagent to be added.*

RNA Sample Requirements

RNA Integrity:
Assess the size and quality of the input RNA by running the RNA sample on an Agilent Bioanalyzer RNA 6000 Nano/Pico Chip to determine the RNA Integrity Number (RIN). For Poly(A) mRNA enrichment, high quality RNA with RIN Score >7 is required.

RNA Sample:
The RNA sample should be free of salts (e.g., Mg^{2+}, or guanidinium salts) or organics (e.g., phenol and ethanol). RNA must be free of DNA. gDNA is a common contaminant in RNA preps. It may be carried over from the interphase of organic extractions or when the silica matrix of solid phase RNA purification methods is overloaded. If the total RNA sample may contain gDNA contamination, treat the sample with DNase I (not provided in this kit) to remove all traces of DNA. After treatment, the DNase I should be removed from the sample. Any residual DNase I may degrade the oligos necessary for the enrichment.

Input Amount:

This protocol has been tested with 100 ng human whole blood total RNA (DNA-free) in a maximum of 50 µl of nuclease-free water, quantified by an RNA-specific dye-assisted fluorometric method (Qubit®) and quality checked by Bioanalyzer.

Keep all buffers on ice, unless otherwise indicated.

1.0. Poly(A) mRNA Enrichment using the NEBNext Poly(A) mRNA Magnetic Isolation Module (NEB #E7490)

1.1. Dilute the total RNA with nuclease-free water to a final volume of 50 µl in a nuclease-free 0.2 ml PCR tube and keep on ice.

1.2. To wash the Oligo (dT) beads, add the components from the table below to a 1.5 ml nuclease-free tube. If preparing multiple libraries, beads for up to 10 samples can be added to a single 1.5 ml tube for subsequent washes (use magnet NEB #S1506 for 1.5 ml tubes). The purpose of this step is to bring the beads from the storage buffer into the binding buffer. The 2X Binding Buffer does not have to be diluted for this step.

COMPONENT	VOLUME PER ONE LIBRARY
NEBNext Oligo d(T)$_{25}$ beads	20 µl
NEBNext RNA Binding Buffer (2X)	100 µl
Total volume	**120 µl**

1.3. Wash the beads by pipetting up and down six times.

1.4. Place the tube on the magnet and incubate at room temperature until the solution is clear (~2 minutes).

1.5. Remove and discard all of the supernatant from the tube. Take care not to disturb the beads.

1.6. Remove the tube from the magnetic rack.

1.7. Add 100 µl RNA Binding Buffer (2X) to the beads and wash by pipetting up and down six times. If preparing multiple libraries, add 100 µl RNA Binding Buffer (2X) per sample. The Binding Buffer does not have to be diluted.

1.8. Place the tubes on the magnet and incubate at room temperature until the solution is clear (~2 minutes).

1.9. Remove and discard the supernatant from the tube. Take care not to disturb the beads.

1.10. Add 50 µl RNA Binding Buffer (2X) to the beads and mix by pipetting up and down until beads are homogenous. If preparing multiple libraries, add 50 µl RNA Binding Buffer (2X) per sample.

1.11. Add 50 µl beads to each RNA sample from Step 1.1 Mix thoroughly by pipetting up and down six times. This binding step removes most of the non-target RNA.

1.12. Place the tube in a thermocycler and close the lid. Heat the sample at **65°C for 5 minutes and cool to 4°C** with the heated lid set at ≥ 75°C. This step will denature the RNA and facilitate binding of the mRNA to the beads.

1.13. Remove the tube from the thermocycler when the temperature reaches 4°C.

1.14. Mix thoroughly by pipetting up and down six times. Place the tube on the bench and incubate at room temperature for 5 minutes to allow the mRNA to bind to the beads.

1.15. Place the tube on the magnetic rack at room temperature until the solution is clear (~2 minutes).

1.16. Remove and discard all of the supernatant. Take care not to disturb the beads.

1.17. Remove the tube from the magnetic rack.

1.18. To remove unbound RNA add 200 µl of Wash Buffer to the tube. Gently pipette the entire volume up and down 6 times to mix thoroughly.

1.19 Spin down the tube briefly to collect the liquid from the wall and lid of the tube.

Note: It is important to spin down the tube to prevent carryover of the Wash Buffer in subsequent steps.

