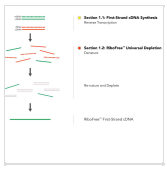

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Ribosomal RNA Depletion and cDNA Synthesis

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ABSTRACT

Ribosomal RNA depletion and cDNA synthesis using the Zymo-Seq RiboFree Universal cDNA Kit. This protocol should follow RNA isolation and purification using the Direct-zol™ RNA Microprep Kit (Zymo) and the RNA Clean & Concentrator Kit (Zymo).

We will follow the manufacturer's instructions for reverse transcription thermocycling and ribosomal depletion.

After completing this protocol, proceed to qPCR using the PrimeTime Gene Expression Kit.

From the manufacturer:
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 RiboFreeTM 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.

RiboFreeTM Universal Depletion (rRNA, beta-globin) is compatible with all biological sample types, tissues (including whole blood), and does not require organism-specific probes.

A streamlined workflow minimizes user manipulation, resulting in a user-friendly protocol with little hands-on time. The RiboFreeTM 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.

ATTACHMENTS

Zymo-Seq RiboFree Universal cDNA Kit.pdf

PROTOCOL CITATION

Alicia M Rich 2021. Ribosomal RNA Depletion and cDNA Synthesis. **protocols.io** https://protocols.io/view/ribosomal-rna-depletion-and-cdna-synthesis-bq3wmype

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GUIDELINES

Carry out this protocol in the DNA/RNA extraction area of the lab using the biosafety cabinet to protect the pre-amplified RNA from contamination.

Ribonucleases (RNases) are very stable and active enzymes that generally do not require cofactors to function. Since RNases are difficult to inactivate and even minute amounts are sufficient to destroy RNA, do not use any plasticware or glassware without first eliminating possible RNase contamination. Great care should be taken to avoid inadvertently introducing RNases into the RNA sample during or after the purification procedure.

Proper microbiological, aseptic technique should always be used when working with RNA. Hands and dust particles may carry bacteria and molds and are the most common sources of RNase contamination. Always wear latex or vinyl gloves while handling reagents and RNA samples to prevent RNase contamination from the surface of the skin or from dusty laboratory equipment. Change gloves frequently and keep tubes closed whenever possible. Keep purified RNA on ice when aliquots are pipetted for downstream applications.

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

MATERIALS TEXT

Zymo-Seq RiboFree Universal cDNA Kit **Zymo**

Research Catalog #R3001 (12 preps)

- R1 Reagent **-80 °C**
- R2 Reagent **-80 °C**
- D1 Reagent **-80 °C**
- D2 Reagent **-80 °C**
- D3 Stop Reagent **-80 °C**

Select-a-Size MagBead Set (10 ml) **Zymo**

- **Research Catalog #D4084-10**
- MagBead Concentrate (2x30µL) **4 °C**
- MagBead Buffer (2x1mL) **4 °C**

Select-a-Size MagBead Set (50 ml) **Zymo**

- **Research Catalog #D4084-50**

Zymo-Seq Wash Buffer **Zymo**

- **Research Catalog #R3004-1-6** Step 22 **Room temperature**

Zymo DNA Elution Buffer **Zymo**

- **Research Catalog #D3004-4-1** Step 24 **Room temperature**

DNase/RNase-Free Water **Zymo**

- **Research Catalog #W1001-1** Step 1.2 **Room temperature**

Specifications:

Sample Input Material: RNA

Recommended Input: 500 ng

Minimum Input: 100 ng

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: **01:30:00** (RNA to single-stranded cDNA)

SAFETY WARNINGS

Wear all proper PPE (lab coat, nitrile gloves).

BEFORE STARTING

1. Thoroughly clean the extraction area with bleach and/or RNase spray. Close the cabinet hood and run the UV light for 15 minutes after you have set up all of your equipment and reagents (except the RNA extracts). Prepare an ice bath or cooling block for RNA extracts.
2. Add **24 mL** of **100 % volume** ethanol (or **26 mL** of **95 % volume** ethanol) to the **6 mL** Zymo-Seq Wash Buffer **Zymo Research Catalog #R3004-1-6** concentrate.
3. Add **30 µl** of **Select-a-Size Magbead Concentrate** to each **1 mL** **Select-a-Size Magbead Buffer**. Resuspend by pipetting up and down.

