ZYMO RESEARCH

RNA - Seq Made Simple

Zymo-Seq RiboFree® Total RNA Library Kit

One for All. Universal rRNA Depletion for Any Organism.

Highlights

- **Universal Depletion:** Novel probe-free technology depletes rRNA from any organism.
- **Simplest Library Prep:** Simultaneous ligation of both adapters reduces hands-on processing.
- **Automation Friendly:** Streamlined protocol for increased scalability.

Catalog Numbers:
R3000, R3003

Scan with your smart-phone camera to view the online protocol/video.

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Product Contents

Zymo-Seq RiboFree® Total RNA Library Kit	R3000 (12 prep)	R3003 (96 prep)	Storage Temperature
cDNA Synthesis Reagent 1	24 µL	8 x 24 µL	-80°C
cDNA Synthesis Reagent 2	120 µL	8 x 120 µL	-80°C
Depletion Reagent 1	120 µL	8 x 120 µL	-80°C
Depletion Reagent 2	120 µL	8 x 120 µL	-80°C
Depletion Reagent 3	120 µL	8 x 120 µL	-80°C
Depletion Reagent 4	24 µL	8 x 24 µL	-20°C
Adapter Ligation Buffer 1	24 µL	8 x 24 µL	-20°C
Adapter Ligation Buffer 2	24 µL	8 x 24 µL	-20°C
Adapter Ligation Buffer 3	24 µL	8 x 24 µL	-20°C
Adapter Ligation Master Mix	312 µL	8 x 312 µL	-20°C
Amplification PreMix	300 µL	8 x 300 µL	-20°C
Zymo-Seq™ UDI Primer Set (1-12)[1]	20 µL/Index	-	-80°C
Zymo-Seq™ UDI Primer Plate (1-96)[2]	-	10 µL/Index	-80°C
Select-a-Size MagBead Concentrate	300 µL	1.5 mL	4 °C
Select-a-Size MagBead Buffer	10 mL	50 mL	4 °C
Library Binding Solution	1 mL	10 mL	4 °C
Zymo-Seq™ Wash Buffer	6 mL	48 mL	Room Temp.
DNA Elution Buffer	10 mL	50 mL	Room Temp.
DNase/RNase-Free Water	1 mL	10 mL	Room Temp.
Instruction Manual	1	1	-

1 The provided **Zymo-Seq™ UDI Primer Set** (Indexes 1-12) (D3008) contains 12 pre-mixed unique dual-index barcode primers in 1.5 mL tubes. See **Appendix B** for primer specifications and index sequences.
2 The provided **Zymo-Seq™ UDI Primer Plate** (Indexes 1-96) (D3096) contains 96 pre-mixed unique dual-index barcode primers in a 96-well plate format. See **Appendix B** for primer specifications and index sequences.

Specifications

- **RNA Input:** 10 – 250 ng of total RNA.
  - For optimal results, please use the recommended 10-250 ng input. Do not use more than 250 ng.
  - If an input below 10 ng is necessary, see **Appendix D** for additional considerations and recommended modifications.
- **Input Quality –** RNA should be free of DNA contamination and enzymatic inhibitors, with A260/A280 and A260/A230 **≥ 1.8**. RNA with lower purity ratios (A260/A280 and A260/A230) should be treated with DNase I and purified with the **RNA Clean & Concentrator™ (Cat. No. R1013)** prior to processing. RNA should be suspended in water, TE, or a low-salt buffer.
  - For optimal results, please use intact RNA (RNA Integrity Number or RIN ≥ 8.0) whenever possible.
  - For degraded RNA input, see **Appendix E** for additional considerations and recommended modifications.
- **Processing Time –** ~4 hours[1]
- **Sequencing Platform Compatibility –** Libraries are compatible with all Illumina® sequencing platforms except HiSeq® X.[2]
- **Equipment Needed (user provided) –** Thermal cycler with heated lid, magnetic stand for 0.2 mL PCR tubes, microcentrifuge for 0.2 mL PCR tubes and 1.5 mL microcentrifuge tubes, and a benchtop vortex mixer.
- **Library Storage –** Libraries eluted in **DNA Elution Buffer** (provided) may be stored at ≤ 4°C overnight or ≤ -20°C for long-term storage.

1 Estimated based on processing ≤ 8 samples at a time using 250 ng of total RNA input. Handling more samples simultaneously or using a lower input will require longer processing time.
2 Illumina® originally limits the applications on HiSeq® X exclusively for whole-genome libraries. Please confirm with the sequencing service provider for acceptability and additional details if expecting to sequence Zymo-Seq RiboFree® Total RNA libraries on HiSeq® X Series sequencers.