1.20. Place the tube on the magnetic rack at room temperature until the solution is clear (~2 minutes).

1.21. Remove and discard all of the supernatant from the tube. Take care not to disturb the beads containing the mRNA.

1.22. Remove the tube from the magnetic rack.

1.23. Repeat steps 1.18.–1.21.

1.24. Add 11 µl of nuclease-free water to each tube. Gently pipette up and down 6 times to mix thoroughly.

1.25. Place the tube in the thermocycler. Close the lid and heat the samples at **80°C for 2 minutes, then cool to 25°C** with the heated lid set at ≥ 90°C to elute the mRNA from the beads.

1.26. Remove the tube from the thermocycler when the temperature reaches 25°C.

1.27 Immediately place the tube on the magnet at room temperature until the solution is clear (~2 minutes).

1.28. Collect the purified mRNA by transferring 10 µl of the supernatant to a clean nuclease-free PCR tube.

1.29. Place the RNA on ice and proceed to the Globin and rRNA Depletion in Section 2.

2.0. Globin and rRNA Depletion using the NEBNext Globin and rRNA Depletion Kit (NEB #E7750/E7755)

2.1 Probe Hybridization to RNA

2.1.2. Assemble the following RNA/Probe hybridization reaction on ice:

RNA/PROBE HYBRIDIZATION REACTION	VOLUME
○ (white) mRNA in nuclease-free water (Step 1.29)	10 µl
○ (white) NEBNext Globin and rRNA Depletion Solution	3 µl
○ (white) NEBNext Probe Hybridization Buffer	2 µl
Total Volume	**15 µl**

2.1.3. Mix thoroughly by gently pipetting up and down at least 10 times. Note: It's crucial to mix well at this step.

2.1.4. Briefly spin down the tube in a microcentrifuge to collect the liquid from the side of the tube.

2.1.5. Place the tube in a pre-heated thermocycler and run the following program with the heated lid set at 105°C. This program will take approximately 15–20 minutes to complete:

TEMPERATURE	TIME
95°C	2 minutes
Ramp down to 22°C	0.1°C/sec
Hold at 22°C	5 minutes

2.1.6. Briefly spin down the tube in a microcentrifuge, and place on ice. Proceed immediately to the RNase H digestion.

2.2. RNase H Digestion

2.2.1. Assemble the following RNase H digestion reaction on ice:

RNA H DIGESTION REACTION	VOLUME
Hybridized RNA (Step 1.1.6)	15 µl
○ (white) NEBNext Thermostable RNase H	2 µl
○ (white) RNase H Reaction Buffer	2 µl
Nuclease-free Water	1 µl
Total Volume	**20 µl**

2.2.2. Mix thoroughly by gently pipetting up and down at least 10 times.

2.2.3. Briefly spin down the tube in a microcentrifuge.

2.2.4. Incubate the tube in a pre-heated thermocycler for **30 minutes at 50°C** with the lid set at 55°C.

2.2.5. Briefly spin down the tube in a microcentrifuge, and place on ice. Proceed immediately to the DNase I digestion.

2.3. DNase I Digestion

2.3.1. Assemble the following DNase I digestion reaction on ice:

DNASE I DIGESTION REACTION	VOLUME
RNase H treated RNA (Step 1.2.5)	20 µl
○ (white) DNase I Reaction Buffer	5 µl
○ (white) NEBNext DNase I	2.5 µl
Nuclease-free Water	22.5 µl
Total Volume	**50 µl**

2.3.2. Mix thoroughly by pipetting up and down at least 10 times.

2.3.3. Briefly spin down the tube in a microcentrifuge.

2.3.4. Incubate the tube in a pre-heated thermocycler for **30 minutes at 37°C** with the lid set at 40°C or off.

2.3.5. Briefly spin down the tube in a microcentrifuge, and place on ice. Proceed immediately to the RNA Purification step.

2.4. RNA Purification Using Agencourt RNAClean XP Beads or NEBNext RNA Sample Purification Beads

2.4.1. Vortex the Agencourt RNAClean XP Beads or NEBNext RNA Sample Purification Beads to resuspend.

2.4.2. Add 90 µl (1.8X) beads to the RNA Sample from Step 2.3.5 and mix thoroughly by pipetting up and down at least 10 times.

