ZYMO RESEARCH

The Beauty of Science is to Make Things Simple

INSTRUCTION MANUAL

Zymo-Seq RiboFree™ Total RNA Library Kit

Cat. No. **R3000S** (Patent Pending)

Highlights

- **The Fastest & Easiest Kit:** Prepare stranded, RiboFree™ libraries from total RNA in 3.5 hours.
- **Compatible With Any Sample:** Probe-free technology depletes rRNA & Globin from any RNA source.
- **The Most Accurate:** Eliminate bias from rRNA depletion.

Contents

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Satisfaction of all Zymo Research products is guaranteed. If you are dissatisfied with this product, please call 1-888-882-9682.

Notes:

Product Contents:

Zymo-Seq RiboFree™ Total RNA Library Kit	Cat. R3000S 6 Prep Sample	Storage Temperature
R1 Reagent	20 µL	-80 °C
R2 Reagent	60 µL	-80 °C
D1 Reagent	60 µL	-80 °C
D2 Reagent	60 µL	-80 °C
D3 Stop Reagent	60 µL	-80 °C
L1 Reagent	60 µL	-80 °C
L2 Reagent	120 µL	-80 °C
L3 Reagent	60 µL	-80 °C
Zymo*Taq* PreMix	300 µL	-80 °C
Zymo-Seq™ Index Primer Sample Set[1]	30 µL / Index	-80 °C
Select-a-Size MagBead Concentrate	3 x 30 µL	4 °C
Select-a-Size MagBead Buffer	3 x 1 mL	4 °C
Zymo-Seq™ Wash Buffer	6 mL	Room Temp.
DNA Elution Buffer	1 mL	Room Temp.
DNase/RNase-Free Water	1 mL	Room Temp.
PCR Strip MagStand	1	-
Instruction Manual	1	-

Note: Integrity of kit components are guaranteed for up to one year from date of purchase. Reagents are routinely tested on a lot-to-lot basis to ensure they provide maximal performance and reliability.

[1] The provided **Zymo-Seq™ Index Primer Sample Set** (Cat. No. D3007S) contains 6 unique pre-mixed single-index barcode primers.

Specifications:

- **Sample Input Material:** RNA
- **Recommended Input:** 500 ng[2]
- **Minimum Input:** 100 ng[2]
- **Maximum Input:** 5 µg
- **Input Quality:** Ensure RNA A_{260}/A_{280} and A_{260}/A_{230} ratios are ≥ 1.8, DNA-free, and PCR inhibitor-free for high-fidelity cDNA transcription and depletion
- **Equipment Required:** Thermocycler, magnet stand (free at checkout), and microcentrifuge
- **Processing Time:** 3.5 hours (RNA to indexed library)[3]
- **Sequencing:** Libraries are stranded and compatible with all Illumina® sequencing platforms. The Read 1 sequence will be antisense to the RNA molecule of origin.

[2] See Appendix B for recommended rRNA depletion incubation times. Lower input will require longer incubation times and may show reduced rRNA depletion efficiency.

[3] Time may increase when performing longer incubation time during rRNA depletion.

Product Description:

Discover the most streamlined, stranded RNA library kit for sequencing total RNA from any biological sample with the **Zymo-Seq RiboFree™ Total RNA Library Prep Kit** (Figure 1).

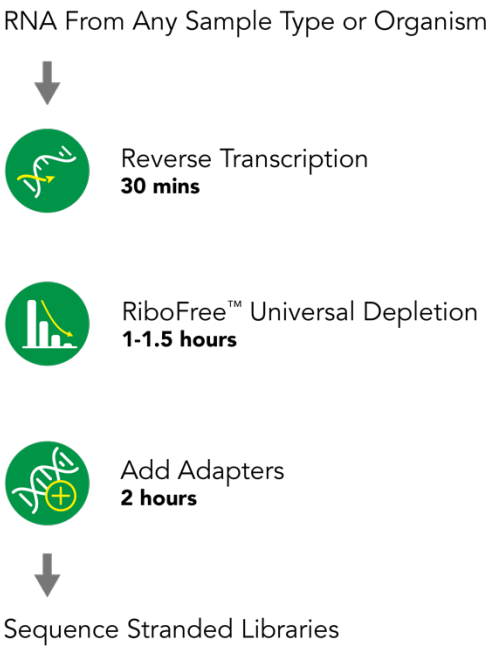


Figure 1: The Zymo-Seq RiboFree™ Total RNA Library Prep Kit is the fastest and easiest Total RNA-Seq workflow. This kit minimizes the number of reagents and steps needed to generate stranded rRNA-depleted total RNA libraries in as little as 3.5 hours.