Product Description

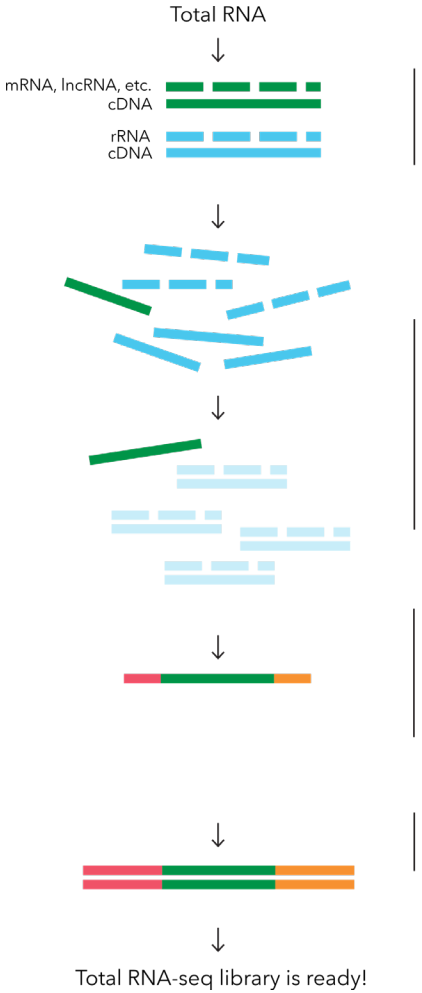
Ribosomal RNAs (rRNA) comprise approximately 90% of total RNA and represents an obstacle to transcriptome enrichment of RNA molecules of interest. **Zymo-Seq RiboFree® Total RNA Library Kit** effectively removes rRNA with a probe-free strategy that is compatible with total RNA from any organism. RiboFree® Universal Depletion uses the input RNA as templates to drive the depletion of the reverse transcribed cDNA from the highly abundant sequences. This eliminates the need for organism-specific probes that may require time-consuming customization and testing and that are often a source of bias due to off-target hybridization.

Compared to mRNA enrichment based methods, total RNA-Seq captures long-noncoding RNAs (lncRNA), intronic RNAs, nucleolar RNAs, and mRNAs with and without intact poly(A) tails. Thus, **Zymo-Seq RiboFree® Total RNA Library Kit** allows the capture of any sample's full transcriptome (both coding and non-coding). Additionally, the reads from Zymo-Seq RiboFree libraries provide even coverage across 5' to 3' ends of the transcripts. This allows the kit to be used for applications such as alternative splicing analysis/novel isoform identification as well as gene expression quantification.

Zymo-Seq RiboFree® Total RNA Library Kit is all-inclusive and cost-effective, with reagents in most sections of the protocol in premixed formats ready for use. The streamlined workflow includes the following main steps: (1) total RNA is reverse transcribed and cDNA from rRNA is depleted; (2) Partial P7 and P5 adapters are directly ligated to the cDNAs simultaneously; and (3) a PCR reaction leverages unique dual indexing to amplify and barcode the stranded library.

See the next page for the detailed overview of Library Preparation Procedure.

Library Preparation Overview



Section 1: cDNA Synthesis
- Random priming based
- Cold reagents with yellow caps
- Processing time: 30 min (including 5 min hands-on)

Section 2: RiboFree® Universal Depletion
- Denature and renature to form rRNA-cDNA hybrids and enzymatically deplete
- Cold reagents with red caps
- Processing time: 75 min to 5 hrs (including 25 min hands-on)
- A safe stopping point

Section 3: Adapter Ligation
- Ligate both adapters to cDNA at the same time
- Cold reagents with green caps
- Processing time: 90 min (including 25 min hands-on)
- A safe stopping point

Section 4: Library Amplification
- Indexing PCR adds full length adapters that contain library barcodes
- Cold reagents with clear caps
- Processing time: ~ 60 min (including 30 min hands-on)

Zymo-Seq RiboFree® Total RNA Library Kit Workflow. Processing time is estimated based on processing ≤ 8 samples at a time. Handling more samples simultaneously adds to the hands-on time and leads to a longer processing time.

Protocol

Buffer Preparation

- ✓ Prepare the **Select-a-Size MagBeads** at least 5 days before library preparation. Add 300 µL of **Select-a-Size MagBead Concentrate** to every 10 mL of the **Select-a-Size MagBead Buffer**. Resuspend by pipetting up and down and vortexing. Store at 4°C to 8°C.
  a. For Cat. No. R3000, 10 mL of **Select-a-Size MagBeads** can be prepared in its entirety.
  b. For Cat. No. R3003, multiple 10-mL **Select-a-Size MagBeads** aliquots can be prepared separately as needed; or 50 mL of **Select-a-Size MagBeads** can be prepared in its entirety.

