ZYMO RESEARCH

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INSTRUCTION MANUAL

Zymo-Seq RiboFree™ Total RNA Library Kit

Cat. No. **R3000 & R3003** (Patent Pending)

Highlights

- **The Fastest & Easiest Kit:** Prepare stranded, RiboFree™ libraries from total RNA in 3.5 hours.
- **Compatible with Any Organism:** 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	R3000 12 Prep Kit	R3003 96 Prep Kit	Storage Temperature
R1 Reagent	24 µL	8 x 24 µL	-80 °C
R2 Reagent	120 µL	8 x 120 µL	-80 °C
D1 Reagent	120 µL	8 x 120 µL	-80 °C
D2 Reagent	120 µL	8 x 120 µL	-80 °C
D3 Stop Reagent	120 µL	8 x 120 µL	-80 °C
L1 Reagent	120 µL	8 x 120 µL	-80 °C
L2 Reagent	240 µL	8 x 240 µL	-80 °C
L3 Reagent	120 µL	8 x 120 µL	-80 °C
Zymo*Taq* PreMix	300 µL	8 x 300 µL	-80 °C
Zymo-Seq™ UDI Primer Set (Indexes 1-12)[1]	20 µL / Index	-	-80 °C
Zymo-Seq™ UDI Primer Plate (Indexes 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
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	-

Note: Integrity of kit components are guaranteed for up to six months 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™ UDI Primer Set (Indexes 1-12)** (Cat. No. D3008) contains 12 pre-mixed unique dual-index barcode primers in 1.5 mL tubes. See Appendix D for primer specifications and index sequences.

[2] The provided **Zymo-Seq™ UDI Primer Set (Indexes 1-96)** (Cat. No. D3096) contains 96 pre-mixed unique dual-index barcode primers in a 96-well plate format. See Appendix D for primer specifications and index sequences.

Specifications:

- **Sample Input Material:** RNA
- **Recommended Input:** 500 ng[3]
- **Minimum Input:** 100 ng[3]
- **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:** As little as 3.5 hours (RNA to indexed library)[4]
- **Sequencing:** Libraries are stranded and compatible with all Illumina® sequencing platforms. The Read 1 sequence will be antisense to the RNA molecule of origin.
- **Barcode Sequences:** Available for download here (USA Only), or by visiting the Documents section of the R3000 and D3096 product pages at www.zymoresearch.com.

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

[4] 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 Kit** (Figure 1)**.**

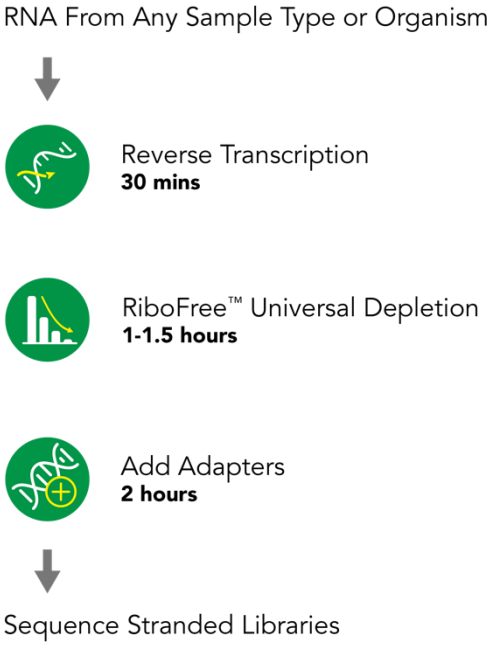


Figure 1: The Zymo-Seq RiboFree™ Total RNA Library Kit is the fastest and easiest Total RNA-Seq workflow. This kit minimizes the number of reagents and steps needed to generate stranded rRNA-depleted total RNA libraries in as little as 3.5 hours.

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™ Universal Depletion effectively removes rRNA and overrepresented transcripts (e.g. globin) without the use of probes that cause off-target digestion compared to popular rRNA removal kits.

RiboFree™ Universal Depletion (rRNA, beta-globin) is probe-free and 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.