Capture any sample's full transcriptome (both coding and non-coding) with one simple RNA library prep kit. Overcome challenges in capturing mRNA from degraded and fragmented samples with this total RNA-seq library prep kit. In contrast to poly-A targeted RNA sequencing, total RNA-Seq also captures long-noncoding RNAs (lncRNA), intronic RNAs, nucleolar RNAs, and mRNAs with degraded poly(A) tails.

Ribosomal RNAs (rRNA) comprise approximately 90% of the total RNA and represent an obstacle to transcriptome enrichment of unique protein coding sequences. Zymo Research's RiboFree™ depletion effectively depletes these rRNA elements and overrepresented transcripts (e.g. globin) without the use of probes that cause off-target digestion compared to popular rRNA removal kits.

The RiboFree™ probe-free depletion (rRNA, beta-globin) is compatible with all biological sample types, tissues (including whole blood), and does not require organism-specific probes (Figure 2).

A streamlined total RNA-Seq library prep workflow minimizes user manipulation, resulting in a user-friendly protocol with little hands-on time. This total RNA-Seq library prep kit is an all-inclusive and cost-effective method for generating stranded libraries with minimal bias (Figure 3) compared to other total RNA-Seq library prep methods.

For **Technical Assistance**, please contact **Zymo** at 1-888-882-9682 or E-mail tech@zymoresearch.com.

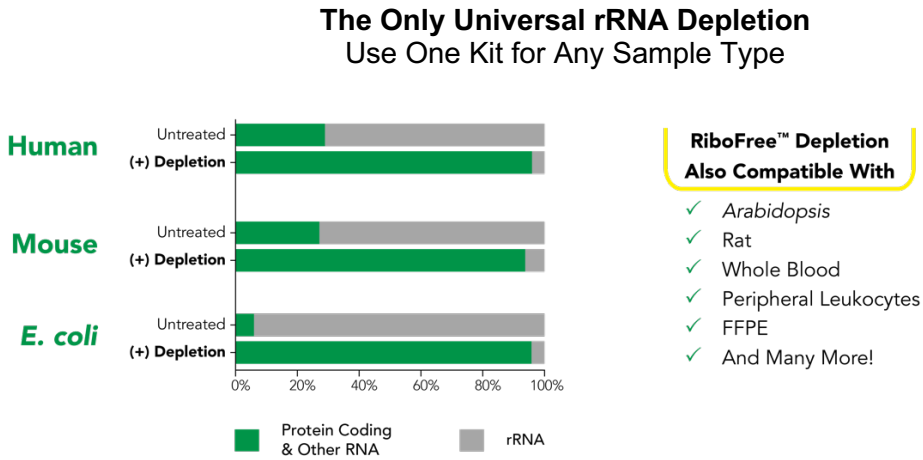


Figure 2: RiboFree™ depletion will enzymatically remove rRNA from any sample type. Paired-end sequencing was performed on stranded total RNA-Seq libraries, both with and without RiboFree™ depletion. Read pairs were aligned to their respective genomes using the STAR aligner. Read classes were defined using a combination of Ensembl GTF gene biotypes and RepBase repeat masker annotations. Number of reads overlapping each annotation class were divided by total reads in that library to calculate percent reads of each annotation class.

