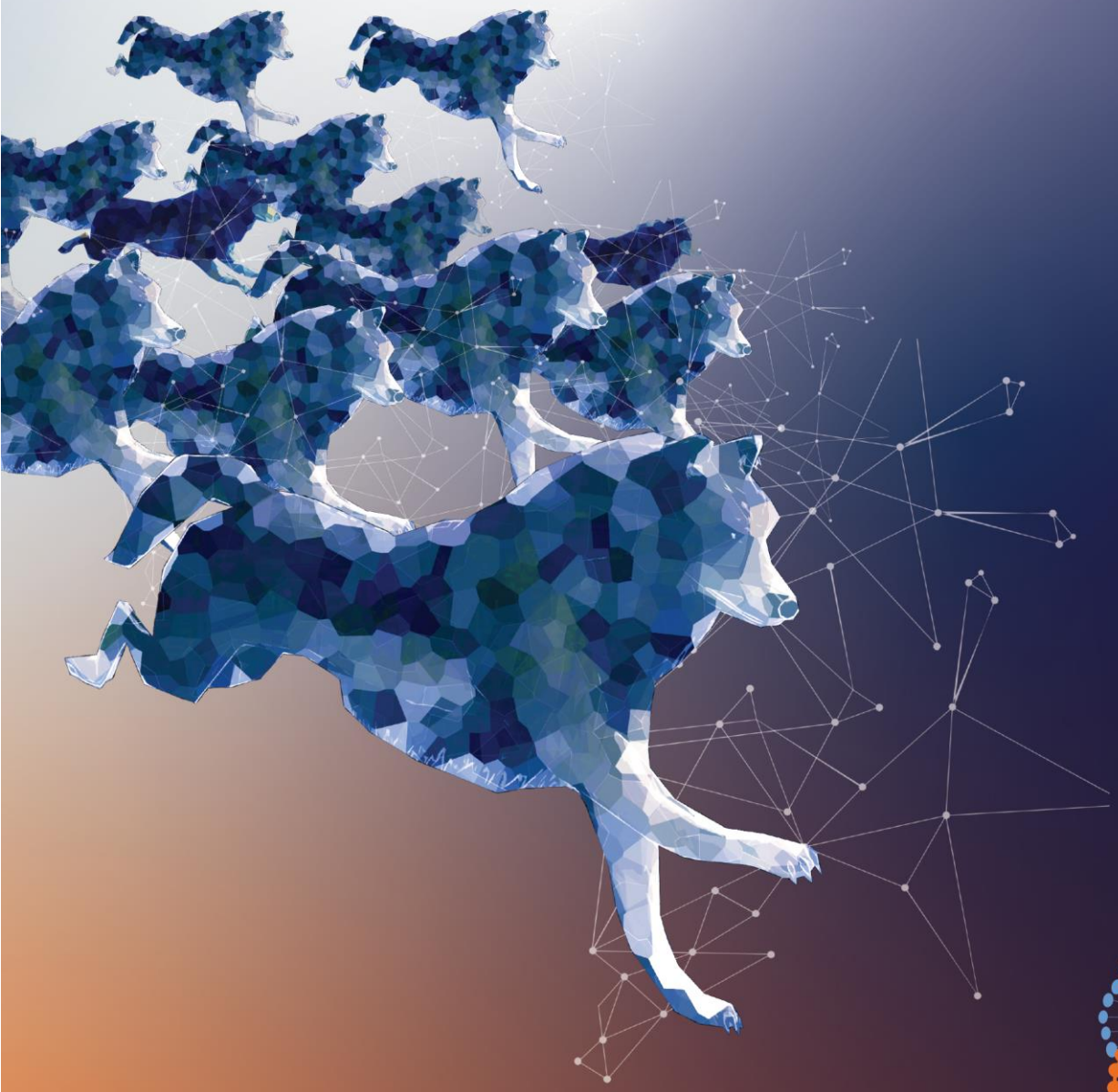
siTOOLs BIOTECH



Manual

riboPOOL™ Kit

Efficient Ribosomal RNA Depletion
For Any Species

Contact Information

siTOOLs Biotech GmbH
www.sitoolsbiotech.com
info@sitools.de
+49 89 12501 4800
@siTOOLsBiotech (Twitter/LinkedIn)
Lochhamer Str. 29A
Planegg/Martinsried, 82152, Germany

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Introduction

Purpose of kit

Ribosomal RNAs (rRNAs) account for up to 90% of total RNA isolated from most organisms including plants, animals and microbes. To enable sensitive and economical detection of scientifically relevant RNAs such as messenger RNA and non-coding RNAs, rRNAs are best removed before RNA analysis by Next-Generation Sequencing (RNA-Seq).

