ZYMO RESEARCH

The Beauty of Science is to Make Things Simple

INSTRUCTION MANUAL

Zymo-Seq RiboFree™ Universal cDNA Kit

Cat. No. **R3001** (Patent Pending)

Highlights

- **The Easiest Kit:** Prepare RiboFree™ cDNA from total RNA in as little as 5 pipetting steps.
- **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™ Universal cDNA Kit	Cat. R3001 12 Prep Kit	Storage Temperature
R1 Reagent	24 µL	-80 °C
R2 Reagent	120 µL	-80 °C
D1 Reagent	120 µL	-80 °C
D2 Reagent	120 µL	-80 °C
D3 Stop Reagent	120 µL	-80 °C
Select-a-Size MagBead Concentrate	2 x 30 µL	4 °C
Select-a-Size MagBead Buffer	2 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.
Instruction Manual	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.

Specifications:

- **Sample Input Material:** RNA
- **Recommended Input:** 500 ng[1]
- **Minimum Input:** 100 ng[1]
- **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 1.5 hours (RNA to single-stranded cDNA)[2]

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

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

Product Description:

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

The **Zymo-Seq RiboFree™ Universal cDNA Kit** is the easiest rRNA and globin depletion kit available. RiboFree™ Universal Depletion is compatible with total RNA from any biological sample for qPCR detection, RNA-Seq library prep, and any other downstream analysis (Figure 1)**.**

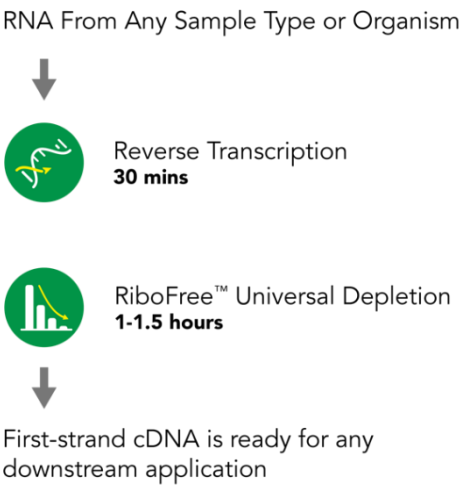

Figure 1: The easiest method to prepare RiboFree™ cDNA. This kit minimizes the number of reagents and steps needed to generate first-strand rRNA-depleted total cDNA in just 5 pipetting steps.

This kit produces first-strand cDNA from any sample's full transcriptome (both coding and non-coding). Overcome challenges in capturing mRNA from degraded and fragmented samples with this kit. In contrast to poly(A) targeted RNA pull-down or oligo (dT) priming, this kit produces cDNA from total RNA, including long-noncoding RNAs (lncRNA), intronic RNAs, nucleolar RNAs, and mRNAs with degraded poly(A) tails.

Ribosomal RNAs (rRNA) can comprise approximately 90% of the total cellular RNA and represents an obstacle when analyzing 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 compatible with all biological sample types, tissues (including whole blood), and does not require organism-specific probes (Figure 2).

A streamlined workflow minimizes user manipulation, resulting in a user-friendly protocol with little hands-on time. The RiboFree™ Universal cDNA Kit is a simple and cost-effective method for generating first-strand cDNA with minimal bias compared to other rRNA depletion or poly(A) enrichment methods (Figure 3).