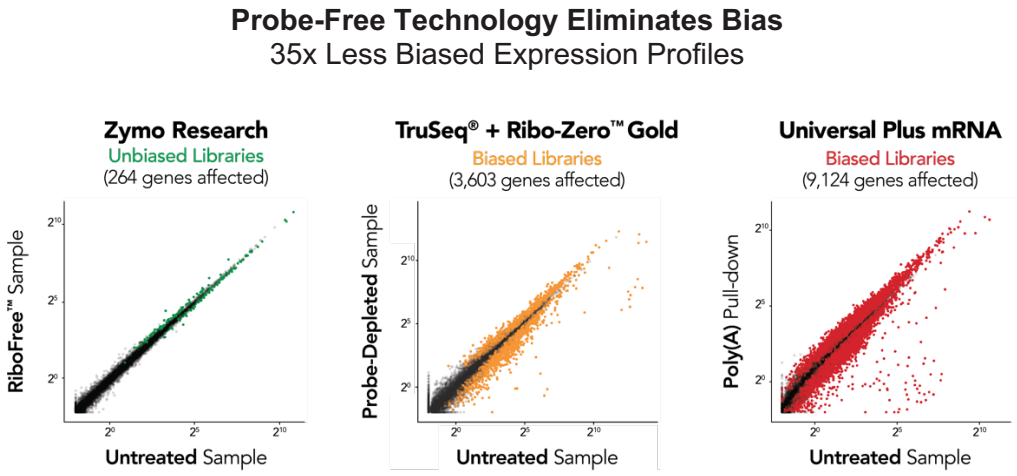
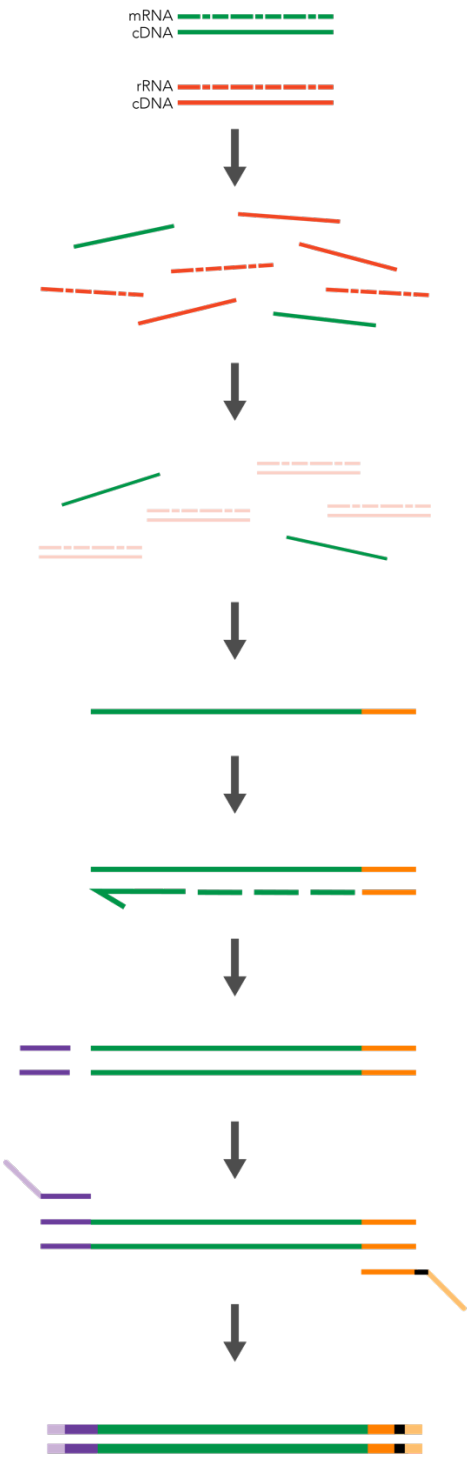


Figure 3: RiboFree™ depletion maintains native expression profiles unlike TruSeq® Total RNA [probe-based Ribo-Zero™ Gold] and Univeral Plus mRNA-Seq [poly(A) enrichment]. Paired-end sequencing was performed on libraries prepared from Universal Human Reference RNA (Invitrogen) containing ERCC Spike-In Mix 1 (Life Technologies), both with and without rRNA removal or poly(A) enrichment. Libraries were sequenced to a depth of ~35 million reads per library, and read pairs were aligned to the hg38 human genome using the STAR aligner. Read classes were defined using Ensembl GTF gene biotypes. The DESeq2 package was used to apply the "apeglm" log-fold-change shrinkage estimator to determine which of the 20,004 protein coding genes and ERCC Spike-In transcripts were significantly affected (p.adj < 0.05) by rRNA removal. Significantly affected transcripts are represented as colored points in the scatterplots.