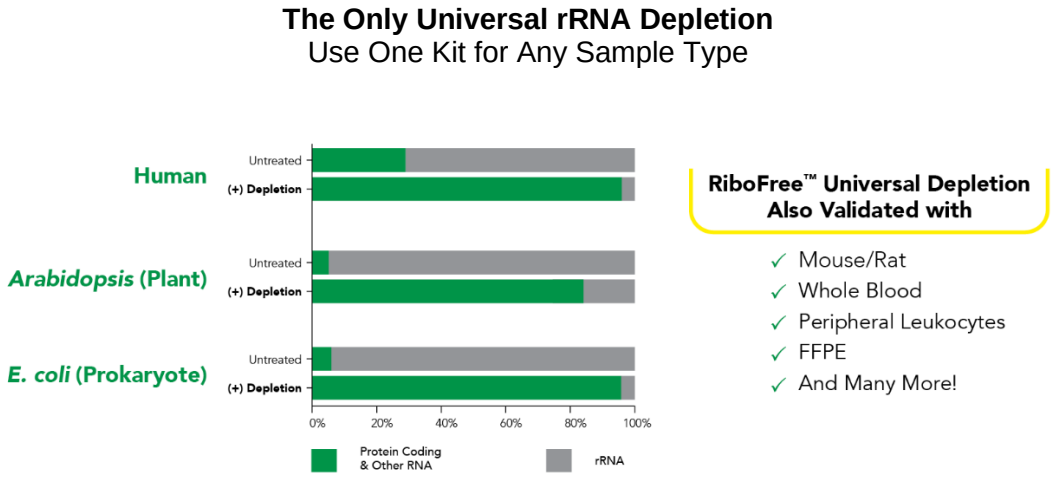


Figure 2: RiboFree™ Universal Depletion will enzymatically remove rRNA from any sample type. Paired-end sequencing was performed on stranded total RNA-Seq libraries, both with and without RiboFree™ Universal Depletion. Read pairs were aligned to their respective genomes using the STAR aligner. Read classes 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.

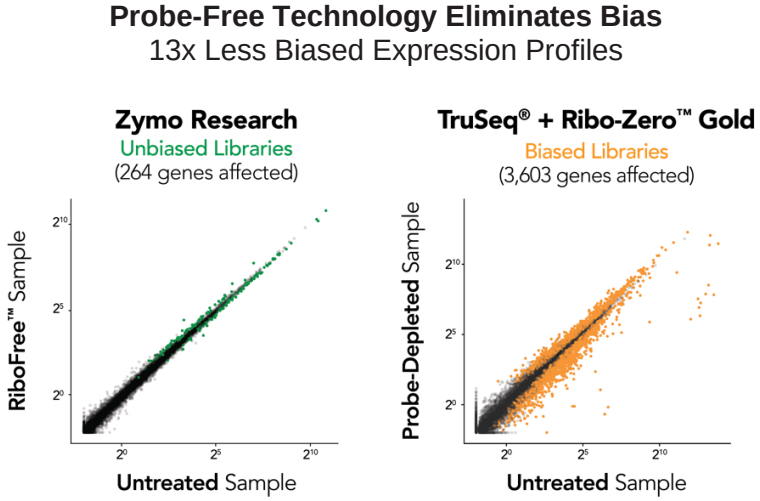
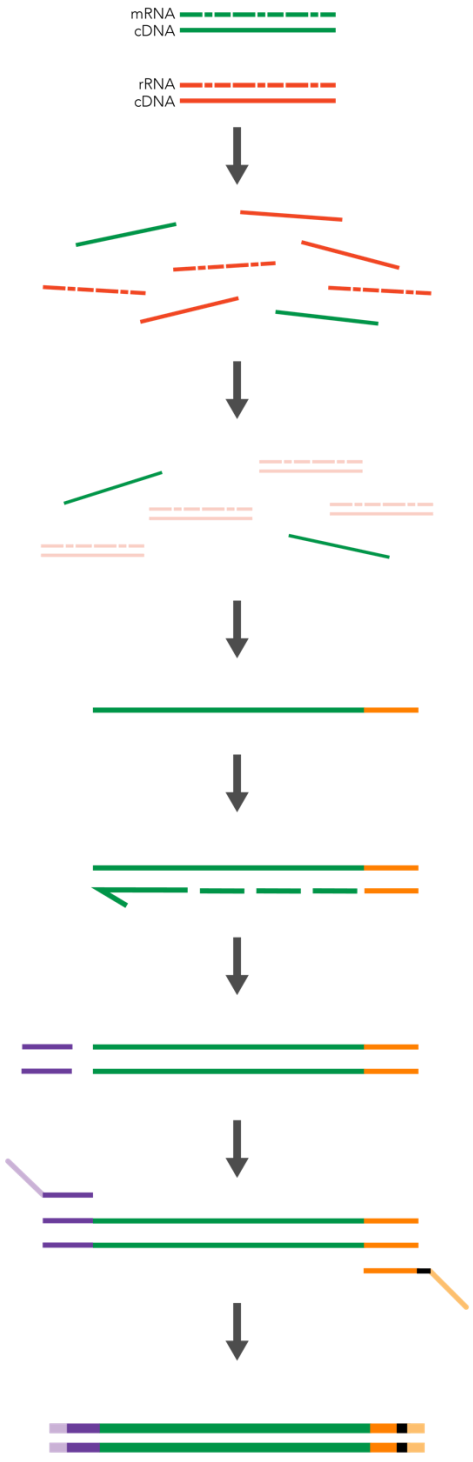


