I. Introduction

This protocol is for use with the PrepX RNA-Seq for Illumina Library Kit, 24 Samples (Cat. No. 640096), which accommodates rapid, walkaway automation of library prep on the Apollo™ Library Prep System. When run with the `PrepX small RNA 8` script, this kit can be used to process **up to 8 samples per batch** into small RNA libraries suitable for sequencing on Illumina platforms. **Read this Protocol-At-A-Glance in its entirety before you begin, with particular attention paid to the Apollo System Best Practices.**

II. Workflow Overview

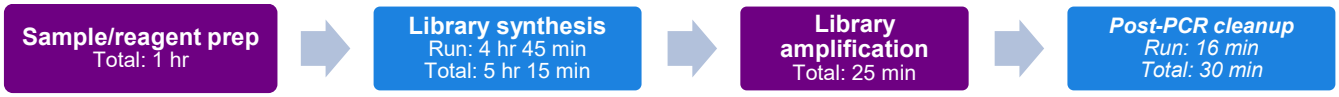


Figure 1. Library preparation workflow overview for one batch of up to 8 samples on the Apollo system with the PrepX Small RNA for Illumina Library Kit, 24 Samples. Blue and purple boxes indicate steps performed on and off the Apollo system, respectively. The italicized text indicates that the post-PCR cleanup requires a separate protocol. Run = the run time in minutes on the Apollo system, if applicable. Total = total time in minutes spent, including thawing of reagents, reagent and equipment setup, heating and cooling of thermal blocks, incubation of reactions, and automated liquid-handling processes, if applicable.

III. List of Components

PrepX RNA-Seq for Illumina Library Kit, 24 samples (400039)	640096
Box 1. (Store at –20°C.)	
PrepX RNA-Seq for Illumina Intermediate Enzyme Strips (blue strips)	24 strips
PrepX RNA-Seq RNase III Enzyme	125 µl
PrepX RNA-Seq RNase III Buffer Solution	125 µl
PrepX RNA-Seq Deoxynucleotide Solution mix	240 µl
PrepX RNA-Seq Murine RNase Inhibitor	65 µl
Box 2. (Store at –20°C.)	
PrepX RNA-Seq for Illumina Intermediate Adapter Primer Kit Sample (orange strips)	24 strips
PrepX RNA-Seq SR Primer*	145 µl
PrepX RNA-Seq Index 1 Primer*	145 µl
Box 3. Store at room temperature	
PrepX Molecular Grade Water	100 ml

*Primers included are sufficient for single-plex reactions **only**. To multiplex libraries, see Appendix A for index primer sequences compatible with this kit.

Additional Materials Required

The following consumables from Takara Bio were used to validate protocols and scripts. **Do not make any substitutions.**

Apollo consumables	Cat. No.	Quantity	Usage/8-rxn run
Apollo Piercing Tips	640085	Box of 1,000 tips	8 tips
Apollo Filter Tips	640084	Box of 960 tips	32 tips
Apollo 0.2 ml PCR 8-Tube Strips, Clear	640082	Box of 125 strips	6 strips
Apollo Caps for 0.2 ml PCR 8-Tube Strips, Clear	640086	Box of 125 strips	6 strips

Reagents

The following reagents were used to validate protocols and scripts. **Do not make any substitutions.**

Product	Supplier	Cat. No.
RNA 6000 Pico Kit	Agilent Technologies	5067-1513
SuperScript III Reverse Transcriptase	Thermo Fisher Scientific	18080044
Agencourt AMPure XP Beads, 450 mL	Beckman Coulter	640084
LongAmp *Taq* 2X Master Mix	NEB	M0287S, M0287L
Index Primers (For a list of additional primer sequences for multiplexing, refer to Appendix A.)	Custom oligo manufacturer	N/A
Agilent High Sensitivity DNA Kit (recommended)	Agilent Technologies	5067-4626

General lab equipment, reagents, and consumables

- Single-channel pipettes: 10 µl, 20 µl, 200 µl, and 1,000 µl
- Eight-channel pipettes (recommended): 20 µl and 200 µl
- Filter pipette tips: 2 µl, 20 µl, 200 µl, and 1,000 µl
- PCR thermal cycler
- DNA LoBind Tubes (Eppendorf, Cat. No. 0030108051)
- 100% ethanol (EtOH; molecular biology grade)