Library Preparation Overview:



Section 1.1: First-Strand cDNA Synthesis
Reverse Transcription

Section 1.2: RiboFree™ Universal Depletion
Denature

Re-nature and Deplete

Section 2.1: P7 Adapter Ligation
Add Partial P7

Second-Strand Synthesis

Section 2.2: P5 Adapter Ligation
Add Partial P5

Section 2.3: Library Index PCR
Index PCR

Stranded Library

Notes:

Protocol:

Important Information:

- Recommended RNA input range is 100 ng – 2 µg, but < 100 ng may be used. Incubation times for the **Depletion Reaction** and **Index PCR Reaction Cycles** will increase inversely with decreased input. Refer to **Appendix B** for recommendations.
- All thermocycler steps should have lid heating **ON**, set to >98°C

Section 1.1: First-Strand cDNA Synthesis (Yellow Caps)

Before Starting:

- Create the following thermocycler program for a total reaction volume of 20 µL:

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

[1] Avoid multiple freeze-thaws (make aliquots if necessary) and keep on ice during use.

1. Thaw the **R1** and **R2 Reagents** on ice[1]. Mix thoroughly by flicking or pipetting before starting. Briefly spin down and keep on ice.
2. Using **DNase/RNase-Free Water**, raise the volume of each RNA input sample to 8 µL in 0.2 mL PCR tube(s) at 4°C or on ice.
3. Add 2 µL of the **R1 Reagent** to each sample for a total of 10 µL. Mix thoroughly by flicking or pipetting. Briefly spin down. (For inputs < 100 ng, 1 µL of the **R1 Reagent** should be used. Supplement with DNase/RNase-free water.)
4. Place tube(s) in thermocycler and run **Steps 1-2** (**Primer Annealing)** of the program.
5. Add 10 µL of the **R2 Reagent** to each sample during the 4°C hold (**Step 2**) or on ice. Mix thoroughly by pipetting.
6. Continue the thermocycler program through **Steps 3-5** (**Reverse Transcription**) of the program.

[2] To skip depletion, raise the sample volume to 50 µL with 30 µL of DNase/RNase-Free Water and skip to Step 8 of Section 1.2.

7. Proceed directly to **Section 1.2**: depletion of ribosomal RNA, globin, or other overrepresented transcripts.[2]

Section 1.2: RiboFree™ Universal Depletion (Red Caps)

Before starting:

- Refer to **Appendix B** for more details and incubation times for RiboFree™ Depletion
- Create the following thermocycler program for a total reaction volume of 50 µL[1]:

Step	Temperature	Time	Input	
1)	98°C	3 min		**Pre-Depletion Incubation**
2)	68°C	5 min		
3)	68°C	**Hold**		
4)	68°C	*30 min* *1 hr* *2 hr*	*(>1 µg)* *(>250 ng)* *(>100 ng)*	**Depletion Reaction**
5)	68°C	**Hold**		
6)	98°C	2 min		**Stop Depletion**
7)	25°C	**Hold**		

1. Thaw the **D1**, **D2**, and **D3 Reagents** on ice[2]. Before starting, mix thoroughly by flicking or pipetting. Briefly spin down and keep on ice.
2. Add 10 µL of the **D1 Reagent** directly to each 20 µL sample on ice for a total of 30 µL. Mix by flicking or pipetting up and down. Briefly spin down.
3. Transfer the samples to the thermocycler and run **Steps 1-3** (**Pre-Depletion Incubation**) of the program. **DO NOT** remove your samples from the thermocycler at the **Step 3** hold.
4. Without removing the tubes, add 10 µL of the **D2 Reagent** to each 30 µL sample during the **Step 3** hold for a total of 40 µL[3]. Mix in the thermocycler by pipetting.
5. Close the thermocycler lid and continue through **Step 4** (**Depletion Reaction**) of the program. **DO NOT** remove your samples from the thermocycler at the **Step 5** hold.
6. Without removing the tubes, add 10 µL of the **D3 Stop Reagent** to each 40 µL sample during the **Step 5** hold for a total of 50 µL[3]. Mix in the thermocycler by pipetting.
7. Close the thermocycler lid and continue through **Steps 6-7** (**Stop Depletion**) of the program. Remove your samples from the thermocycler.
8. Add 25 µL of 95% ethanol to each 50 µL sample for a total of 75 µL. Mix by pipetting.
9. Follow the clean-up protocol (**Appendix A**) using 150 µL of **Select-a-Size DNA MagBeads**[4]. Resuspend beads in 10 µL of **DNA Elution Buffer** and incubate at 95°C for 5 minutes then cool the tubes to room temperature for elution**.**

This is a safe stopping point. Cleaned-up cDNA can be safely stored at ≤ 4°C overnight or ≤ −20°C for up to one week.