2.4.3. Incubate the tube for **15 minutes on ice** to bind the RNA to the beads.

2.4.4. Place the tube on a magnetic rack to separate the beads from the supernatant.

2.4.5. After the solution is clear, carefully remove and discard the supernatant. Be careful not to disturb the beads which contain the RNA.

2.4.6. Add 200 µl of freshly prepared 80% ethanol to the tube while in the magnetic rack. Incubate at room temperature for 30 seconds, and then carefully remove and discard the supernatant. Be careful not to disturb the beads, which contain the RNA.

2.4.7. Repeat Step 2.4.6 once for a total of two washes.

2.4.8. Completely remove residual ethanol, and air dry the beads for up to 5 minutes while the tube is on the magnetic rack with the lid open.

Caution: Do not over-dry the beads. This may result in lower recovery of RNA target. Elute the samples when the beads are still dark brown and glossy looking, but when all visible liquid has evaporated. When the beads turn lighter brown and start to crack they are too dry.

2.4.9. Remove the tube from the magnetic rack. Elute the RNA from the beads by adding 7 µl of nuclease-free water. Mix thoroughly by pipetting up and down at least 10 times and briefly spin the tube.

2.4.10. Incubate the tube for 2 minutes at room temperature.

2.4.11. Place the tube on the magnetic rack until the solution is clear (~ 2 minutes).

2.4.12. Remove 5 µl of the supernatant containing RNA and transfer to a nuclease-free tube.

2.4.13. Place the tube on ice and proceed with RNA-Seq library construction (protocol below) or other downstream application. Alternatively, the sample can be stored at -80°C.

3.0 Library preparation using the NEBNext Ultra II Directional RNA Library Prep for Illumina (NEB #E7760)

3.1. RNA Fragmentation and Priming

3.1.1 Assemble the following fragmentation and priming reaction on ice:

FRAGMENTATION AND PRIMING REACTION	VOLUME
Enriched mRNA Sample (Step 2.4.13)	5 µl
(lilac) NEBNext First Strand Synthesis Reaction Buffer	4 µl
(lilac) Random Primers	1 µl
Total Volume	**10 µl**

3.1.2. Mix thoroughly by pipetting up and down 10 times.

⚠️ **Note: The next step provides a fragmentation incubation time resulting in an RNA insert of ~200nt. Refer to Appendix (Section 4 of the NEBNext Ultra II Directional RNA Library Prep for Illumina Manual) for fragmentation conditions if you are preparing libraries with large inserts (>200 bp).**

3.1.3. Incubate the sample for **15 minutes at 94°C** in a thermocycler with the heated lid set at 105°C.

3.1.4. Immediately transfer the tube to ice for 1 minute.

3.1.5 Perform a quick spin to collect all liquid from the sides of the tube and proceed to First Strand cDNA Synthesis.

3.2. First Strand cDNA Synthesis

3.2.1. Assemble the first strand synthesis reaction on ice by adding the following components to the fragmented and primed RNA from Step 3.1.5:

FIRST STRAND SYNTHESIS REACTION	VOLUME
Fragmented and primed RNA (Step 3.1.5)	10 µl
(brown) NEBNext Strand Specificity Reagent	8 µl
(lilac) NEBNext First Strand Synthesis Enzyme Mix	2 µl
Total Volume	**20 µl**

3.2.2. Mix thoroughly by pipetting up and down 10 times.

3.2.3. Incubate the sample in a preheated thermocycler with the heated lid set at ≥ 80°C as follows:

Note: If you are following recommendations in Section 4 of the NEBNext Ultra II Directional RNA Library Prep for Illumina Manual for libraries with longer inserts (>200 bases), increase the incubation at 42°C from 15 minutes to 50 minutes at Step 2 below.

Step 1: 10 minutes at 25°C

Step 2: 15 minutes at 42°C

Step 3: 15 minutes at 70°C

Step 4: Hold at 4°C

3.2.4. Proceed directly to Second Strand cDNA Synthesis.

3.3. Second Strand cDNA Synthesis

3.3.1. Assemble the second strand cDNA synthesis reaction on ice by adding the following components into the first strand synthesis product from Step 3.2.4.