Ribosomal RNA depletion pools (riboPOOLs) developed by siTOOLs Biotech present an efficient, affordable and flexible solution that gives scientists the freedom to deplete rRNAs from any species.

The workflow with riboPOOLs can be completed within 70 min, is enzyme-free and compatible with high-throughput automation. As it does not rely on polyA-selection, it can be used to detect non-polyadenylated RNA that includes non-coding RNA, histones and prokaryotic RNA with uniform transcript coverage.

Product description

Composed of high complexity pools of optimally designed biotinylated DNA probes, riboPOOLs specifically hybridize with cytoplasmic and mitochondrial rRNAs, enabling their removal with streptavidin-coated magnetic beads (I. Kim, 2019, BMC Genomics).

Following the biotin-streptavidin magnetic bead-based removal of rRNAs, remaining RNA is cleared of salts and buffer concentrates by either ethanol precipitation, silica column purification or SPRI bead-based purification. This kit contains reagents for ethanol precipitation, but is compatible with column or bead-based purification kits, available from various commercial vendors.

RiboPOOLs have been made against a diverse array of organisms (Table 1). Custom riboPOOLs can also be tailor-made towards ribosomal RNA or other abundant RNA from any species.

Product performance

Human and mouse riboPOOLs were demonstrated by RNA-Seq to deplete > 95 % rRNA with high reproducibility between biological replicates (refer riboPOOL Product Page on website). Most riboPOOLs including custom riboPOOLs perform similarly, depleting rRNA with > 95% efficiency (as reported by customers).

For multi-species-targeting riboPOOLs such as the Pan-Prokaryote riboPOOL which provides rRNA depletion for a broad spectrum of microbes from major phyla, depletion efficiencies may vary between different microbial species (refer Pan-Prokaryote Brochure on website for more information).

The high complexity of riboPOOLs make them suitable for use with degraded samples. Best performance is obtained however with high quality RNA samples of RNA Integrity Number (RIN) ≥ 7.

List of Ready-Made riboPOOLs

Ready-Made riboPOOLs for Eukaryotes
Homo sapiens (human)
Mus musculus/Rattus norvegicus (mouse/rat)
Arabidopsis thaliana
Amphimedon queenslandica (sponge)
Bombyx morii (silkworm)
Chinchilla laginera (rodent)
Danio rerio (zebrafish)
Drosophila melanogaster (fruitfly)
Ixodes scapulars (ticks)
Pichia pastoris (yeast)
Plautia stali (brown winged green bug)
Sacchromyces cerevisiae (yeast)
Schmidtea mediterranea (planaria)
Ustilago maydis (fungus)
Pan Plant (Angiospermae)
Pan Fungi
Ready-Made riboPOOLs for Prokaryotes
Pan-Prokaryote (universal microbe rRNA depletion)
Escherichia coli
Pseudomonas aeruginosa
Staphylococcus aureus
Bacillus subtilis
Salmonella enterica
Stenotrophomonas sp.
Caulobacter crescentus
Haloferax volcanii
Clostridium perfringens
Ready-Made riboPOOLs for Abundant RNA
Human globin

Table 1. List of Ready-Made riboPOOLs

Special applications

Metatranscriptomics with Pan-Prokaryote riboPOOL

The Pan-Prokaryote riboPOOL is designed to deplete rRNA from a broad spectrum of microbes from all major phyla, enabling its use as a universal microbial rRNA depletion tool. It can be used for rRNA depletion of environmental or microbiome samples containing complex mixtures of known and unknown microbes (e.g. soil, sludge, pathogen-infected tissue).

Depletion of abundant RNAs

Aside from rRNA, depletion of abundant tissue-specific mRNAs (e.g. globin) or other abundant RNAs found in sequencing data can be performed with riboPOOLs.

Ribosome profiling

Ribosome profiling involves the sequencing of ribosome-bound RNA to assess the translatome. Ribosome-protected fragments are isolated and rRNA depletion is carried out prior to RNA-Seq. As these fragments range from 17-35 nucleotides, rRNA depletion efficiencies are expected to be lower than typical RNA samples. The human riboPOOL has been applied with rRNA depletion efficiencies ranging from 50-80%. To further enhance rRNA removal, custom riboPOOLs have also been applied against rRNA sequences remaining after rRNA depletion.