- ✓ Add 24 mL of 100% ethanol (26 mL of 95% ethanol) to the 6 mL **Zymo-Seq™ Wash Buffer** concentrate (provided with Cat. No. R3000), or 192 mL of 100% ethanol (208 mL of 95% ethanol) to the 48 mL **Zymo-Seq™ Wash Buffer** concentrate (provided with Cat. No. R3003).

Before Starting

- ✓ Set the thermal cycler lid temperature to 105°C for each program unless instructed otherwise.
- ✓ Thaw and keep -80°C and -20°C components on ice during use unless instructed otherwise. Mix reagents by flicking and centrifuge briefly.
  - ✓ Refer to Section 3, Before Starting (page 9) for additional considerations for handling the adapter ligation reagents.
- ✓ Allow the **Select-a-Size MagBeads** to equilibrate to room temperature for ≥30 minutes prior to use.
- ✓ Resuspend the magnetic particles immediately before each use by vigorously inverting and vortexing the **Select-a-Size MagBeads** until homogenous.
- ✓ Avoid multiple freeze-thaws of -80°C and -20°C components. Make aliquots as necessary.
- ✓ Use a multichannel pipette for reagent transfer to minimize hands-on time when processing multiple samples.
- ✓ **For new users, please read Appendix A: Select-a-Size MagBead Clean-up Protocol carefully before performing the protocol.**

Section 1: cDNA Synthesis (Yellow Caps)

1. Set up the following thermal cycler program (reaction volume: 20 µL).

Program	Step	Temperature	Time
Primer Annealing	1	98°C	3 min
	2	4°C	Hold
Reverse Transcription	3	25°C	5 min
	4	48°C	15 min
	5	4°C	Hold

2. Transfer the input RNA (10 – 250 ng at a volume ≤ 8 µL)[1] into a 0.2 mL PCR tube. If the input volume is < 8 µL, use **DNase/RNase-Free Water** to raise the volume up to 8 µL. Place the sample on ice.

3. For input ≥ 50ng, add 2 µL of **cDNA Synthesis Reagent 1** to the sample tube for a total of 10 µL. For input < 50 ng, add 1 µL of **cDNA Synthesis Reagent 1** and supplement with 1 µL of **DNase/RNase-Free Water** to the sample tube for a total of 10 µL. Mix thoroughly by flicking or pipetting. Centrifuge briefly.

4. Place the tube in the thermal cycler and run **Steps 1-2** (**Primer Annealing**) of the program.

5. Add 10 µL of **cDNA Synthesis Reagent 2** to each sample during the 4°C hold (**Step 2**) or on ice. Mix thoroughly by pipetting. Centrifuge briefly.

6. Close the thermal cycler lid and continue **Steps 3-5** (**Reverse Transcription**) of the program.

7. Proceed directly to **Section 2**: **RiboFree® Universal Depletion** for the depletion of ribosomal RNA.

1 If using an input lower than the kit's recommended minimum (10 ng) or desiring to skip depletion, refer to **Appendix D** for additional considerations before continuing the protocol. If using degraded RNA as input, refer to **Appendix E** for recommendations before continuing the protocol.

Section 2: RiboFree® Universal Depletion (Red Caps)

1. Set up the following thermal cycler program (reaction volume: 50 µL). Adjust the "Depletion Reaction" time based on the RNA input amount as listed on the right side of the table[1]. *Please note that this section involves adding reagents promptly to tubes inside a thermal cycler.*

Program	Step	Temperature	Time
Pre-Depletion Incubation	1	98°C	3 min
	2	68°C	5 min
	3	68°C	Hold
Depletion Reaction	4	68°C	X min
Stop Depletion	5	68°C	Hold
	6	98°C	2 min
	7	25°C	Hold
Depletion Cleanup	8	55°C	15 min
	9	95°C	5 min
	10	25°C	Hold
DNA Elution	11	95°C	5 min
	12	25°C	Hold

250 ng: X = 15
150 ng: X = 30
100 ng: X = 60
50 ng: X = 90
25 ng: X = 120
10 ng: X = 240

2. Add 10 µL of **Depletion Reagent 1** to the tube containing the 20 µL sample from Section 1, Step 7 on ice for a total of 30 µL. Mix thoroughly by pipetting. Centrifuge briefly.