Notes:

[1] Section 1.2: Depletion will involve transferring reagents to tubes inside the thermocycler.

[2] Avoid multiple freeze-thaws (make aliquots if necessary) and keep on ice after thawing between storage.

[3] **Tip:** Using a multichannel pipette will minimize hands-on time when performing multiple reactions.

[4] Sample and bead volumes are optimized for Select-a-Size MagBead-based cleanups. Recommended volumes will minimize pipetting error.

Notes:

Section 2.1: P7 Adapter Ligation (Green Caps)

Before starting:

- Create the following thermocycler program for a total reaction volume of 40 µL:

Step	Temperature	Time	
1)	37°C	15 min	**P7 Ligation Reaction**
2)	95°C	2 min	
3)	4°C	**Hold**	
4)	95°C	10 min	**Second Strand Synthesis**
5)	63°C	30 sec	
6)	72°C	7 min	
7)	4°C	**Hold**	

1. Thaw the **L1** and **L2 Reagents** on ice[1]. Before starting, mix thoroughly by flicking or pipetting. Briefly spin down and keep on ice.
2. Add 10 µL of **L1 Reagent** to each 10 µL cDNA sample in a 0.2 mL PCR tube on ice for a total of 20 µL. Mix by gently pipetting up and down. Briefly spin down and keep on ice.
3. Place tube(s) in thermocycler and run **Steps 1-3** (**P7 Ligation Reaction**).
4. Add 20 µL of **L2 Reagent** to each 20 µL sample for a total of 40 µL. Mix by flicking or pipetting up and down. Briefly spin down.
5. Place tube(s) in thermocycler and continue the program through **Steps 4-7** (**Second Strand Synthesis**).
6. Follow the clean-up protocol (**Appendix A**) using 60 µL of **Select-a-Size DNA MagBeads**[2]. Resuspend beads in 10 µL of **DNA Elution Buffer** at room temperature for elution (no need to incubate).

This is a safe stopping point. Cleaned-up cDNA can be safely stored at ≤ 4°C overnight or ≤ −20°C for up to one week.

[1] Avoid multiple freeze-thaws (make aliquots if necessary) and keep on ice after thawing between storage.

[2] Sample and bead volumes are optimized for Select-a-Size MagBead-based cleanups. Recommended volumes will minimize pipetting error.

Section 2.2: P5 Adapter Ligation (Green Caps)

Before starting:

- Create the following thermocycler program for a total reaction volume of 20 µL:

Step	Temperature	Time	
1)	25°C	15 min	**P5 Ligation Reaction**
2)	4°C	**Hold**	

1. Add 10 µL of **L3 Reagent** to each 10 µL sample in a 0.2 mL PCR tube on ice for a total of 20 µL. Mix by gently pipetting up and down. Briefly spin down.

2. Place tube(s) in thermocycler and run **Steps 1-2** (**P5 Ligation Reaction**).

3. Spin down and raise the volume of each reaction to 100 µL by adding 80 µL of **DNA Elution Buffer**.

4. Follow the clean-up protocol (**Appendix A**) using 100 µL of **Select-a-Size DNA MagBeads**[1]. Resuspend beads in 20 µL of **DNA Elution Buffer** at room temperature for elution (no need to incubate)**.**

This is a safe stopping point. Cleaned-up cDNA can be safely stored at ≤ 4°C overnight or ≤ −20°C for up to one week.

Notes:

[1] Sample and bead volumes are optimized for Select-a-Size MagBead-based cleanups. Recommended volumes will minimize pipetting error.

Notes:

Section 2.3: Library Index PCR (Green Caps)

Before starting:

- Create the following thermocycler program for a total reaction volume of 50 µL:

Step	Temperature	Time		
1)	95°C	10 min		
2)	95°C	30 sec	> 1µg	= 10 cycles
3)	60°C	30 sec	250 ng – 1 µg	= 11 cycles
4)	72°C	1 min	100 ng – 250 ng	= 12 cycles
	-- Go to step 2 --		< 100 ng	= 13+ cycles
5)	72°C	7 min		
6)	4°C	Hold		

1. Add 5 µL of the appropriate **Zymo-Seq Index Primers**[1] to each 20 µL sample in a 0.2 mL PCR tube for a total of 25 µL.
2. Add 25 µL of **Zymo*Taq* Premix** to each 25 µL sample for a total of 50 µL. Mix by gently pipetting up and down. Briefly spin down.
3. Place tube(s) in the thermocycler and run the thermocycler program above.
4. Spin down and raise volume of each reaction to 100 µL by adding 50 µL of **DNA Elution Buffer**.
5. Follow the clean-up protocol (**Appendix A**) by using 85 µL of **Select-a-Size DNA MagBeads**[2]. Resuspend beads in 15-25 µL of **DNA Elution Buffer** at room temperature for elution (no need to incubate)**.**

The eluate is your final RNA-Seq library[3]. Libraries may be stored at ≤ 4°C overnight or ≤ -20°C for long-term storage.