Depletion of rRNAs from multiple species with Combination riboPOOLs

To deplete rRNA from samples containing multiple species or for convenient use of one kit across samples from different species, Combination riboPOOLs may be applied. These are mixtures of up to four Ready-Made riboPOOLs combined in a ratio specified by the customer.

In-house tests on high quality RNA samples show the Combination riboPOOL (Human/Mouse/Pan-Prokaryote) performed similarly to species-specific riboPOOLs to deplete rRNA from Human, Mouse and Escherichia coli RNA (refer riboPOOL Application Note – *Efficient ribosomal RNA depletion across multiple species and input amounts in 70 min*).

However, siTOOLs Biotech cannot guarantee that Combination riboPOOLs will perform with similar depletion efficiencies across all sample types as depletion efficiency is expected to be highly dependent on sample quality.

Contents of riboPOOL kit

Code	Component	6 reaction kit (Trial)	12 reaction kit	24 reaction kit	96 reaction kit
RP	riboPOOL	1 x lyophilized powder	1 x lyophilized powder	1 x lyophilized powder	1 x lyophilized powder
H2O	Nuclease-free water	1 x 1 ml	1 x 1 ml	1 x 1 ml	1 x 1 ml
HB	Hybridization buffer	1 x 1 ml	1 x 1 ml	1 x 1 ml	1 x 1 ml
DB	Depletion buffer	1 x 8 ml	1 x 8 ml	1 x 8 ml	2 x 8 ml
SMB	Streptavidin-coated magnetic beads	1 x 0.6 ml	1 x 1.2 ml	2 x 1.2 ml	8 x 1.2 ml
	1.5 ml Reaction tubes	1 x 25 tubes	1 x 50 tubes	2 x 50 tubes	4 x 50 tubes
LA	Linear acrylamide	1 x 10 µl	1 x 30 µl	1 x 30 µl	1 x 110 µl
SA	Sodium acetate, 3M	1 x 100 µl	1 x 300 µl	1 x 300 µl	1 x 1.1 ml
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Storage instructions

The riboPOOL kit is shipped at room temperature.

Upon receipt, please store buffers (HB, DB), sodium acetate (SA) and reaction tubes at room temperature (r.t.). The solutions would be stable up to a year.

Streptavidin-coated magnetic beads (SMB) should be stored at 4°C and not be frozen.

Linear acrylamide (LA) is best stored at -20°C.

The riboPOOL (RP) when lyophilized, is stable at r.t. for up to a year, but is best stored at -20°C upon receipt. Upon resuspension in nuclease-free water (H2O), riboPOOLs are stable up to 6 months when stored at or below -20°C. Please store in aliquots to avoid freeze-thaw cycles.

Additional material required

(not supplied in kit)

- Magnetic rack or plate
- Temperature-controlled mixer or thermal cycler
- RNase inhibitor (optional)
- Sterile, low-retention pipette tips for minimal surface binding of RNA and beads
- 96-well PCR plate and sealing foil or caps (for 96 reaction kit)
- Common laboratory equipment – benchtop centrifuge, vortex, pipettes
- 100% research-grade ethanol
- 70% research-grade ethanol
- Personal protection equipment – lab coat, gloves

Application tips

- Use highest quality RNA (RIN > 7) as input for best results. Degraded RNA may also be applied but could result in lower depletion efficiencies.
- RNA input should be DNA-free.
- RNA input amount may range from 100 ng to 5 µg. For larger amounts, split into aliquots ≤ 5 µg and follow protocol accordingly.
- During protocol, avoid leaving tubes with RNA open or at r.t. over an extended time.
- Take necessary precautions to avoid RNase contamination i.e. keep work area clean, wear gloves, do not speak or work above open tubes.
- After rRNA depletion, expect to lose ≥ 90% of initial RNA amount due to high abundance of rRNA. Expected yield for 1 µg of input RNA is < 80 ng.
- Equilibrate all reagents to room temperature before use.
- To agitate tubes containing beads, flick the tube gently till solution becomes homogenous. Alternatively, vortex the tube at medium speed.
- Follow recommended volumes of reagents for RNA input 100 ng – 5 µg as modifying volumes may result in decreased efficiency. For input amount < 100 ng, reduce probe amount to 30 pmol/reaction.