3. Place the tube to the thermal cycler and run **Steps 1-3** (**Pre-Depletion Incubation**) of the program. **DO NOT** remove the tube from the thermal cycler at the **Step 3** hold.

4. Without removing the tube, add 10 µL of **Depletion Reagent 2** to the 30 µL sample for a total of 40 µL. Mix thoroughly by pipetting.

Continue Section 2 protocol on the next page.

1 RiboFree® Universal Depletion of rRNA uses the input RNA as templates to drive the enzymatic digestion of reverse transcribed cDNA from the highly abundant rRNA sequences. Therefore, lower input requires longer depletion reaction time, and the listed duration serves as a starting point. For good practice, optimization of depletion reaction time is recommended, especially prior to working with precious samples.

5. Close the thermal cycler lid and continue **Step 4** (**Depletion Reaction**) of the program. **DO NOT** remove the tube from the thermal cycler at the **Step 5** hold.

6. Without removing the tube, add 10 µL of **Depletion Reagent 3** to the 40 µL sample for a total of 50 µL. Mix thoroughly by pipetting.

7. Close the thermal cycler lid and continue through **Steps 6-7** (**Stop Depletion**) of the program.

8. Add 2 µL of **Depletion Reagent 4** to the 50 µL sample for a total of 52 µL. Quickly remove the tube from the thermal cycler and gently flick to mix thoroughly. Centrifuge briefly to collect the reaction. Immediately return the tube to the thermal cycler to continue.

9. Close the thermal cycler lid and continue through **Steps 8-10** (**Depletion Cleanup**) of the program.

10. Remove the tube from the thermal cycler and centrifuge briefly. At room temperature, add 26 µL of 95% ethanol to the 52 µL sample for a total of 78 µL. Mix thoroughly by pipetting.

11. Follow the clean-up protocol (**Appendix A**, page 13) using 156 µL of **Select-a-Size MagBeads** (2× sample volume) to obtain purified cDNA with the following modification for elution (Appendix A, Steps 5-6).

    a. For elution, resuspend the beads in 11 µL of **DNA Elution Buffer** and transfer the tube to the thermal cycler to complete **Steps 11-12** (**DNA Elution**) of the program.

    b. Remove the tube from the thermal cycler. Centrifuge briefly to collect all the eluate before placing the tube on the magnetic stand to elute. Transfer 10 µL of the eluate to a new 0.2-mL PCR tube[1].

This is a safe stopping point. Purified cDNA can be safely stored at −20°C over the weekend.

1 The side wall of the tube may have a brown shade from heating the beads. This is not a concern and the eluate will be clear after placing it on the magnet.

Section 3: Adapter Ligation (Green Caps)

Before Starting:

- ✓ Thaw the **Adapter Ligation Master Mix** to room temperature. Once thawed, vortex for at least 30 seconds and invert to mix well.
- ✓ The **Adapter Ligation Buffer 2**, and **Adapter Ligation Buffer 3**, and **Adapter Ligation Master Mix** should only be thawed 4 times maximum. Make additional aliquots as necessary upon first thaw.

1. Preheat a thermal cycler to 98°C (lid temperature at 105°C) for a 3-minute incubation.

2. Combine the following on ice in the 0.2 mL PCR tube containing the 10 µL eluate from Section 2, Step 11. Mix the entire reaction by pipetting thoroughly and centrifuge briefly to ensure there are no droplets in the cap or on the sides of the tube.

Component	Volume
Eluate from Section 2, Step 11	10 µL
Adapter Ligation Buffer 1	2 µL
DNase/RNase-Free Water	8 µL
Total Volume	**20 µL**

3. Incubate the tube on ice for 2 minutes.

4. Heat shock by immediately placing the tube at 98°C (lid temperature at 105°C) for 3 minutes.

5. Immediately return the tube to ice and incubate for at least 2 minutes. *During this incubation, reset the thermal cycler to 37°C (lid temperature at 45°C) for a 1-hour incubation. Leave the lid open to cool faster.*

6. Thoroughly mix the **Adapter Ligation Master Mix** by vortexing for at least 30 seconds and inverting several times.

Continue Section 3 protocol on the next page.

7. Add the following on ice in the order defined below to the sample tube:

Component	Volume
Reaction from Section 3, Step 5	20 µL
Adapter Ligation Buffer 2	2 µL
Adapter Ligation Buffer 3	2 µL
Adapter Ligation Master Mix	26 µL
Total Volume	**50 µL**

8. Mix the entire reaction thoroughly by vortexing for 1 minute to ensure complete homogenization. Centrifuge briefly to ensure there are no droplets in the cap or on the sides of the tube.