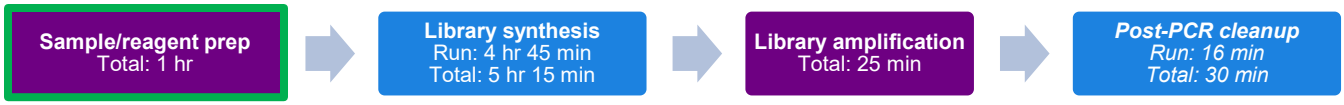
IV. Apollo System Best Practices

- **Read this Protocol-At-A-Glance in its entirety before you begin.**
- Clean the work surfaces, including the retention plates, with 70% ethanol at least once a week.

  NOTE: While the standard cleaning procedure is sufficient to clean Apollo system surfaces for RNA work, the user may wish to use additional decontamination solutions to remove nucleases. As these are known to be corrosive and may damage the system, ensure any nuclease decontamination is immediately followed by a cleanup with 70% ethanol.
- **Restart the instrument before every run. Also, between each subprotocol, perform a power cycle by turning the instrument off, waiting 1 min, and then turning it back on.**
- Discard any deformed plastics.
- Separate partial tube strips with scissors and remove resulting plastic overhangs.
- Spin down reagents before placing them on the deck to avoid air bubbles. **Bubbles at the bottoms of tubes must be removed to ensure accurate volume delivery.**
- Ensure plastics are properly seated on the deck surface **with caps/lids removed**. Be sure to push any tubes down completely and evenly prior to installing the metal retention plates.
- Empty the waste box before every run. **An accumulation of tips in the waste box may cause the run to fail.**

V. Protocols

A. Protocol: Sample and Reagent Prep



For each protocol, the corresponding step in the workflow diagram is indicated with a green outline.

Materials Required

Reagents	Storage conditions	Source
Sample (RNA)	–80°C	User
5X First Strand (FS) Buffer	–20°C	Thermo Fisher Scientific
0.1 M DTT	–20°C	Thermo Fisher Scientific
SuperScript III Reverse Transcriptase	–20°C	Thermo Fisher Scientific
Murine RNAse Inhibitors	–20°C	Takara Bio
Deoxynucleotide Solution Mix	–20°C	Takara Bio
PrepX Molecular Grade Water	4°C	Takara Bio
70% ethanol (prepared fresh)	Room temperature	User

Apollo consumables	Cat. No.	Quantity	Usage/48-rxn run
Apollo 0.2 ml PCR 8-Tube Strips, Clear	640082	Box of 125 strips	6 strips
Apollo Caps for 0.2 ml PCR 8-Tube Strips, Clear	640086	Box of 125 strips	6 strips

1. For each sample, prepare 100 ng–5 µg of total RNA in a 7-µl volume (for a concentration of 14.28 ng/µl–0.71 µg/µl).

   NOTE: We recommend QC of RNA samples with a Bioanalyzer instrument (Agilent Genomics) prior to use with this kit. RNA with an RNA integrity number (RIN) <9.0 will not give optimal results when used with these protocols.

2. Prepare the RT Reaction Mix as follows:

Reagents	Volume/rxn (µl)	Volume/8 rxns + 1 rxn excess (µl)
5X First Strand (FS) Buffer	16	144
0.1 M DTT	8	72
Deoxynucleotide Solution Mix	4	36
Superscript III Reverse Transcriptase	2	18
Murine RNAse Inhibitors	1	9
Total	31	279

3. On the benchtop, aliquot the reagents into the consumables as described in the table below:

Component	Consumable	Volume/tube
Total RNA Sample	Apollo PCR Tubes	7 µl
RT Reaction Mix	Apollo PCR Tubes	31 µl

NOTE: The system is calibrated for Apollo PCR tubes only. Using other tubes may cause the run to fail.