Protocol

Protocol Snapshot

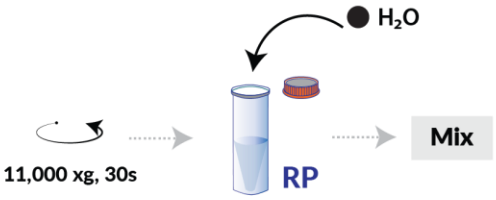
1 *Prepare riboPOOL™*

Components required

RP
H_2O

1.1

Centrifuge **RP** contents
Add **H_2O**



Reaction kit	H_2O
6	10 µl
12	15 µl
24	30 µl
96	110 µl

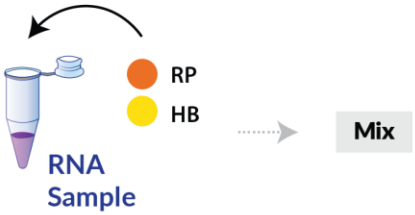
2 *Hybridization*

RNA Sample
RP
HB

2.1

Combine

14 µl RNA sample *(100 ng – 5 µg)*
1 µl resuspended RP
5 µl HB
x µl RNase inhibitor (optional)



2.2

Perform Annealing

3 *Prepare Beads*

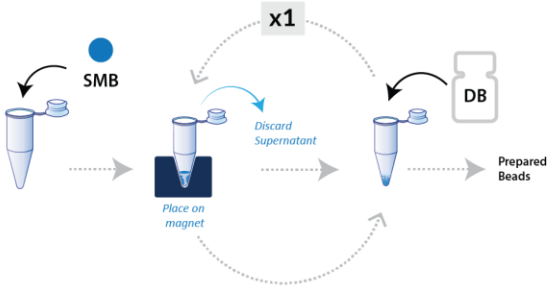
DB
SMB

3.1

Aliquot **90 µl** SMB per sample
(6 samples: 540 µl)

3.2

Discard Supernatant
& Add **80 µl** DB per sample.
Repeat step
(6 samples: 480 µl)



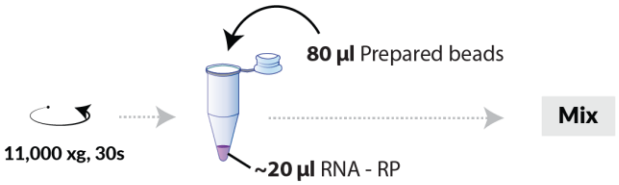
4 Depletion

Hybridized RNA - riboPOOL from stage 2.2 | Prepared beads from stage 3.2

4.1

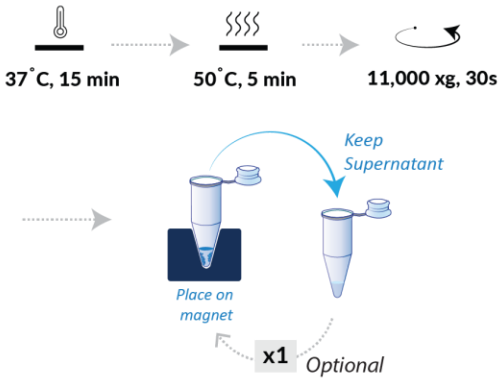
Centrifuge hybridized RNA - RP *(Step 2.2)*