SECOND STRAND SYNTHESIS REACTION	VOLUME
First-Strand Synthesis Product (Step 3.2.4)	20 µl

SECOND STRAND SYNTHESIS REACTION	VOLUME
(orange) NEBNext Second Strand Synthesis Reaction Buffer with dUTP Mix (10X)	8 µl
(orange) NEBNext Second Strand Synthesis Enzyme Mix	4 µl
Nuclease-free Water	48 µl
Total Volume	**80 µl**

3.3.2. Keeping the tube on ice, mix thoroughly by pipetting up and down at least 10 times.

3.3.3. Incubate in a thermocycler for **1 hour at 16°C** with the heated lid set at ≤ 40°C (or off).

3.4. Purification of Double-stranded cDNA Using SPRIselect Beads or NEBNext Sample Purification Beads

3.4.1. Vortex SPRIselect Beads or NEBNext Sample Purification Beads to resuspend.

3.4.2. Add 144 µl (1.8X) of resuspended 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.

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

3.4.4. Briefly spin the tube in a microcentrifuge to collect any sample on the sides of the tube. Place the tube on a magnetic rack to separate beads from the supernatant. After the solution is clear, carefully remove and discard the supernatant. Be careful not to disturb the beads, which contain DNA. **Caution: do not discard beads.**

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

3.4.6. Repeat Step 3.4.5 once for a total of 2 washing steps.

3.4.7. Air dry the beads for up to 5 minutes while the tube is on the magnetic rack with lid open.

Caution: Do not over-dry the beads. This may result in lower recovery of DNA target. Elute the samples when the beads are still dark brown and glossy looking, but when all visible liquid has evaporated. When the beads turn lighter brown and start to crack they are too dry.

3.4.8. Remove the tube from the magnetic rack. Elute the DNA from the beads by adding 53 µl 0.1X TE Buffer (provided) to the beads. Mix well on a vortex mixer or by pipetting up and down at least 10 times. Quickly spin the tube and incubate for 2 minutes at room temperature. Place the tube on the magnetic rack until the solution is clear.

3.4.9. Remove 50 µl of the supernatant and transfer to a clean nuclease-free PCR tube.

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

3.5. End Prep of cDNA Library

3.5.1. Assemble the End Prep reaction on ice by adding the following components to the second strand synthesis product from Step 3.4.9.

END PREP REACTION	VOLUME
Second Strand cDNA Synthesis Product (Step 3.4.9)	50 µl
🟢 (green) NEBNext Ultra II End Prep Reaction Buffer	7 µl
🟢 (green) NEBNext Ultra II End Prep Enzyme Mix	3 µl
Total Volume	**60 µl**

If a master mix is made, add 10 µl of master mix to 50 µl of cDNA for the End Prep reaction.

3.5.2. Set a 100 µl or 200 µl pipette to 50 µl and then pipette the entire volume up and down at least 10 times to mix thoroughly. Perform a quick spin to collect all liquid from the sides of the tube.

Note: It is important to mix well. The presence of a small amount of bubbles will not interfere with performance.

3.5.3. Incubate the sample in a thermocycler with the heated lid set at ≥ 75°C as follows.

30 minutes at 20°C

30 minutes at 65°C

Hold at 4°C.

3.5.4. Proceed immediately to Adaptor Ligation.

3.6. Adaptor Ligation

⚠️

3.6.1. Dilute the 🔴 (red) NEBNext Adaptor* prior to setting up the ligation reaction in ice-cold Adaptor Dilution Buffer and keep the adaptor on ice.

TOTAL RNA INPUT	DILUTION REQUIRED
100 ng	25-fold dilution in Adaptor Dilution Buffer

*The NEBNext adaptor is provided in NEBNext oligo kit. NEB has several oligo kit options, which are supplied separately from the library prep kit.

3.6.2. Assemble the ligation reaction on ice by adding the following components, in the order given, to the end prep reaction product from Step 3.5.4.

LIGATION REACTION	VOLUME
End Prepped DNA (Step 2.9.4)	60 µl
Diluted Adaptor (Step 2.10.1)	2.5 µl
🔴 (red) NEBNext Ligation Enhancer	1 µl

LIGATION REACTION	VOLUME
(red) NEBNext Ultra II Ligation Master Mix	30 µl
Total Volume	**93.5 µl**

Note: The Ligation Master Mix and Ligation Enhancer can be mixed ahead of time and is stable for at least 8 hours @ 4°C. We do not recommend premixing the Ligation Master Mix, Ligation Enhancer and adaptor prior to use in the Adaptor Ligation Step.