0.0 Estimate Starting RNA concentration

1 Estimate the starting RNA concentration of each extract using the Nanodrop.

1.1 **On ice** Bring RNA extracts and RNase-free water on a cold block to the spectrophotometer downstairs.

1.2 DNase/RNase-Free Water **Zymo Research Catalog #W1001-1**

Wash the sample reader with and dry with a KimWipe.

1.3 Load **2 µl** of elution water (blank) and initialize the system.

1.4 Change the computer's setting to RNA and click the "blank" button.

1.5 Load **2 µl** of sample and click the "measure" button.

1.6 After the read is complete, record the A_{260}/A_{280} and A_{260}/A_{230} ratios as well as the amount of RNA recovered (in **0 µg/µl**).

1.7 Wipe the sample reader with a clean, dry KimWipe between samples. **go to step #1.3**

1.8 Interpret your results.

- As a reference, collagen gels seeded with ~700,000 cells typically yield around 50-150 ng/µL of RNA when eluted in 30-35 µL of water
- A lower than expected concentration of RNA indicates low cell numbers in the sample, poor homogenization of samples, or too much volume of water used in the elution step of RNA purification.
- Very pure RNA will have an **A_{260}/A_{280}** ratio of ~2.1. Anything higher than 1.8 is considered to be of acceptable purity, and a ratio of <1.8 indicates potential DNA or protein contamination. A low A_{260}/A_{280} ratio is likely due to mixing phases when removing the upper aqueous phase of the Trizol separation or is also more common in samples with a very low yield of RNA.
- The **A_{260}/A_{230}** ratio should also be above 2.0. A low A_{260}/A_{230} ratio indicates contamination with the wash solutions, chaotropic salts, phenols, or protein. A low A_{260}/A_{230} ratio is most likely due to contamination of the samples with washing buffers during the spin column washes. Be more careful when handling the tubes, especially when adding a wash buffer or removing the spin-through. Try to gently pour out the flow-through and then carefully wipe away drops on the outer rim of the collection tube with a KimWipe.

1.1 First-Strand cDNA Synthesis (Yellow Caps)

2 Create the following thermocycler program for a total reaction volume of **20 µl**:

Temperature	Time	Stage
98 C	3 min	Primer Annealing
4 C	Hold	Primer Annealing
25 C	5 min	Reverse Transcription
48 C	15 min	Reverse Transcription
4 C	Hold	Reverse Transcription

This program should be in the thermocycler under "cDNA synth"

SimpliAmp Thermal Cycler
PCR

Applied Biosystems **A24811**

Any standard PCR thermocycler will suffice

3 **On ice** Thaw the R1 and R2 Reagents. Mix thoroughly using one of the mini-vortexes or by flicking. Briefly spin down on the mini-centrifuge.

Avoid multiple freeze-thaws (make aliquots if necessary) and always keep samples on ice or cooling block during use.

4 **On ice** Using RNase/RNase-Free Water, raise the volume of each RNA input sample to **8 µl** in 0.2 mL PCR tubes or plates.

5 **On ice** Add **2 µl** of the **R1 Reagent** to each sample for a total of **10 µl** . Mix thoroughly using a mini-vortex. Briefly spin down using the mini-centrifuge.

For inputs < **100 ng** , **1 µl** of the **R1 Reagent** should be used. Supplement with DNase/RNase-free water.

6 Place tubes or plate in the thermocycler. Run Steps 1-2 (Primer Annealing Stage) of the program.

Temp	Time	Stage
98 C	3 min	Primer Annealing
4 C	Hold	Primer Annealing

SimpliAmp Thermal Cycler
PCR

Applied Biosystems **A24811**

Any standard PCR thermocycler will suffice

7 **On ice or just keep on 4C hold in thermocycler** Add **10 µl** of the **R2 Reagent** to each sample.