The Only Universal rRNA Depletion
Use One Kit for Any Sample Type

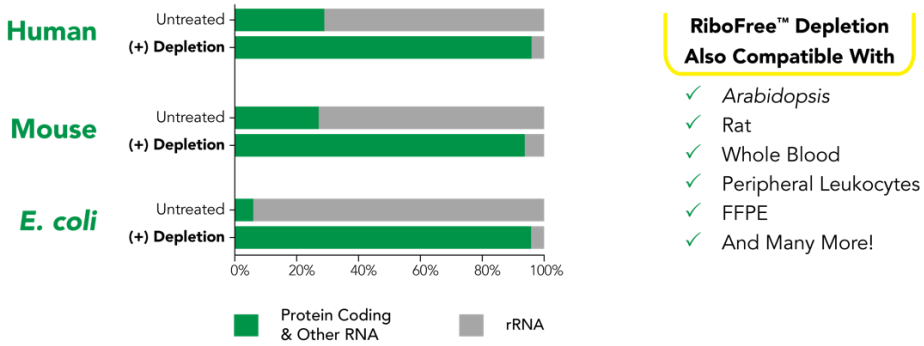

Figure 2: RiboFree™ Universal Depletion will enzymatically remove rRNA from any sample type. Paired-end sequencing was performed on stranded total RNA-Seq libraries, both with and without RiboFree™ Universal Depletion. Read pairs were aligned to their respective genomes using the STAR aligner. Read classes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

Probe-Free Technology Eliminates Bias
35x Less Biased Expression Profiles

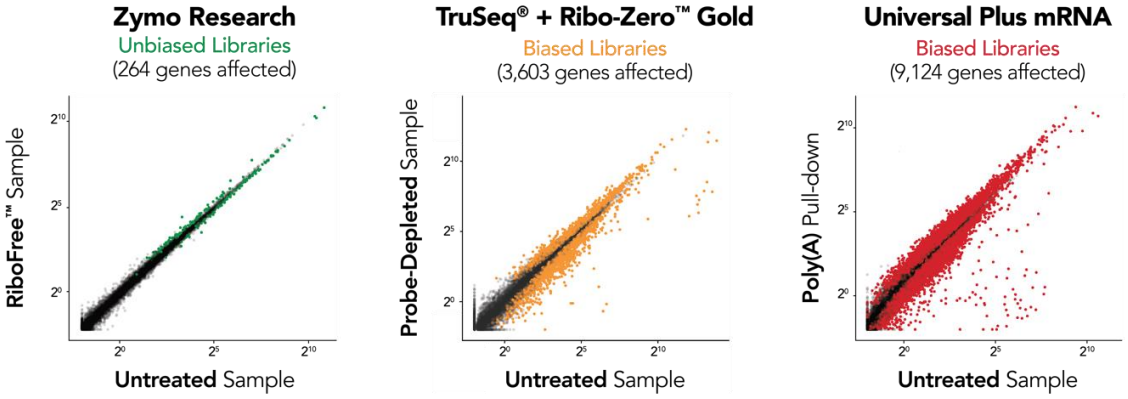

Figure 3: RiboFree™ Universal Depletion maintains native expression profiles unlike TruSeq® Total RNA [probe-based Ribo-Zero™ Gold] and Universal Plus mRNA-Seq [poly(A) enrichment]. Paired-end sequencing was performed on libraries prepared from Universal Human Reference RNA (Invitrogen) containing ERCC 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.

RiboFree™ Universal cDNA Kit Overview:

Section 1.1: First-Strand cDNA Synthesis
Reverse Transcription

Section 1.2: RiboFree™ Universal Depletion
Denature

Re-nature and Deplete

RiboFree™ First-Strand cDNA

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

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 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 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**.**

The eluate is your final RiboFree™ first-strand cDNA, and may be stored at ≤ 4°C overnight or ≤ -20°C for long-term storage.

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.

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.
- 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 MagBeads** until homogenous.

1. Add 150 µL 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 ≥10 µL of **DNA Elution Buffer** to the beads and mix thoroughly by pipetting up and down until homogenous. Incubate at 95°C for 5 minutes.

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.

Recommended incubation times for standard applications:

RNA Input	RiboFree™ Universal Depletion
> 1 µg	30 min
> 250 ng	1 hour
> 100 ng	2 hours
< 100 ng	3-16 hours

Ordering Information

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

For Individual Sale	Catalog No.	Amount
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	*1

*Inquire for access to a free PCR Strip MagStand (3DP-1002) with a purchase of R3000 and R30001. Limited supplies available.

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