*See **Appendix C** for representative size distribution and Tapestation® (Agilent) profile for assessing library quality. Please refer to sequencing platform-specific guidelines for library quantification and normalization.*

[1] See Appendix D for index primer sequences and barcodes.

[2] Sample and bead volumes are optimized for Select-a-Size MagBead-based cleanups. Recommended volumes will minimize pipetting error.

[3] Recommended**:** Remove an aliquot (e.g. 5 µL) for analysis and quantitation.

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

Before starting:

- Add 24 mL of 100% ethanol (26 mL of 95% ethanol) to the 6 mL **Zymo-Seq™ Wash Buffer** concentrate.
- Add 30 µL of **Select-a-Size Magbead Concentrate** to each 1 mL **Select-a-Size Magbead Buffer**. Resuspend by pipetting.
- Resuspend the magnetic particles immediately before use by vigorously inverting the **Select-a-Size DNA MagBeads** until homogenous.

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

2. Place the samples on a magnetic rack (provided) until the beads have fully separated from the solution, then discard the supernatant[1].

3. While the beads are still on the magnetic rack, add 200 µL of **Zymo-Seq™ Wash Buffer**. Remove and discard the supernatant. Repeat this step (2 washes total)[2].

4. Remove tubes from the magnetic rack and keep the tops open for 3 minutes to dry the beads.

5. Add the appropriate volume of **DNA Elution Buffer** to the beads and mix thoroughly by pipetting up and down until homogenous. Incubate at the indicated temperature.

6. Place the tubes on a magnetic rack to separate the magnetic beads from the suspension, and transfer eluate to a new tube. *Some beads may carry over into the eluate. Bead carry-over will not affect downstream reactions.*

Notes:

[1] Avoid aspirating any beads when removing the supernatant.

[2] Aspirate any residual **Zymo-Seq™ Wash Buffer**.

Appendix B: Depletion Reaction Incubation Guide

Depletion of highly abundant transcripts is based on the enzymatic digestion of high-concentration complementary sequences. The duration of the depletion incubation is inversely proportional to the input concentration. Lower inputs require longer incubation.

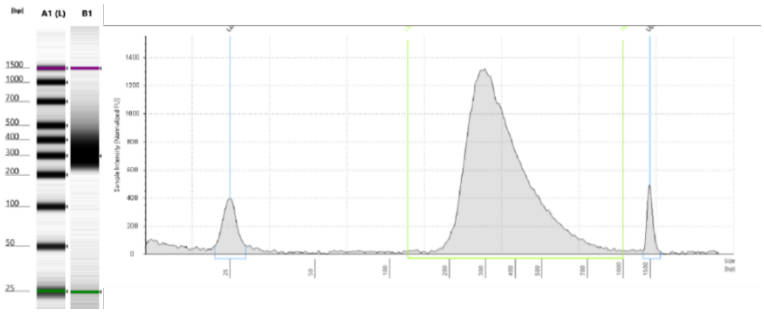
We have found that longer incubation times (up to 16 hours) do not adversely affect the sample. Optional: Users may optimize the depletion incubation time for various input amounts and unique sample types to improve rRNA depletion efficiency.

The PCR cycle number for the final Library Index Reaction will vary with RNA input. The cycle numbers provided are guidelines when depletion is performed. Users should determine the optimal cycle numbers empirically.

Recommended incubation times for standard applications:

RNA Input	Depletion	Index PCR cycles
> 1 µg	30 min	10 cycles
> 250 ng	1 hour	11 cycles
> 100 ng	2 hours	12 cycles
< 100 ng	3-16 hours	13+ cycles

Appendix C: RiboFree™ Total RNA-Seq Library Profile



Agilent 2200 TapeStation® D1000 electropherogram of a typical Zymo-Seq RiboFree™ Total RNA Library Prep using 500 ng of Universal Human Reference RNA and indexed using 11 PCR cycles. Fragment sizes range from 200 bp – 600 bp, for libraries prepared from intact RNA (RIN > 9.0).