Figure 3: RiboFree™ Universal Depletion maintains native expression profiles unlike TruSeq® Total RNA [probe-based Ribo-Zero™ Gold]. Paired-end sequencing was performed on libraries prepared from Universal Human Reference RNA (Invitrogen) containing ERCC Spike-In Mix 1 (Life Technologies), both with 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 – 5 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.
- Allow the **Select-a-Size MagBeads** to equilibrate to room temperature for 30 minutes prior to use. Refer to **Appendix A** for additional details.
- 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.
7. Proceed directly to **Section 1.2**: depletion of ribosomal RNA, globin, or other overrepresented transcripts.[2]

[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.

Section 1.2: RiboFree™ Universal Depletion (Red Caps)

Before starting:

- Refer to **Appendix B** for further RiboFree™ Univeral Depletion incubation times
- 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 mins*	*(>1 µg)*	**Depletion Reaction**
		1 hr	*(500 ng-1 µg)*	
		1-2 hrs	*(250-500 ng)*	
		2-4 hrs	*(100-250 ng)*	
5)	68°C	**Hold**		**Stop Depletion**
6)	98°C	2 min		
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 MagBeads**[4]. For elution, resuspend the beads in 10 µL of **DNA Elution Buffer** and incubate at 95°C for 5 minutes then cool the tubes to room temperature**.**

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: RiboFree™ Universal 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 MagBeads**[2]. For elution, resuspend the beads in 10 µL of **DNA Elution Buffer** at room temperature (no need to incubate).

This is a safe stopping point. Cleaned-up DNA 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 MagBeads**[1]. For elution, resuspend the beads in 20 µL of **DNA Elution Buffer** at room temperature (no need to incubate)**.**

This is a safe stopping point. Cleaned-up DNA 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	500 ng - 1 µg = 11 cycles
4)	72°C	1 min	250 ng - 500 ng = 12 cycles
	-- Go to step 2 --		100 ng - 250 ng = 13 cycles
			< 100 ng = 13+ cycles
5)	72°C	7 min	
6)	4°C	Hold	

1. Add 5 µL of the appropriate pre-mixed **Zymo-Seq UDI 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 MagBeads**[2]. For elution, resuspend the beads in 15-25 µL of **DNA Elution Buffer** at room temperature (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 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 (R3000), or 192 mL of 100% ethanol (208 mL of 95% ethanol) to the 48 mL **Zymo-Seq™ Wash Buffer** concentrate (R3003).
- Add 300 µL of **Select-a-Size Magbead Concentrate** to each 10 mL **Select-a-Size Magbead Buffer** (R3000), or add 1.5 mL of **Select-a-Size Magbead Concentrate** to each 50 mL **Select-a-Size Magbead Buffer** (R3003). Resuspend by pipetting.
- Allow the **Select-a-Size MagBeads** to equilibrate to room temperature for 30 minutes prior to use.
- Resuspend the magnetic particles immediately before use by vigorously shaking the **Select-a-Size 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: RiboFree™ Universal Depletion 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.