8 Continue the thermocycler program through **Steps 3-5 (Reverse Transcription)** of the program.

Temp	Time	Stage
25 C	5 min	Reverse Transcription
48 C	15 min	Reverse Transcription
4 C	Hold	Reverse Transcription

1.2 RiboFree Universal Depletion (Red Caps)

9

Create the following thermocycler program for a total reaction volume of **50 µl** .

Temp	Time	Stage
98 C	3 min	Pre-Depletion Incubation
68 C	5 min	Pre-Depletion Incubation
68 C	Hold	Pre-Depletion Incubation
68 C	Depends on input - 30 min if >1 ug, 1 hr if >250 ng, or 2 hr if >100 ng	Depletion Rxn
68 C	Hold	Depletion Rxn
98 C	2 min	Stop Depletion
25 C	Hold	Stop Depletion

This program should be in thermocycler under "ribo depletion"

Refer to **Appendix B** of manufacturer protocol (table below) for further RiboFree Universal Depletion Incubation Times if troubleshooting is needed.

RNA Input	Depletion Time (Step 4)
> 1 ug	30 min
> 250 ng	1 hr
> 100 ng	2 hrs
< 100 ng	3-16 hrs

Optional depletion time modifications for Step 4 of "ribo depletion" program.

This universal depletion stage will involve transferring reagents to tubes while they are still inside the thermocycler. Make sure you are not overlapping with someone's scheduled use of the machine.

10 **On ice** Thaw the **D1, D2, and D3 Reagents**. Before starting, mix thoroughly using the mini-vortex and then spin down briefly using the mini-centrifuge.

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

11 **On ice** Add **10 µl** of the **D1 Reagent** directly to each **20 µl** sample for a total of **30 µl** . Mix thoroughly using the mini-vortex and then spin down briefly using the mini-centrifuge.

12 Transfer the samples to the thermocycler and run **Steps 1-3 (Pre-Depletion Incubation)** of the "ribo depletion" program.

DO NOT REMOVE YOUR SAMPLES FROM THE THERMOCYCLER AT THE STEP 3 HOLD.

13 Without removing the tubes or plate, add **10 µl** of the **D2 Reagent** to each **30 µl** sample during the **Step 3** hold for a total volume of **40 µl** . Mix in the thermocycler by pipetting up and down.

Optional: if available, you can use a multichannel pipette to minimize hands-on time for this step.

14 Close the thermocycler lid and continue through the **Depletion Reaction** of the "ribo depletion" program.

DO NOT REMOVE YOUR SAMPLES AT THE STEP 5 HOLD

15 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 volume of **50 µl** . Mix in the thermocycler by pipetting.

16 Close the thermocycler lid and continue through the **Stop Depletion** stage of the "ribo depletion" program. Remove samples from the thermocycler.

17 **On ice** Add **25 µl** of **[M]95 % volume** ethanol to each **50 µl** sample for a total volume of **75 µl** . Mix by pipetting up and down.

Select-a-Size MagBead Clean-up 13m

18 Resuspend the magnetic particles immediately before use by vigorously inverting the **Select-a-Size MagBeads** until homogenous.

19 Add **150 µl** of **Select-a-Size MagBeads** to each sample. Mix thoroughly by pipetting until homogenous.

5m

20 **Room temperature** Incubate for **00:05:00**

21 Place the samples on a magnetic rack (included in kit) until the beads have fully separated from the solution. Then discard the supernatant.

Avoid aspirating any beads when removing the supernatant.

22 While the beads are still on the magnetic rack, add **200 µl** of Zymo-Seq Wash Buffer **Zymo Research Catalog #R3004-1-6** . Remove and discard the supernatant.

22.1 **go to step #22**

Aspirate any residual wash buffer when repeating the step (2 washes total).

3m

23 Remove the tubes from the magnetic rack and keep the tops open for **00:03:00** to dry the beads.

24 Zymo DNA Elution Buffer **Zymo**

Add ≥ **10 µl** of **Research Catalog #D3004-4-1** to the beads and mix thoroughly by pipetting up and down until homogenous.

25 Incubate at **95 °C** for **00:05:00** . 5m

26 Place the tubes on a magnetic rack to separate the magnetic beads from the suspension, and transfer the eluate to a new tube.

Some beads may carry over into the eluate. Bead carry-over will not affect downstream reactions.