Yields will vary depending on the total quantity and quality of sample input RNA.

Appendix D: Single Index Primer Sequences

Indexes in the **Zymo-Seq™ Index Primer Set** (Cat. No. D3007) come as pre-mixes. The universal forward primer and a reverse indexing primer are provided at 5 µM total concentration (2.5 µM each). Index primers generate sequencing libraries that are compatible with Illumina® platforms. The indexed reverse primer sequences contain a 6 bp barcode (underlined in red).

Universal Forward Primer Sequence:

5' – AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT – 3'

Indexed Reverse Primer Sequences:

Cat. No. (Forward + Reverse)	Name	Reverse Primer Sequence	i7 Bases for Samplesheet
D3007-02	Index 2 (A002)	5′ – CAAGCAGAAGACGGCATACGAGATACATCGGTGACTGGAGTTCAGACGTGT GCTCTTCCGATCT – 3′	CGATGT
D3007-04	Index 4 (A004)	5′ – CAAGCAGAAGACGGCATACGAGATTGGTCAGTGACTGGAGTTCAGACGTGT GCTCTTCCGATCT – 3′	TGACCA
D3007-05	Index 5 (A005)	5′ – CAAGCAGAAGACGGCATACGAGATCACTGTGTGACTGGAGTTCAGACGTGT GCTCTTCCGATCT – 3′	ACAGTG
D3007-06	Index 6 (A006)	5′ – CAAGCAGAAGACGGCATACGAGATATTGGCGTGACTGGAGTTCAGACGTGT GCTCTTCCGATCT – 3′	GCCAAT
D3007-07	Index 7 (A007)	5′ – CAAGCAGAAGACGGCATACGAGATGATCTGGTGACTGGAGTTCAGACGTGT GCTCTTCCGATCT – 3′	CAGATC
D3007-12	Index 12 (A012)	5′ – CAAGCAGAAGACGGCATACGAGATTACAAGGTGACTGGAGTTCAGACGTGT GCTCTTCCGATCT – 3′	CTTGTA

For additional index primer sets, please contact our technical support team at **tech@zymoresearch.com**

Appendix E: Illumina® Platform Compatibility

Illumina® sequencing chemistry uses the initial bases of the P5 read (Read 1) to identify sequence clusters and to establish focus and color balance. When sequencing libraries on the MiSeq®, please use the MiSeq Software Updater v2.2.0.2 (containing RTA v.1.17.28 rel. 3/18/2013) or later.

When sequencing, adding a high complexity spike-in is recommended.

Appendix F: Bioinformatics

The **Zymo-Seq RiboFree™ Total RNA Library Prep Kit** employs a low-complexity bridge to ligate the Illumina® P7 adapter sequence to the library inserts. This sequence can extend up to 10 nucleotides, and should not affect sequencing alignment with standard modern workflows, such as those using STAR alignment. If desired, an additional 10 bases at the P7-proximal end of library inserts may be trimmed using tools such as Fastp or Cutadapt before standard adapter-trimming.

Please note that QC analysis software, such as FastQC (Babraham Bioinformatics) or RSeQC (Wang *et al.* 2012), may raise “Per base sequence content” or “Per base GC content” flags at the beginning of Read 2. These flags are expected due to this low complexity bridge sequence.

Libraries generated with the **Zymo-Seq RiboFree™ Total RNA Library Prep Kit** begin with random hexamer priming to generate an initial cDNA library from total RNA. The position of these primers will correspond to the library insert sequence immediately following the P5 adapter.

Libraries are minus-stranded: The Read 1 sequence will be antisense to the RNA transcript from which it originates.

Ordering Information

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

For Individual Sale	Catalog No.	Amount
Zymo*Taq*™ PreMix	E2003 E2004	50 rxns 200 rxns
Select-a-Size MagBead Set	D4084-10 D4084-50	10 mL 50 mL
Zymo-Seq™ Wash Buffer	R3004-1-6	6 mL
DNA Elution Buffer	D3004-4-1	1 mL
DNase/RNase-Free Water	W1001-1	1 mL
PCR Strip MagStand	3DP-1002	Free at Checkout

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