Add **80 µl** prepared beads *(Step 3.2)*



4.2

Perform Depletion

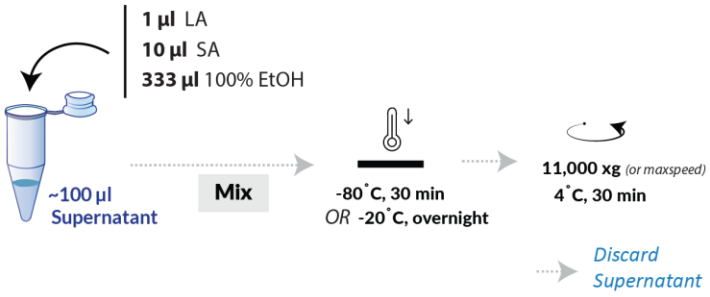


5 Clean Up by EtOH precipitation

LA ○ SA ○ H2O ●

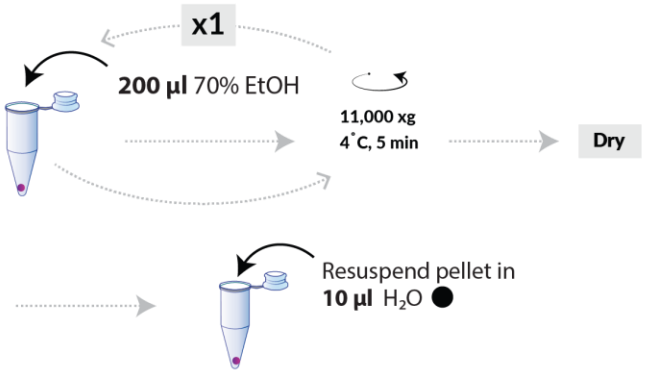
5.1

Precipitate RNA



5.2

Wash and resuspend pellet



Detailed Protocol

Notes before starting:

- Make sure all reagents are equilibrated to room temperature before use.
- RNA sample should always be stored on ice until hybridization.
- Set heat block or thermal cycler to 68°C.

1. Resuspension of riboPOOL RP, H2O (Kit components required)

a. Centrifuge **riboPOOL (RP)** at 11 000 x *g* for 30s before opening.

b. For respective kit size, add the following amount of **nuclease-free water (H2O)** provided into **RP** tube:

Kit size	H2O to add (µl)
6 reaction (1 nmol)	10
12 reaction (1.5 nmol)	15
24 reaction (3 nmol)	30
96 reaction (11 nmol)	110

c. Vortex well.

d. Spin down contents of tube before using.

2. Hybridization of riboPOOL to RNA RP, HB, RNA sample

a. To 14 µl of RNA sample (containing 100 ng - 5 µg of total RNA), add:
If sample volume is > 14 µl, adjust HB volume accordingly to 0.25X total volume. Total volume however should not exceed 40 µl.

- 1 µl of **resuspended RP** (from step 1; i.e. 100 pmol)
- 5 µl of **Hybridization Buffer (HB)**
- RNase inhibitor (optional)
  Follow manufacturer's instructions for volume required and ensure enzyme is active at 68°C. RNase inhibitor may also be introduced during bead preparation.

b. Vortex well and spin down droplets.

c. Incubate at **68°C for 10 min** to denature RNA.

d. Allow to **cool slowly** from **68°C to 37°C** for optimal hybridization.
To do this, shut off the heating block and let temperature fall naturally to 37°C. If using temperature-controlled ramping, cool at 3°C/min.

3. Preparation of beads SMB, DB

a. Resuspend the **streptavidin-coated magnetic beads (SMB)** by carefully vortexing tube at medium speed.

b. Transfer 90 µl of bead suspension per sample into a fresh tube.
For batch washing of beads for multiple samples, aliquot bead suspension for up to 6 (i.e. 540 µl) or 12 samples (i.e. 1080 µl) in a single tube.

c. Place tube on magnetic rack and wait for 1 min.
Beads may stick to sides of tube making solution appear brown. Aspirated solution however, should be clear.

d. Aspirate and discard all supernatant.

e. Add 80 µl per sample (i.e. 480 µl for 6 samples, 960 µl for 12 samples) of **Depletion Buffer (DB)** and agitate the tube well to resuspend beads.

f. Repeat steps c to e.

4. Ribosomal RNA depletion

a. Briefly centrifuge the tube containing ~20 µl hybridized riboPOOL and total RNA (from step 2) to spin down droplets.

b. Pipette 80 µl of the prepared beads (from step 3) into the tube containing hybridized riboPOOL-RNA solution. Agitate the tube to resuspend well.

c. Incubate the tube at **37°C for 15 min**, followed by a **50°C incubation for 5 min**.

d. Briefly spin down droplets.

e. Place on magnet for 2 min then carefully transfer the supernatant to a new tube.

f. Place tube on magnet for 1 min to get rid of trace amounts of beads (optional).

g. Carefully transfer the supernatant to a new tube (optional).
Steps f and g are recommended to remove any potential trace amount of beads but can be left out if desired.

At this point, RNA can be stored at -20°C overnight or -80°C for up to a month.

5. RNA Clean-up

RNA samples subject to rRNA depletion **must** be purified before sequencing library preparation to remove salts and buffer concentrates.