9. Incubate the tube at 37°C (lid temperature at 45°C)[1] for 1 hour.

10. Remove the tube from the thermal cycler. At room temperature, add 85 µL of **DNA Elution Buffer** to the sample to bring the volume up to 135 µL and mix thoroughly by pipetting.

11. Follow the clean-up protocol (**Appendix A**, page 13) using 48.6 µL of **Select-a-Size MagBeads** (0.36× sample volume) to obtain purified adapter-ligated DNA. Elute with 15 µL of **DNA Elution Buffer** at room temperature. Transfer the entire eluate into a new 0.2-mL PCR tube.

This is a safe stopping point. Purified DNA can be safely stored at −20°C over the weekend.

1 Ensure that the temperature has reached 37°C (lid temperature at 45°C) before starting this incubation. If it is still cooling down, leave the sample tube on ice until the thermal cycler is ready.

Section 4: Library Amplification (Clear Caps)

Before Starting:

✓ Allow the **Library Binding Solution** to equilibrate to room temperature for ≥30 minutes prior to use.

1. Set up the following thermal cycler program (reaction volume: 50 µL). Adjust the total cycles based on the RNA input amount as listed on the right side of the table below.

Step	Temperature	Time	
1	98°C	45 sec	
2	98°C	15 sec	250 ng = 10 cycles 100 – 249 ng = 11 cycles 10 – 99 ng = 12 cycles
3	60°C	30 sec	
4	72°C	1 min	
	-- Go to Step 2 --		
5	72°C	1 min	
6	4°C	Hold	

2. Add 10 µL of the appropriate **Zymo-Seq UDI Primers**[1] to the tube containing the 15 µL eluate from Section 3, Step 11 for a total of 25 µL. Mix thoroughly by pipetting.

3. Add 25 µL of **Amplification PreMix** to the tube for a total of 50 µL. Mix thoroughly by gently pipetting and centrifuge briefly.

4. Place the tube in the thermal cycler and run the program above.

5. Remove the tube from the thermal cycler. Centrifuge briefly. At room temperature, add 50 µL of **DNA Elution Buffer** to the tube to raise the volume to 100 µL.

6. Add 80 µL of **Select-a Size MagBeads** (0.8× sample volume) to the tube. Mix thoroughly by pipetting until homogenous. Incubate for 5 minutes at room temperature.

Continue Section 4 protocol on the next page.

1 Refer to **Appendix B** for guidance on selecting compatible index primer sets for multiplexing.

7. Place the sample tube on a magnetic stand for 5 minutes, or until the beads have fully separated from the solution. Without dislodging the bead pellet, aspirate slowly and discard the supernatant.

8. Remove the sample tube from the magnetic stand. Add 100 µL of **DNA Elution Buffer** to the beads and mix thoroughly by pipetting up and down until homogenous.

9. Add 80 µL of **Library Binding Solution** to the tube. Mix thoroughly by pipetting up and down until homogenous. Incubate for 5 minutes at room temperature.

10. Follow the clean-up protocol (**Appendix A**, page 13) starting from Step 2 to obtain purified library. Elute with 20 µL of **DNA Elution Buffer** at room temperature.

The eluate is your final RNA-Seq library[1]. Libraries may be stored at ≤ 4°C overnight or ≤ -20°C for long-term storage.

1 Refer to **Appendix C** for characterizing libraries generated with the kit.

Appendices

Appendix A: Select-a-Size MagBead Clean-up Protocol

Before Starting:

- ✓ Allow the **Select-a-Size MagBeads** to equilibrate to room temperature for ≥30 minutes prior to use.
- ✓ Resuspend the magnetic particles immediately before each use by vigorously shaking or vortexing the **Select-a-Size MagBeads** until homogenous.

1. Add the specified volume of **Select-a-Size MagBeads** to each sample. Mix thoroughly by pipetting until homogenous. Incubate for 5 minutes at room temperature.

2. Place the sample on a magnetic stand for 5 minutes, or until the beads have fully separated from the solution. Without dislodging the bead pellet, aspirate slowly and discard the supernatant.

3. While the sample is still on the magnetic stand, add 200 µL of **Zymo-Seq™ Wash Buffer** without disturbing the bead pellet. Aspirate slowly and discard the supernatant without dislodging the bead pellet. Repeat this step for a total of 2 washes.