B. Protocol: Library Synthesis

Materials Required

Reagents	Current temperature	Source
Sample	On ice	User
RT Reaction Mix	On ice	Section V.A.
Adapter Strips (orange strips)	On ice	Takara Bio
Enzyme Strips (blue strips)	On ice	Takara Bio

Apollo consumables	Cat. No.	Quantity	Usage/8-rxn run
Apollo Piercing Tips	640085	Box of 1,000 tips	8 tips
Apollo Filter Tips	640084	Box of 960 tips	32 tips
Apollo 0.2 ml PCR 8-Tube Strips, Clear	640082	Box of 125 strips	6 strips

1. Turn on the instrument or, if the instrument is already on, perform a power cycle by turning the instrument off, waiting 1 min, and then turning it back on.
2. Load consumables onto the Apollo system work surface according to the layout in Figure 2. First, load the consumables that do not initially hold reagents (table above). Just before the run, load the consumables containing reagents, but not samples, onto the system. For information on the deck layout when processing fewer than 8 samples, please see Appendix B.
3. To access the `PrepX small RNA 8` script, press **Library Prep** > **RNA > ILM > Small RNA > PrepX small RNA 8**. The **Cooling** indicator will appear.
4. When the **Cooling** indicator has disappeared, and the **Run** button has appeared, load the samples and the correctly oriented blue Enzyme Strips and orange Adapter/Primer strips (AdP Strips) onto the Apollo deck according to the instructions shown on the touch screen and the layout shown in Figure 2.

   NOTE: Ensure plastics are properly seated on the deck surface with caps carefully removed. Be sure to push any tubes down completely and evenly prior to installing the metal retention plates.
5. Install the metal retention plates on Blocks 3 and 4.
6. Empty the waste box and remove any used consumables from the system.

   NOTE: An accumulation of tips in the waste box may cause the run to fail.
7. Close the instrument door and press **Run.**

   NOTE: The run time is 4 hours and 45 minutes.
8. When the run is complete, remove the libraries from Block 3, Row 5, and put them on ice. The final product volume should be ~50 µl per tube.
9. Turn off the instrument.

   SAFE STOPPING POINT: While we recommend continuing directly to PCR, if you do not plan to proceed immediately to the Library Amplification protocol, the products in Block 3 can be capped and stored at –20°C for up to one week.

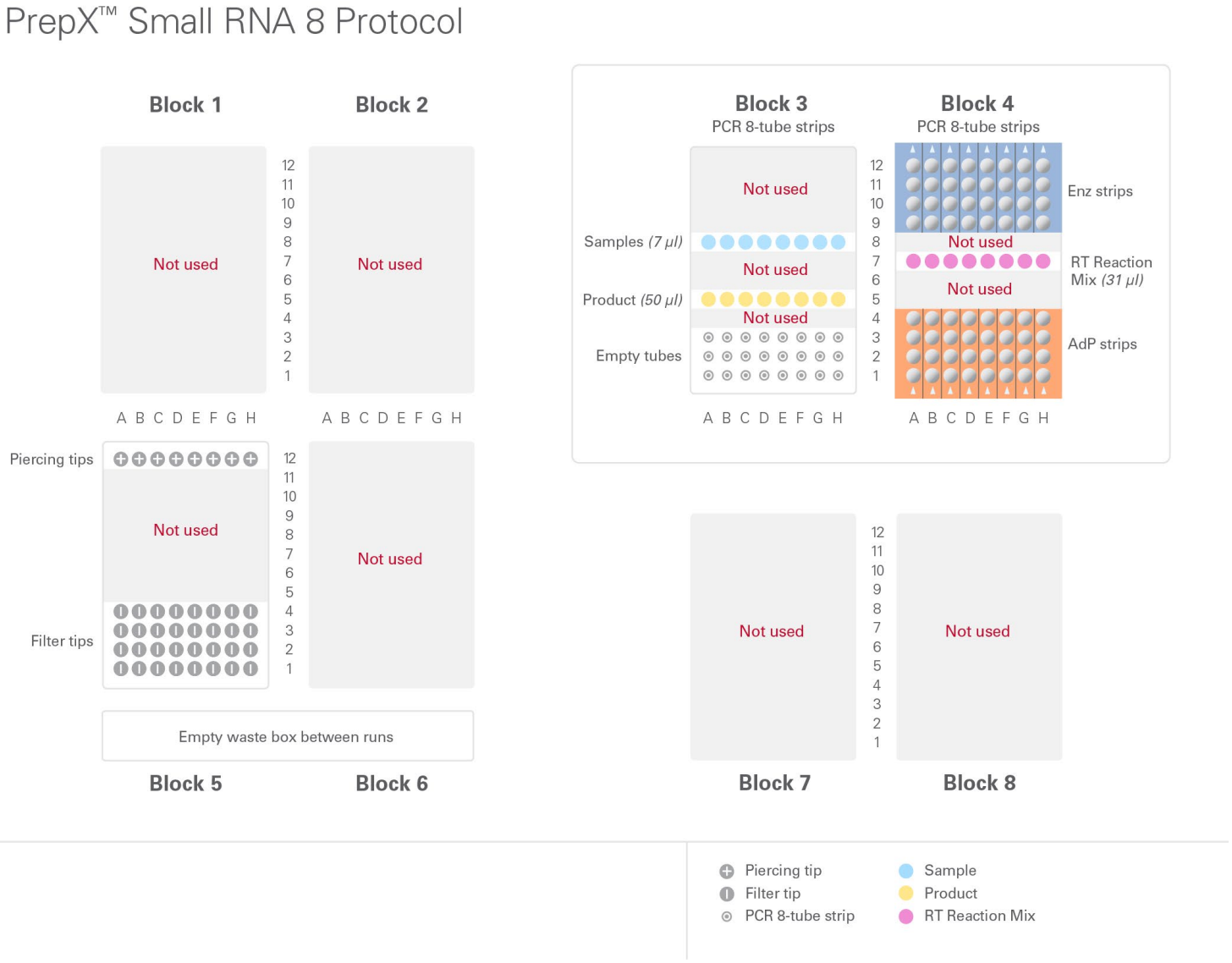
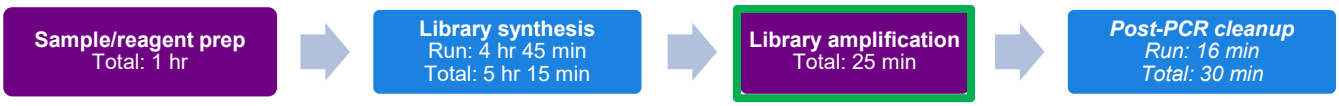