The choice of clean-up method may employ a size-selection of RNA fragments that can affect RNA-Seq results. The riboPOOL kit includes reagents for ethanol precipitation which will recover small and large RNAs including tRNAs, miRNAs, mRNA and large non-coding RNA.

Other clean-up methods such as silica column-based or SPRI bead-based RNA purification may only recover RNA fragments > 200 nt (which excludes tRNAs and small RNAs) dependent on specific buffer conditions used. Hence, careful attention should be paid to product specifications and accompanying protocols.

The following RNA clean-up methods have been successfully applied with riboPOOLs:

- RNA Clean & Concentrator kit from Zymo Research (#R1013)
- Nucleospin RNA Clean-up XS from Macharey Nagel (#740903)
- Agencourt RNAClean XP from Beckman Coulter (#A63987, #A66514)

RNA Clean-up by Ethanol Precipitation SA, LA, H2O

a. Add 10 µl of **3M sodium acetate (SA)** to final solution (~110 µl) from step 4.
b. Add 1 µl of **Linear acrylamide (LA).**
c. Vortex well.
d. Add 333 µl of 100% ethanol.
e. Vortex well.
f. Incubate tube at -80°C for 30 min OR -20°C overnight.
g. Centrifuge at 11 000 x *g* (or max speed) for 30 min at 4°C.
h. Carefully remove and discard all supernatant, making sure not to disrupt pellet.
i. Add 200 µl of 70% ethanol to wash pellet.
j. Centrifuge at 11 000 x *g* for 5 min at 4°C.
k. Carefully remove and discard all supernatant, making sure not to disrupt pellet.
l. Repeat ethanol wash (steps i to k).
m. Dry pellet at room temperature for 5 min.
n. Resuspend the pellet in 10 µl of **nuclease-free water (H2O)** or appropriate elution buffer for library preparation.

Expected Results

1. Yield assessment

The expected yield is typically < 10% of the RNA input due to the abundance of rRNA (expected yield for 1 µg RNA: < 80 ng). Yields can be assessed on a Bioanalyzer® with an RNA 6000 Pico Kit, High Sensitivity RNA ScreenTape assay (both from Agilent Biosystems), or Qubit RNA HS Assay kit with Qubit Fluorometer (Invitrogen, Thermo Fisher).

***Note that RNA should undergo clean-up before yield assessment to avoid interference by buffer/salts/remaining riboPOOL.*

To assess yield with Bioanalyzer**:**

a. Prepare RNA 6000 pico chip with gel-dye mix, conditioning solution and marker.
b. Load 1 µl of RNA sample that has not undergone riboPOOL depletion as Input. This should be diluted to 25-50 ng/µl with nuclease-free water.
c. In another sample well, load 1 µl of riboPOOL-depleted RNA without dilution.

Bioanalyzer Results

Depleted samples should show loss of 18S/28S rRNA peaks. Yields from low input amounts may be below detection sensitivity and can be assessed by rtqPCR or after cDNA amplification with Bioanalyzer High Sensitivity DNA analysis.

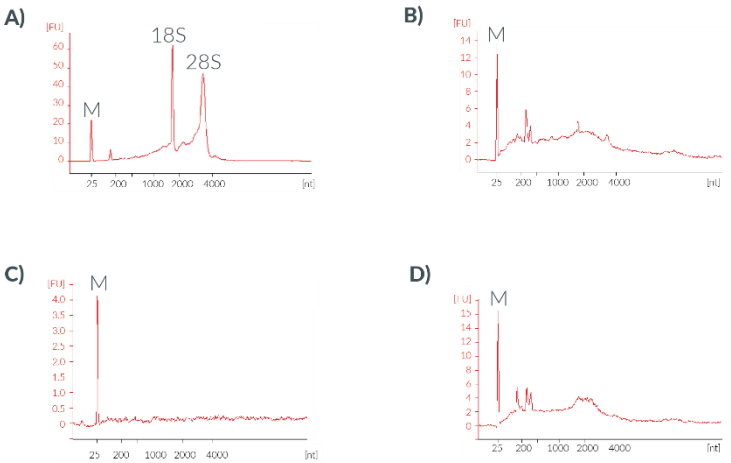


Figure 1. Bioanalyzer analysis of riboPOOL-mediated rRNA depletion. A) 1 µg human RNA input diluted 1:20; B) 1 µg human RNA subject to human riboPOOL depletion; C) 100 ng human RNA subject to human riboPOOL depletion; D) 5 µg human RNA subject to human riboPOOL depletion. (M: 25 nt RNA Marker).