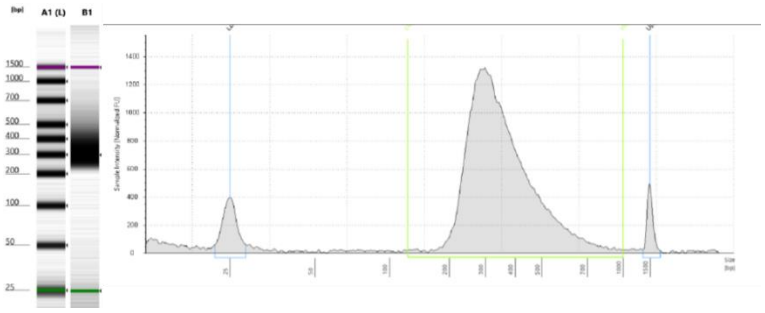
We have found that longer incubation times (up to 16 hours) do not adversely affect the sample. Optional: Users may optimize the depletion incubation time for various input amounts and unique sample types to improve rRNA depletion efficiency.

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	RiboFree™ Universal Depletion	Index PCR Amplification
> 1 µg	30 min	10 cycles
500 ng - 1 µg	1 hour	11 cycles
250 ng - 500 ng	1-2 hours	12 cycles
100 ng - 250 ng	2-4 hours	13 cycles
< 100 ng	4-16 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 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: Unique Dual Index Primer Sequences

Indexes in the **Zymo-Seq™ UDI Primer Set (Indexes 1-12)** are dispensed in 1.5 mL tubes (Cat. No. D3008), and the **Zymo-Seq™ UDI Primer Plate (Indexes 1-96)** are dispensed in single-use foil-sealed 96-well plates (Ca. No. D3096). Indexes come as pre-mixes, and the forward and reverse primers are provided at 5 µM total concentration (2.5 µM each). Zymo-Seq™ index primers generate sequencing libraries that are compatible with all Illumina® platforms.

The complete index sample sheet (Pages 13-14) are availalble for downloaded here (USA Only), or by visiting the Documents section of the R3000 and D3096 product pages at www.zymoresearch.com.

Primer Sequences:

Forward Primer Sequence (i5):

5′-AATGATACGGCGACCACCGAGATCTACAC**NNNNNNNN**ACACTCTTTCCCTACACGACGCTCTTCCGATCT-3′

Reverse Primer Sequence (i7):

5′-CAAGCAGAAGACGGCATACGAGAT**NNNNNNNN**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT-3′

Note: The **NNNNNNNN** sequences correspond to the "Bases in Adapter" columns in the table below.

UDI Primer Plate (D3096) Setup:

To use UDI primers, pool ≥ 2 libraries 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

Unique Dual Index Barcode Sequences:

Index (Fwd + Rev)	i5 Bases in Adapter / for NovaSeq, MiSeq, and HiSeq 2000/2500	i5 Bases for iSeq, NextSeq, and HiSeq 3000/3500	i7 Bases in Adapter	i7 Bases for all sample sheets
UDI_01	AGCGCTAG	CTAGCGCT	AACCGCGG	CCGCGGTT
UDI_02	GATATCGA	TCGATATC	GGTTATAA	TTATAACC
UDI_03	CGCAGACG	CGTCTGCG	CCAAGTCC	GGACTTGG
UDI_04	TATGAGTA	TACTCATA	TTGGACTT	AAGTCCAA
UDI_05	AGGTGCGT	ACGCACCT	CAGTGGAT	ATCCACTG
UDI_06	GAACATAC	GTATGTTC	TGACAAGC	GCTTGTCA
UDI_07	ACATAGCG	CGCTATGT	CTAGCTTG	CAAGCTAG
UDI_08	GTGCGATA	TATCGCAC	TCGATCCA	TGGATCGA
UDI_09	CCAACAGA	TCTGTTGG	CCTGAACT	AGTTCAGG
UDI_10	TTGGTGAG	CTCACCAA	TTCAGGTC	GACCTGAA
UDI_11	CGCGGTTC	GAACCGCG	AGTAGAGA	TCTCTACT
UDI_12	TATAACCT	AGGTTATA	GACGAGAG	CTCTCGTC
UDI_13	AAGGATGA	TCATCCTT	AGACTTGG	CCAAGTCT
UDI_14	GGAAGCAG	CTGCTTCC	GAGTCCAA	TTGGACTC
UDI_15	TCGTGACC	GGTCACGA	CTTAAGCC	GGCTTAAG
UDI_16	CTACAGTT	AACTGTAG	TCCGGATT	AATCCGGA
UDI_17	ATATTCAC	GTGAATAT	CTGTATTA	TAATACAG
UDI_18	GCGCCTGT	ACAGGCGC	TCACGCCG	CGGCGTGA
UDI_19	ACTCTATG	CATAGAGT	ACTTACAT	ATGTAAGT
UDI_20	GTCTCGCA	TGCGAGAC	GTCCGTGC	GCACGGAC
UDI_21	AAGACGTC	GACGTCTT	AAGGTACC	GGTACCTT
UDI_22	GGAGTACT	AGTACTCC	GGAACGTT	AACGTTCC
UDI_23	ACCGGCCA	TGGCCGGT	AATTCTGC	GCAGAATT
UDI_24	GTTAATTG	CAATTAAC	GGCCTCAT	ATGAGGCC
UDI_25	AACCGCGG	CCGCGGTT	ATCTTAGT	ACTAAGAT
UDI_26	GGTTATAA	TTATAACC	GCTCCGAC	GTCGGAGC
UDI_27	CCAAGTCC	GGACTTGG	ATACCAAG	CTTGGTAT
UDI_28	TTGGACTT	AAGTCCAA	GCGTTGGA	TCCAACGC
UDI_29	CAGTGGAT	ATCCACTG	CTTCACGG	CCGTGAAG
UDI_30	TGACAAGC	GCTTGTCA	TCCTGTAA	TTACAGGA
UDI_31	CTAGCTTG	CAAGCTAG	AGAATGCC	GGCATTCT
UDI_32	TCGATCCA	TGGATCGA	GAGGCATT	AATGCCTC
UDI_33	CCTGAACT	AGTTCAGG	CCTCGGTA	TACCGAGG
UDI_34	TTCAGGTC	GACCTGAA	TTCTAACG	CGTTAGAA
UDI_35	AGTAGAGA	TCTCTACT	ATGAGGCT	AGCCTCAT
UDI_36	GACGAGAG	CTCTCGTC	GCAGAATC	GATTCTGC
UDI_37	AGACTTGG	CCAAGTCT	CACTACGA	TCGTAGTG
UDI_38	GAGTCCAA	TTGGACTC	TGTCGTAG	CTACGACA
UDI_39	CTTAAGCC	GGCTTAAG	ACCACTTA	TAAGTGGT
UDI_40	TCCGGATT	AATCCGGA	GTTGTCCG	CGGACAAC
UDI_41	CTGTATTA	TAATACAG	ATCCATAT	ATATGGAT
UDI_42	TCACGCCG	CGGCGTGA	GCTTGCGC	GCGCAAGC
UDI_43	ACTTACAT	ATGTAAGT	AGTATCTT	AAGATACT
UDI_44	GTCCGTGC	GCACGGAC	GACGCTCC	GGAGCGTC
UDI_45	AAGGTACC	GGTACCTT	CATGCCAT	ATGGCATG
UDI_46	GGAACGTT	AACGTTCC	TGCATTGC	GCAATGCA