Figure 2. Deck layout for library synthesis from 8 samples. For this protocol, use script `PrepX small RNA 8`. Run time is 4 hours and 45 minutes.

C. Protocol: Library Amplification



Materials Required

Reagents	Current temperature	Source
cDNA products (from Block 3, Row 5)	On ice	Section V.B.
SR Primer	On ice	Takara Bio
Index 1 Primer	On ice	Takara Bio
LongAmp *Taq* 2X Master Mix	On ice	NEB

1. On ice, prepare the PCR Master mix:

Reagents	Volume/rxn (µl)	Volume/8 rxns +1 rxn excess (µl)
LongAmp *Taq* 2X Master Mix	50.0	450.0
SR Primer	2.5	22.5
Total	52.5	472.5

2. On ice, prepare the Reaction Mixture:

Reagents	Volume/rxn (µl)
PCR Master Mix	52.5
cDNA from PrepX Small RNA 24 protocol	45.0
Index 1 Primer	2.5
Total	100.0

3. Perform PCR using the following program:

94°C	30 sec	
12–15 cycles		
94°C	15 sec	12 cycles (0.5–1 µg total RNA) 15 cycles (100–500 ng total RNA) *Depending on sample quality and expression level, 15 PCR cycles may be required for total RNA input at 500 ng.
62°C	30 sec	
70°C	15 sec	
70°C	300 sec	
4°C	forever	

4. (Optional) We recommend gel size selection following PCR using a 6% PAGE gel.
5. Proceed to the **PrepX PCR Cleanup 8** protocol immediately **OR** PCR products may be stored at 4°C overnight.

Appendix A. Index Primers and Sequences

Table I. Index Primers and Sequences for PrepX mRNA Protocols. The index sequence within a primer is highlighted in red. The "s" near the 3' end of each primer sequence indicates a phosphothiorate bond between the last two nucleotides. Refer to your oligo manufacturer's guidelines for how to appropriately indicate this in your order.