3.6.3. Set a 100 µl or 200 µl pipette to 80 µl and then pipette the entire volume up and down at least 10 times to mix thoroughly. Perform a quick spin to collect all liquid from the sides of the tube.

Caution: The NEBNext Ultra II Ligation Master Mix is very viscous. Care should be taken to ensure adequate mixing of the ligation reaction, as incomplete mixing will result in reduced ligation efficiency. The presence of a small amount of bubbles will not interfere with performance.

3.6.4. Incubate **15 minutes at 20°C** in a thermocycler.

3.6.5. Add 3 µl (blue) USER® Enzyme to the ligation mixture from Step 2.10.4, resulting in total volume of 96.5 µl.

3.6.6. Mix well and incubate at **37°C for 15 minutes** with the heated lid set to ≥ 45°C.

3.6.7. Proceed immediately to Purification of the Ligation Reaction.

3.7. Purification of the Ligation Reaction Using SPRIselect Beads or NEBNext Sample Purification Beads

⚠ **Note: If you are selecting for libraries with larger insert size (>200 nt) follow the size selection recommendations in Appendix, Section 4 of the NEBNext Ultra II Directional RNA Library Prep for Illumina Manual.**

3.7.1. Add 87 µl (0.9X) resuspended SPRIselect Beads or NEBNext Sample Purification Beads and mix well on a vortex mixer or by pipetting up and down at least 10 times.

3.7.2. Incubate for **10 minutes at room temperature**.

3.7.3. Quickly spin the tube in a microcentrifuge and place the tube on a magnetic rack to separate beads from the supernatant. After the solution is clear (~ 5 minutes), discard the supernatant that contains unwanted fragments. **Caution: do not discard beads.**

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

3.7.5. Repeat Step 3.7.4 once for a total of 2 washing steps.

3.7.6. Briefly spin the tube and put the tube back in the magnetic rack.

3.7.7. Completely remove the residual ethanol, and air-dry beads until the beads are dry for up to 5 minutes while the tube is on the magnetic rack with the lid open.

Caution: Do not over-dry the beads. This may result in lower recovery of DNA target. Elute the samples when the beads are still dark brown and glossy looking, but when all visible liquid has evaporated. When the beads turn lighter brown and start to crack they are too dry.

3.7.8. Remove the tube from the magnetic rack. Elute DNA target from the beads by adding 17 µl 0.1X TE (provided) to the beads. Mix well on a vortex or by pipetting up and down. Quickly spin the tube and incubate for 2 minutes at room temperature. Place the tube in the magnet until the solution is clear.

3.7.9. Without disturbing the bead pellet, transfer 15 µl of the supernatant to a clean PCR tube and proceed to PCR enrichment.

Note: If you need to stop at this point in the protocol, samples can be stored at –20°C.

3.8. PCR Enrichment of Adaptor Ligated DNA

⚠️ Check and verify that the concentration of your oligos is 10 µM on the label.

⚠️ Use Option A for any NEBNext Oligos kit where index primers are supplied in tubes. These kits have the forward and reverse primers supplied in separate tubes.

Use Option B for any NEBNext Oligos kit where index primers are supplied in a 96-well plate format. These kits have the forward and reverse primers (i7 and i5) combined.

3.8.1. Set up the PCR reaction as described below based on the type of oligos (PCR primers) used.

3.8.1A. Forward and Reverse Primers Separate

COMPONENT	VOLUME PER ONE LIBRARY
Adaptor Ligated DNA (Step 2.11.9)	15 µl
(blue) NEBNext Ultra II Q5® Master Mix	25 µl
Universal PCR Primer/i5 Primer*,**	5 µl
Index (X) Primer/i7 Primer*, **	5 µl
Total Volume	**50 µl**

* NEBNext Oligos must be purchased separately from the library prep kit. Refer to the corresponding NEBNext Oligo kit manual for determining valid barcode combinations.

** Use only one i7 primer/ index primer per sample. Use only one i5 primer (or the universal primer for single index kits) per sample.

3.8.1B. Forward and Reverse Primers Combined

COMPONENT	VOLUME PER ONE LIBRARY
Adaptor Ligated DNA (Step 2.11.9)	15 µl
(blue) NEBNext Ultra II Q5 Master Mix	25 µl
Index (X) Primer/i7 Primer Mix*	10 µl
Total Volume	**50 µl**

* NEBNext Oligos must be purchased separately from the library prep kit. Refer to the corresponding NEBNext Oligo kit manual for determining valid barcode combinations.