4. While the sample is still on the magnetic stand, keep the tube cap open to air-dry the beads. After 1 minute, aspirate any residual **Zymo-Seq™ Wash Buffer** that has collected at the bottom of the tube. Continue to air-dry until the bead pellet appears matte without cracking. See an example image on the next page for reference.

5. Remove the sample from the magnetic stand. Add the specified volume of **DNA Elution Buffer** to the beads and mix thoroughly by pipetting up and down until homogenous.

6. Place the sample on the magnetic stand for 1-2 minutes or until the eluate is clear. Transfer the eluate to a new 0.2-mL PCR tube for each sample.

Further Information on Bead Pellet Air-dry

The optimal air-dry time can vary depending on the humidity and temperature. Optimally dried beads should appear matte without cracking (e.g., the one in the middle in Figure 1 below).

Start with 5 minutes of air-dry time and adjust the time as needed to achieve optimally dried beads. Wash buffer carryover from insufficiently dried beads or overdried, cracked beads may reduce nucleic acid recovery.

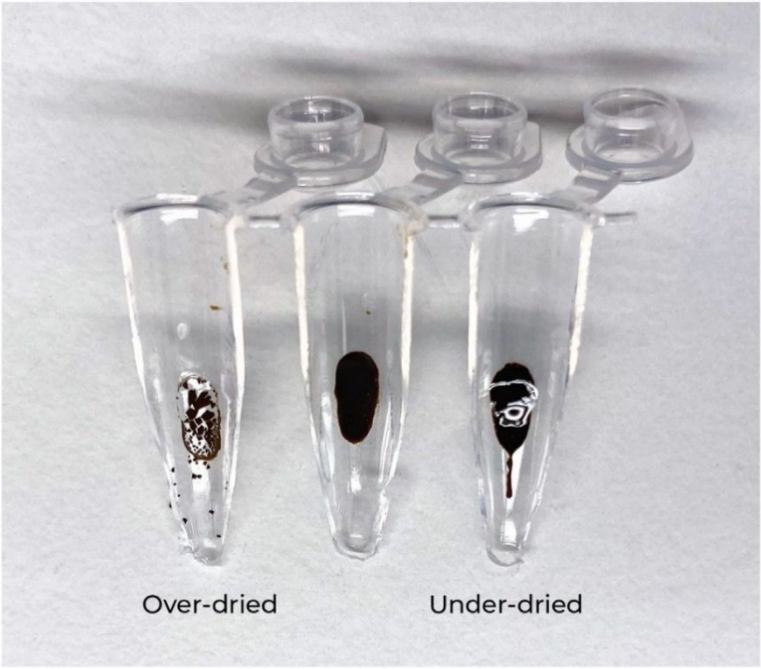


Figure 1. Over-dried beads are cracked and flakey, resembling dried mud. Under-dried beads are glossy and wet, like saturated mud. Optimally dried beads appear damp, but lack gloss.

Appendix B: Unique Dual Index (UDI) Primer Sets

Indexes in the **Zymo-Seq™ UDI Primer Set (Indexes 1-12)** are dispensed in 1.5 mL tubes (D3008), and indexes in the **Zymo-Seq™ UDI Primer Plate (Indexes 1-96)** are dispensed in single-use foil-sealed 96-well plates (D3096). Indexes come as pre-mixes, and the forward and reverse primers are provided at 5 µM total concentration (2.5 µM each).

The complete index sample sheet is available for download here (USA Only), or by visiting the Documents section of the D3008 and D3096 product pages at www.zymoresearch.com.

Primer Sequences:

Forward Primer Sequence (i5):
5′-AATGATACGGCGACCACCGAGATCTACAC**NNNNNNNN**ACACTC TTTCCCTACACGACGCTCTTCCGATCT-3’

Reverse Primer Sequence (i7):
5′-CAAGCAGAAGACGGCATACGAGAT**NNNNNNNN**GTGACTGGAG TTCAGACGTGTGCTCTTCCGATCT-3’

Note: **NNNNNNNN** correspond to the “Bases in Adapter” columns in the index sample sheet mentioned above.

UDI Primer Plate (D3096) Setup:

To use UDI primers, choose ≥ 2 sets down a column not across a row.