Index	Primer sequence (5´–3´)	Index sequence
1	CAAGCAGAAGACGGCATACGAGATCGTGATGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC-s-T	ATCACG
2	CAAGCAGAAGACGGCATACGAGATACATCGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC-s-T	CGATGT
3	CAAGCAGAAGACGGCATACGAGATGCCTAAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC-s-T	TTAGGC
4	CAAGCAGAAGACGGCATACGAGATTGGTCAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC-s-T	TGACCA
5	CAAGCAGAAGACGGCATACGAGATCACTGTGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC-s-T	ACAGTG
6	CAAGCAGAAGACGGCATACGAGATATTGGCGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC-s-T	GCCAAT
7	CAAGCAGAAGACGGCATACGAGATGATCTGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC-s-T	CAGATC
8	CAAGCAGAAGACGGCATACGAGATTCAAGTGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC-s-T	ACTTGA
9	CAAGCAGAAGACGGCATACGAGATCTGATCGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC-s-T	GATCAG
10	CAAGCAGAAGACGGCATACGAGATAAGCTAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC-s-T	TAGCTT
11	CAAGCAGAAGACGGCATACGAGATGTAGCCGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC-s-T	GGCTAC
12	CAAGCAGAAGACGGCATACGAGATTACAAGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC-s-T	CTTGTA
13	CAAGCAGAAGACGGCATACGAGATTTGACTGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC-s-T	AGTCAA
14	CAAGCAGAAGACGGCATACGAGATGGAACTGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC-s-T	AGTTCC
15	CAAGCAGAAGACGGCATACGAGATTGACATGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC-s-T	ATGTCA
16	CAAGCAGAAGACGGCATACGAGATGGACGGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC-s-T	CCGTCC
17	CAAGCAGAAGACGGCATACGAGATCTCTACGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC-s-T	GTAGAG
18	CAAGCAGAAGACGGCATACGAGATGCGGACGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC-s-T	GTCCGC
19	CAAGCAGAAGACGGCATACGAGATTTTCACGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC-s-T	GTGAAA
20	CAAGCAGAAGACGGCATACGAGATGGCCACGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC-s-T	GTGGCC
21	CAAGCAGAAGACGGCATACGAGATCGAAACGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC-s-T	GTTTCG
22	CAAGCAGAAGACGGCATACGAGATCGTACGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC-s-T	CGTACG
23	CAAGCAGAAGACGGCATACGAGATCCACTCGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC-s-T	GAGTGG
24	CAAGCAGAAGACGGCATACGAGATGCTACCGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC-s-T	GGTAGC
25	CAAGCAGAAGACGGCATACGAGATATCAGTGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC-s-T	ACTGAT
26	CAAGCAGAAGACGGCATACGAGATGCTCATGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC-s-T	ATGAGC
27	CAAGCAGAAGACGGCATACGAGATAGGAATGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC-s-T	ATTCCT
28	CAAGCAGAAGACGGCATACGAGATCTTTTGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC-s-T	CAAAAG
29	CAAGCAGAAGACGGCATACGAGATTAGTTGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC-s-T	CAACTA
30	CAAGCAGAAGACGGCATACGAGATCCGGTGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC-s-T	CACCGG
31	CAAGCAGAAGACGGCATACGAGATATCGTGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC-s-T	CACGAT
32	CAAGCAGAAGACGGCATACGAGATTGAGTGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC-s-T	CACTCA

33	CAAGCAGAAGACGGCATACGAGATCGCCTGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC-s-T	CAGGCG
34	CAAGCAGAAGACGGCATACGAGATGCCATGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC-s-T	CATGGC
35	CAAGCAGAAGACGGCATACGAGATAAAATGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC-s-T	CATTTT
36	CAAGCAGAAGACGGCATACGAGATTGTTGGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC-s-T	CCAACA
37	CAAGCAGAAGACGGCATACGAGATATTCCGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC-s-T	CGGAAT
38	CAAGCAGAAGACGGCATACGAGATAGCTAGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC-s-T	CTAGCT
39	CAAGCAGAAGACGGCATACGAGATGTATAGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC-s-T	CTATAC
40	CAAGCAGAAGACGGCATACGAGATTCTGAGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC-s-T	CTCAGA
41	CAAGCAGAAGACGGCATACGAGATGTCGTCGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC-s-T	GACGAC
42	CAAGCAGAAGACGGCATACGAGATCGATTAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC-s-T	TAATCG
43	CAAGCAGAAGACGGCATACGAGATGCTGTAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC-s-T	TACAGC
44	CAAGCAGAAGACGGCATACGAGATATTATAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC-s-T	TATAAT
45	CAAGCAGAAGACGGCATACGAGATGAATGAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC-s-T	TCATTC
46	CAAGCAGAAGACGGCATACGAGATTCGGGAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC-s-T	TCCCGA
47	CAAGCAGAAGACGGCATACGAGATCTTCGAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC-s-T	TCGAAG
48	CAAGCAGAAGACGGCATACGAGATTGCCGAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC-s-T	TCGGCA