2. Checking rRNA depletion by real-time quantitative PCR

Ribosomal RNA removal may be monitored by real-time quantitative PCR (rtqPCR) with primers designed against 18S and/or 28S RNA and a house-keeping gene.

***Note that RNA should undergo clean-up before rtqPCR to avoid interference by buffer/salts/remaining riboPOOL.*

To assess rRNA removal by rtqPCR:

a. Perform cDNA amplification with 1 µl of RNA input and riboPOOL-depleted RNA using a reverse transcription kit (e.g. Superscript IV kit from Thermo Fisher).
b. Dilute cDNA obtained 1:20 and combine with primers and rtqPCR enzyme mix at recommended volumes of rtqPCR kit (e.g. SYBR® No-ROX kit from Bioline).
   Optimal cDNA dilution may depend on primers and kit used.
c. Perform rtqPCR in triplicates with a real-time PCR detection system (e.g. CFX Connect Real-time PCR Detection System by Bio-Rad).

rtqPCR results

Amount of 18S/28S rRNA should be < 0.5% compared to RNA input following riboPOOL-mediated depletion. In contrast, house-keeping gene (e.g. GAPDH) RNA levels should not be significantly depleted compared to input.

Actual depletion efficiency values observed with rtqPCR may differ from that obtained by RNA-Seq.

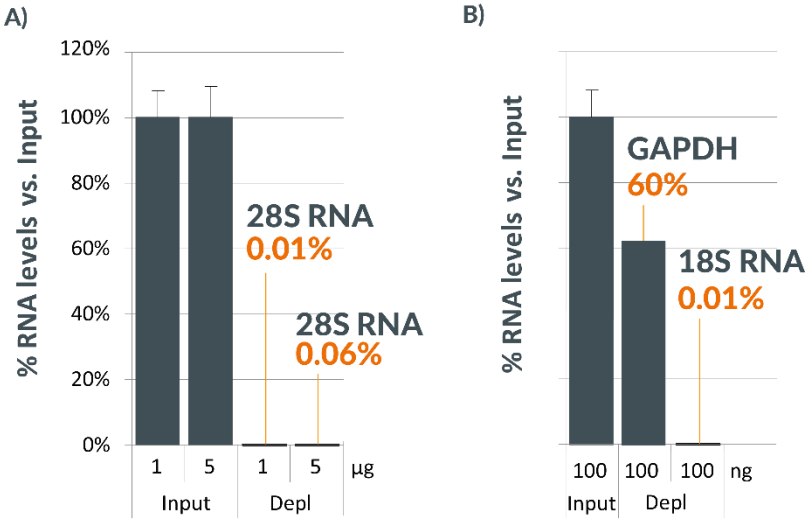


Figure 2. Real-time quantitative PCR analysis of riboPOOL-mediated rRNA depletion. A) Relative 28S RNA levels of 1 or 5 µg human RNA input before and after human riboPOOL depletion; B) Relative 18S and GAPDH levels of 100 ng human RNA before and after human riboPOOL depletion.

Manual version and appropriate use

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The instructions within this manual should be strictly followed by qualified personnel for safe and proper use of the product(s) described herein. Failure to completely read and perform the protocol in an adequate test environment may result in damage to the product(s), injury to persons, including to users or others, and damage to other property. siTOOLs Biotech does not assume any liability arising out of the improper use of the product(s) in any form or environment.

The riboPOOL kit is developed, designed, produced and sold FOR RESEARCH PURPOSES only. No claim or representations is intended for clinical use (included, but not limited to diagnostic, prognostic, therapeutic purposes). It is rather the responsibility of the user to inspect and assure the use of the riboPOOL kit for a well-defined and specific application.

For other general terms of business and safety documentation, please refer to the siTOOLs Biotech website (www.sitoolsbiotech.com) under Resources > Other Downloads.

This manual, referred to hereby as riboPOOLKitManual_v1-2, was first created on 30th September 2019 and revised on 13th November 2019 and on 11th February 2020. It may be subject to future revisions. Please refer to our website for latest updates.

Contact Information

siTOOLs Biotech GmbH
www.sitoolsbiotech.com
info@sitools.de
+49 89 12501 4800
@siTOOLsBiotech (Twitter)
Lochhamer Str. 29A
Planegg/Martinsried, 82152, Germany

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