** Use only one i7 primer/ index primer per sample. Use only one i5 primer (or the universal primer for single index kits) per sample

3.8.2. Mix well by gently pipetting up and down 10 times. Quickly spin the tube in a microcentrifuge.

3.8.3. Place the tube on a thermocycler with the heated lid set to 105°C and perform PCR amplification using the following PCR cycling conditions (refer to Table 3.8.3A and Table 3.8.3B):

Table 3.8.3A:

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

* The number of PCR cycles should be adjusted based on RNA input.

** It is important to limit the number of PCR cycles to avoid overamplification.
If overamplification occurs, a second peak ~ 1,000 bp will appear on the Bioanalyzer trace (See Figure 5.2 of the NEBNext Ultra II Directional RNA Library Prep for Illumina Manual).

Table 3.8.3B: Recommended PCR cycles based on total RNA input amount:

TOTAL RNA INPUT	**RECOMMENDED PCR CYCLES***
100 ng	14-15

***** The PCR cycles are recommended based on high quality human whole blood total RNA. To prevent over-amplification, the number of cycles may require optimization based on the sample quality and the fraction of globin mRNA. For RNA where globin mRNA is > than 50% of the transcripts (once rRNA is removed), follow the higher cycle recommendation for that input.

3.9. Purification of the PCR Reaction using SPRIselect Beads or NEBNext Sample Purification Beads

3.9.1. Vortex SPRIselect Beads or NEBNext Sample Purification Beads to resuspend.

3.9.2. Add 45 µl (0.9X) of resuspended beads to the PCR reaction (~ 50 µl). Mix well on a vortex mixer or by pipetting up and down at least 10 times.

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

3.9.4. Quickly spin the tube in a microcentrifuge and place the tube on a magnetic rack to separate beads from the supernatant. After the solution is clear (about 5 minutes), carefully remove and discard the supernatant. Be careful not to disturb the beads that contain DNA targets. **Caution: do not discard beads.**

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

3.9.6. Repeat Step 3.9.5 once for a total of 2 washing steps.

3.9.7. Air dry the beads for up to 5 minutes while the tube is on the magnetic rack with the lid open.

Caution: Do not over-dry the beads. This may result in lower recovery of DNA target. Elute the samples when the beads are still dark brown and glossy looking, but when all visible liquid has evaporated. When the beads turn lighter brown and start to crack they are too dry.

3.9.8. Remove the tube from the magnetic rack. Elute the DNA target from the beads by adding 23 µl 0.1X TE (provided) to the beads. Mix well on a vortex mixer or by pipetting up and down ten times. Quickly spin the tube in a microcentrifuge and incubate for 2 minutes at room temperature. Place the tube in the magnetic rack until the solution is clear.

3.9.9. Transfer 20 µl of the supernatant to a clean PCR tube and store at –20°C.

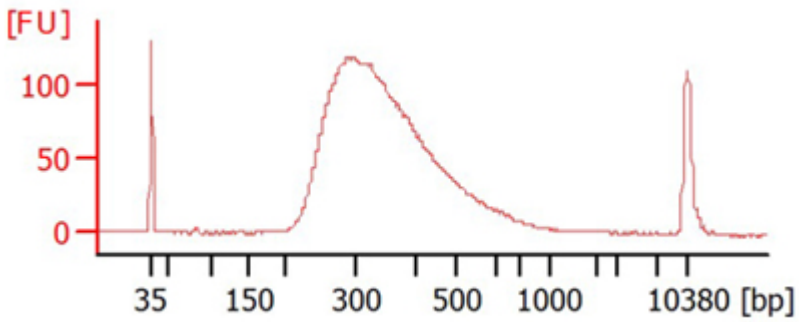
3.10. Library Quantification

3.10.1. Use a Bioanalyzer or TapeStation to determine the size distribution and concentration of the libraries.

3.10.2. 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 visible in the bioanalyzer traces, bring up the sample volume (from Step 3.9.9) to 50 µl with 0.1X TE buffer and repeat the SPRIselect Bead or NEBNext Sample Purification Bead Cleanup Step (Section 3.9).

Figure 3.9.1 Example of RNA library size distribution on a Bioanalyzer.



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