Appendix B. Deck Setup for Additional Reaction Sizes

Using the `PrepX small RNA 8` script, the Apollo system can run up to eight samples per batch. If you are performing fewer than eight reactions, please use the deck layout listed below based on the number of samples:

Setting up less than 8 samples	
# of samples	Columns to load
1	D or E*
2	D, E
3	C, D, E or D, E, F
4	C, D, E, F
5	B, C, D, E, F or C, D, E, F, G
6	B, C, D, E, F, G
7	A, B, C, D, E, F, G or B, C, D, E, F, G, H

*The user can choose between a deck setup centered around column D or E. The scripts are designed to accommodate either layout

Appendix C. Library Sizing and Quantitation

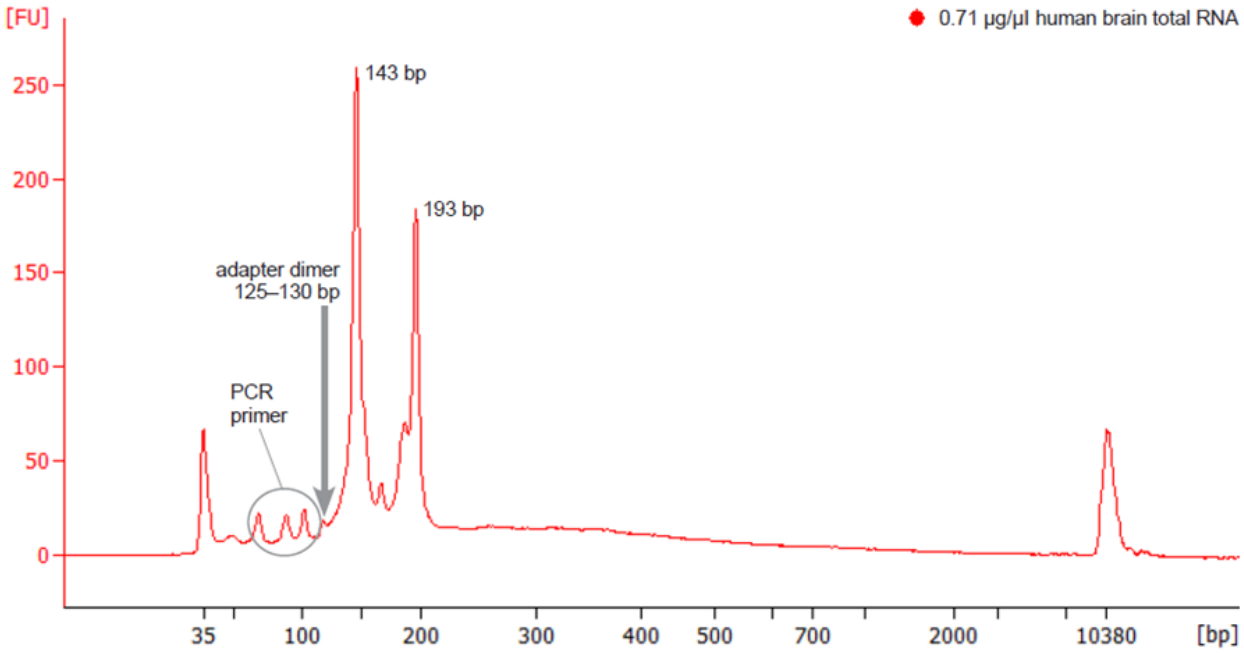


Figure 3. Bioanalyzer traces of post-PCR library outputs. Human brain total RNA was processed through the library amplification protocol (Section V.C) and analyzed on an Agilent 2100 Bioanalyzer. The 143-bp peak corresponds to the miRNAs, while the 193-bp peak corresponds to ribosomal RNAs.

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