	1	2	3	4	5	6	7	8	9	10	11	12
A	UDI_01	UDI_09	UDI_17	UDI_25	UDI_33	UDI_41	UDI_49	UDI_57	UDI_65	UDI_73	UDI_81	UDI_89
B	UDI_02	UDI_10	UDI_18	UDI_26	UDI_34	UDI_42	UDI_50	UDI_58	UDI_66	UDI_74	UDI_82	UDI_90
C	UDI_03	UDI_11	UDI_19	UDI_27	UDI_35	UDI_43	UDI_51	UDI_59	UDI_67	UDI_75	UDI_83	UDI_91
D	UDI_04	UDI_12	UDI_20	UDI_28	UDI_36	UDI_44	UDI_52	UDI_60	UDI_68	UDI_76	UDI_84	UDI_92
E	UDI_05	UDI_13	UDI_21	UDI_29	UDI_37	UDI_45	UDI_53	UDI_61	UDI_69	UDI_77	UDI_85	UDI_93
F	UDI_06	UDI_14	UDI_22	UDI_30	UDI_38	UDI_46	UDI_54	UDI_62	UDI_70	UDI_78	UDI_86	UDI_94
G	UDI_07	UDI_15	UDI_23	UDI_31	UDI_39	UDI_47	UDI_55	UDI_63	UDI_71	UDI_79	UDI_87	UDI_95
H	UDI_08	UDI_16	UDI_24	UDI_32	UDI_40	UDI_48	UDI_56	UDI_64	UDI_72	UDI_80	UDI_88	UDI_96

Appendix C: Library Characterization

Libraries should be visualized by running an agarose gel or using an automated electrophoresis instrument (i.e., Agilent TapeStation®, Agilent Bioanalyzer®, etc.) to determine that the correct library size is present (e.g., Figure 1).

Yields and size distributions may vary depending on the quantity, quality, and source of the input RNA and the number of indexing PCR cycles**.**

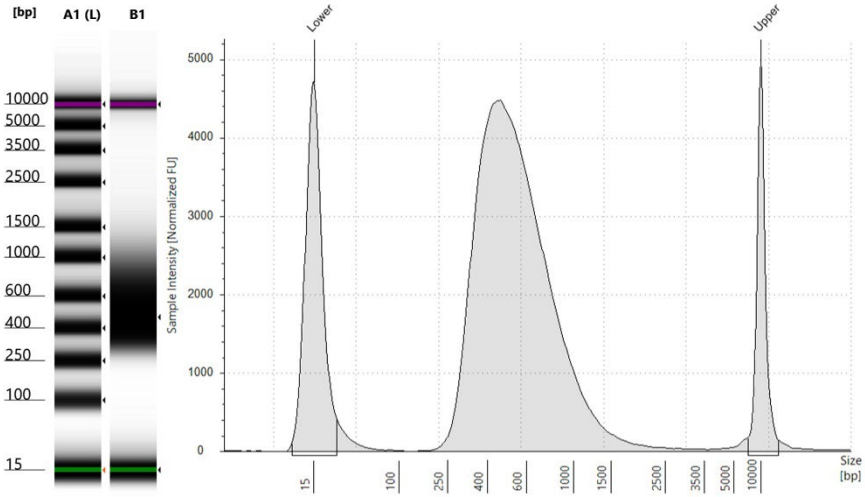


Figure 1. Agilent 4150 TapeStation® D5000 electropherograms of a typical Zymo-Seq RiboFree® Total RNA Library using 100 ng of Universal Human Reference RNA (UHRR, RIN > 8.0) as input and indexed with 11 PCR cycles. The library is with an average size of ~ 560 bp with no adapter dimers (~ 180 bp) present.

Appendix D: Considerations for Low Input or No Depletion

For Library Preparation with Low Input (1 – 9 ng)

It is possible to prepare libraries with input lower than the kit's minimum input requirement (10 ng); however, the rRNA depletion efficiency may be negatively impacted. Use the recommended modifications below if the total RNA input is below 10 ng.

1. For 5 – 9 ng of total RNA input:
   - At Section 1, Step 3 (page 6): Add 1 µL of **cDNA Synthesis Reagent 1** and supplement with 1 µL of **DNase/RNase-Free Water** to the sample tube for a total of 10 µL.
   - At Section 2, Step 1 (page 7): Adjust "Depletion Reaction" to be 240 mins (i.e., 4 hours).
   - At Section 4 (page 11): Amplify with 14 cycles.

2. For 1 – 4 ng of total RNA input:
   - At Section 1, Step 3 (page 6):
     a. Dilute 1 µL of **cDNA Synthesis Reagent 1** with 4 µL of **DNA Elution Buffer**.
     b. Add 1 µL of the diluted cDNA Synthesis Reagent 1 and supplement with 1 µL of **DNase/RNase-Free Water** to the sample tube for a total of 10 µL.
   - At Section 2, Step 1 (page 7): Adjust "Depletion Reaction" to be 240 mins (i.e., 4 hours).
   - At Section 4 (page 11): Amplify with 15 cycles.