UDI_47	AATTCTGC	GCAGAATT	ATTGGAAC	GTTCCAAT
UDI_48	GGCCTCAT	ATGAGGCC	GCCAAGGT	ACCTTGGC
UDI_49	ATCTTAGT	ACTAAGAT	CGAGATAT	ATATCTCG
UDI_50	GCTCCGAC	GTCGGAGC	TAGAGCGC	GCGCTCTA
UDI_51	ATACCAAG	CTTGGTAT	AACCTGTT	AACAGGTT
UDI_52	GCGTTGGA	TCCAACGC	GGTTCACC	GGTGAACC
UDI_53	CTTCACGG	CCGTGAAG	CATTGTTG	CAACAATG
UDI_54	TCCTGTAA	TTACAGGA	TGCCACCA	TGGTGGCA
UDI_55	AGAATGCC	GGCATTCT	CTCTGCCT	AGGCAGAG
UDI_56	GAGGCATT	AATGCCTC	TCTCATTC	GAATGAGA
UDI_57	CCTCGGTA	TACCGAGG	ACGCCGCA	TGCGGCGT
UDI_58	TTCTAACG	CGTTAGAA	GTATTATG	CATAATAC
UDI_59	ATGAGGCT	AGCCTCAT	GATAGATC	GATCTATC
UDI_60	GCAGAATC	GATTCTGC	AGCGAGCT	AGCTCGCT
UDI_61	CACTACGA	TCGTAGTG	CAGTTCCG	CGGAACTG
UDI_62	TGTCGTAG	CTACGACA	TGACCTTA	TAAGGTCA
UDI_63	ACCACTTA	TAAGTGGT	CTAGGCAA	TTGCCTAG
UDI_64	GTTGTCCG	CGGACAAC	TCGAATGG	CCATTCGA
UDI_65	ATCCATAT	ATATGGAT	CTTAGTGT	ACACTAAG
UDI_66	GCTTGCGC	GCGCAAGC	TCCGACAC	GTGTCGGA
UDI_67	AGTATCTT	AAGATACT	AACAGGAA	TTCCTGTT
UDI_68	GACGCTCC	GGAGCGTC	GGTGAAGG	CCTTCACC
UDI_69	CATGCCAT	ATGGCATG	CCTGTGGC	GCCACAGG
UDI_70	TGCATTGC	GCAATGCA	TTCACAAT	ATTGTGAA
UDI_71	ATTGGAAC	GTTCCAAT	ACACGAGT	ACTCGTGT
UDI_72	GCCAAGGT	ACCTTGGC	GTGTAGAC	GTCTACAC
UDI_73	CGAGATAT	ATATCTCG	GTTAATTG	CAATTAAC
UDI_74	TAGAGCGC	GCGCTCTA	ACCGGCCA	TGGCCGGT
UDI_75	AACCTGTT	AACAGGTT	GGAGTACT	AGTACTCC
UDI_76	GGTTCACC	GGTGAACC	AAGACGTC	GACGTCTT
UDI_77	CATTGTTG	CAACAATG	GTCTCGCA	TGCGAGAC
UDI_78	TGCCACCA	TGGTGGCA	ACTCTATG	CATAGAGT
UDI_79	CTCTGCCT	AGGCAGAG	GCGCCTGT	ACAGGCGC
UDI_80	TCTCATTC	GAATGAGA	ATATTCAC	GTGAATAT
UDI_81	ACGCCGCA	TGCGGCGT	CTACAGTT	AACTGTAG
UDI_82	GTATTATG	CATAATAC	TCGTGACC	GGTCACGA
UDI_83	GATAGATC	GATCTATC	GGAAGCAG	CTGCTTCC
UDI_84	AGCGAGCT	AGCTCGCT	AAGGATGA	TCATCCTT
UDI_85	CAGTTCCG	CGGAACTG	TATAACCT	AGGTTATA
UDI_86	TGACCTTA	TAAGGTCA	CGCGGTTC	GAACCGCG
UDI_87	CTAGGCAA	TTGCCTAG	TTGGTGAG	CTCACCAA
UDI_88	TCGAATGG	CCATTCGA	CCAACAGA	TCTGTTGG
UDI_89	CTTAGTGT	ACACTAAG	GTGCGATA	TATCGCAC
UDI_90	TCCGACAC	GTGTCGGA	ACATAGCG	CGCTATGT
UDI_91	AACAGGAA	TTCCTGTT	GAACATAC	GTATGTTC
UDI_92	GGTGAAGG	CCTTCACC	AGGTGCGT	ACGCACCT
UDI_93	CCTGTGGC	GCCACAGG	TATGAGTA	TACTCATA
UDI_94	TTCACAAT	ATTGTGAA	CGCAGACG	CGTCTGCG
UDI_95	ACACGAGT	ACTCGTGT	GATATCGA	TCGATATC
UDI_96	GTGTAGAC	GTCTACAC	AGCGCTAG	CTAGCGCT

Appendix E: Illumina® Platform Compatibility

Illumina® sequencing chemistry uses the initial bases of 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 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.

Library preparation using the **Zymo-Seq RiboFree™ Total RNA Library Kit** begins with random 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™ 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

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 R3004-1-48	6 mL 48 mL
DNA Elution Buffer	D3004-4-10 D3004-4-50	10 mL 50 mL
DNase/RNase-Free Water	W1001-1 W1001-10	1 mL 10 mL
PCR Strip MagStand	3DP-1002	Free at Checkout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