For Library Preparation without Depletion

It is possible to prepare libraries without rRNA depletion using this kit. For such a need, please follow the recommended modifications below.

- Use < 10 ng of total RNA as input.
- Upon completing Section 1, Step 6 (page 6), add 32 µL of **DNase/RNase-Free Water** to the 20 µL reaction. Mix thoroughly by pipetting.
- Skip to Section 2, Step 10 (page 8) to continue the protocol with no further modifications.

Appendix E: Considerations for Degraded RNA Input

It is possible to prepare libraries using degraded RNA with this kit; however, the rRNA depletion efficiency may be negatively impacted. Use the recommended modifications below if RNA input is degraded.

1. For RNA with a RIN ~4 or peak ≥ 500 nt:
    - Use more than the minimum input whenever possible.
    - At Section 4 (page 11): Amplify with 1 more cycle than recommended.

2. For RNA with a RIN < 4 or peak < 500 nt:
    - Use ≥ 50 ng as input whenever possible.
    - At Section 1, Step 3 (page 6):
        a. Dilute 1 µL of **cDNA Synthesis Reagent 1** with 4 µL of **DNA Elution Buffer**.
        b. Add 1 µL of the diluted cDNA Synthesis Reagent 1 and supplement with 1 µL of **DNase/RNase-Free Water** to the sample tube for a total of 10 µL.
    - At Section 4 (page 11): Amplify with 1 more cycle than recommended.

Appendix F: Considerations for Sequencing and Bioinformatics

Instrument Compatibility

The libraries prepared with this kit are compatible with all Illumina® sequencing platforms except the HiSeq® X Series.[1] Follow the instruction manual of the specific sequencer to determine important parameters such as library loading concentrations and PhiX spike-in percentages.

Library Quantification

For optimal clustering and sequencing results, please use a qPCR-based method designed for Illumina platforms (e.g., KAPA® Library Quantification Kit) to quantify the concentrations of individual libraries or library pools prior to sequencing.

Trimming Reads

We recommend trimming adapter sequences from the raw reads prior to subsequent analysis. An example using Trim Galore![2] for such trimming is as below:

```
trim_galore --paired -a
AGATCGGAAGAGCACACGTCTGAACTCCAGTCAC -a2
AGATCGGAAGAGCGTCGTGTAGGGAAAGA sample.R1.fastq.gz
sample.R2.fastq.gz
```

Library Strand Information

The Read 1 sequence will be antisense to the RNA transcript from which it originates. Therefore, the strandedness of the library should be set as reverse stranded for applicable bioinformatics tools.

1 Illumina® originally limits the applications on HiSeq® X exclusively for whole-genome libraries. Please confirm with the sequencing service provider for acceptability and additional details if expecting to sequence Zymo-Seq RiboFree® Total RNA libraries on HiSeq® X Series sequencers.

2 Trim Galore! is a publicly available software and accessible at https://github.com/FelixKrueger/TrimGalore.

Ordering Information

Product Description	Catalog No.	Size
Zymo-Seq RiboFree® Total RNA Library Kit	R3000	12 preps
Zymo-Seq RiboFree® Total RNA Library Kit	R3003	96 preps
Zymo-Seq RiboFree® Universal cDNA Kit	R3001	12 preps
Zymo-Seq™ UDI Primer Set (Indexes 1-12)	D3008	12 Indexes
Zymo-Seq™ UDI Primer Plate (Indexes 1-96)	D3096	96 Indexes

Individual Kit Components	Catalog No.	Amount
DNase/RNase-Free Water	W1001-1 W1001-4 W1001-6 W1001-10 W1001-30	1 ml 4 ml 6 ml 10 ml 30 ml
Zymo-Seq™ Wash Buffer	R3004-1-6 R3004-1-48	6 mL 48 mL
DNA Elution Buffer	D3004-4-10 D3004-4-50	10 mL 50 mL

Complete Your Workflow

RNA-Seq *Made Simple*®

Sample to NGS Library in a Single Day

Sample Collection

Prevent RNA Degradation

DNA/RNA Shield [Cat. No. R1100-50]

NGS-Grade RNA Extraction

RNA Isolation from Any Sample Type

Quick-RNA Miniprep Plus [Cat. No. R1057]

RNA-Seq Library Prep Kit

Featuring Universal rRNA Depletion for Any Organism

Zymo-Seq RiboFree Total RNA Library Kit [Cat. No. R3000]

Stranded Libraries

In as little as 4 hours

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Reagents are routinely tested on a lot-to-lot basis to ensure they provide the highest performance and reliability.

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