siTOOLs BIOTECH

Efficient and rapid rRNA-depletion solution for total RNA

riboPOOL™ Kit: User Guide

For Research Use Only.

Content

Introduction

Purpose of kit

Ribosomal RNAs (rRNAs) constitute a significant portion, up to 90%, of the RNA extracted from the majority of organisms. This applies to RNA samples used in both total RNA-Seq analyses and ribosome profiling (Ribo-Seq). Thus, it is necessary to remove rRNAs prior to sequencing in order to enable sensitive and cost-effective detection of target RNAs.

Ribosomal RNA depletion pools for RNA Sequencing (riboPOOLs) developed by siTOOLs Biotech represent an efficient, affordable, and flexible solution offering scientists the freedom to deplete rRNAs from any species, thereby increasing abundance of RNAs of interest.

RiboPOOLs are available and can be utilized for rRNA depletion in total RNA-Seq and Ribo-Seq samples.

Since all riboPOOLs are specifically optimized for their respective application, we do recommend using Standard RNA-Seq riboPOOLs for common RNA-Seq workflows of high-quality RNA (RIN>7), FFPE/degr. riboPOOLs for degraded RNA (RIN<6) and Ribo-Seq riboPOOLs for ribosome profiling applications.

Ribodepletion with riboPOOLs can be completed within 70 minutes, without the need for enzymes, and is compatible with high-throughput automation.

Product description

Composed of high complexity pools of optimally designed biotinylated DNA probes, riboPOOLs specifically hybridize with cytoplasmic, mitochondrial, and/ or plastid rRNAs, enabling their removal with streptavidin-coated magnetic beads.

Following the biotin-streptavidin magnetic bead-based removal of rRNAs, remaining RNA is cleared of salts and buffer concentrates by either SPRI bead-based purification (recommended for total RNA-Seq) or ethanol precipitation (recommended for Ribo-Seq).

All riboPOOL kits include reagents for both purification approaches.

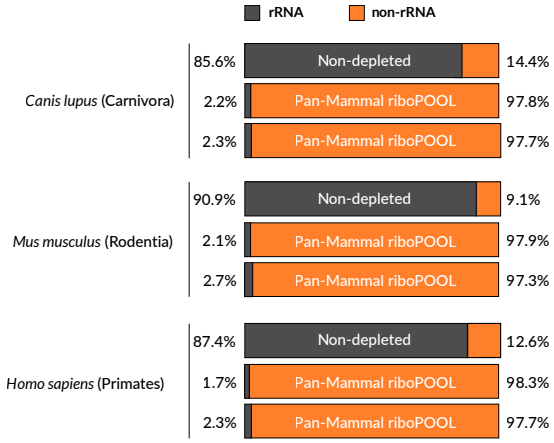
riboPOOLs are available for a diverse array of organisms. www.sitoolsbiotech.com/products/ribopools/available-ribopools

Product performance

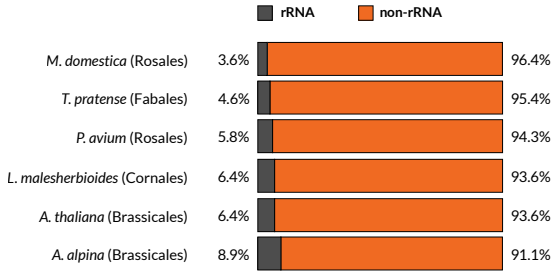
riboPOOLs were demonstrated by RNA-Seq to reliably deplete > 95 % rRNA with high reproducibility between biological replicates.

For multi-species-targeting, riboPOOLs such as the Pan-Bacteria riboPOOL provide rRNA depletion for a broad spectrum of microbes from all phyla.

Pan-Mammal riboPOOL



Pan-Plant riboPOOL



Pan-Bacteria riboPOOL

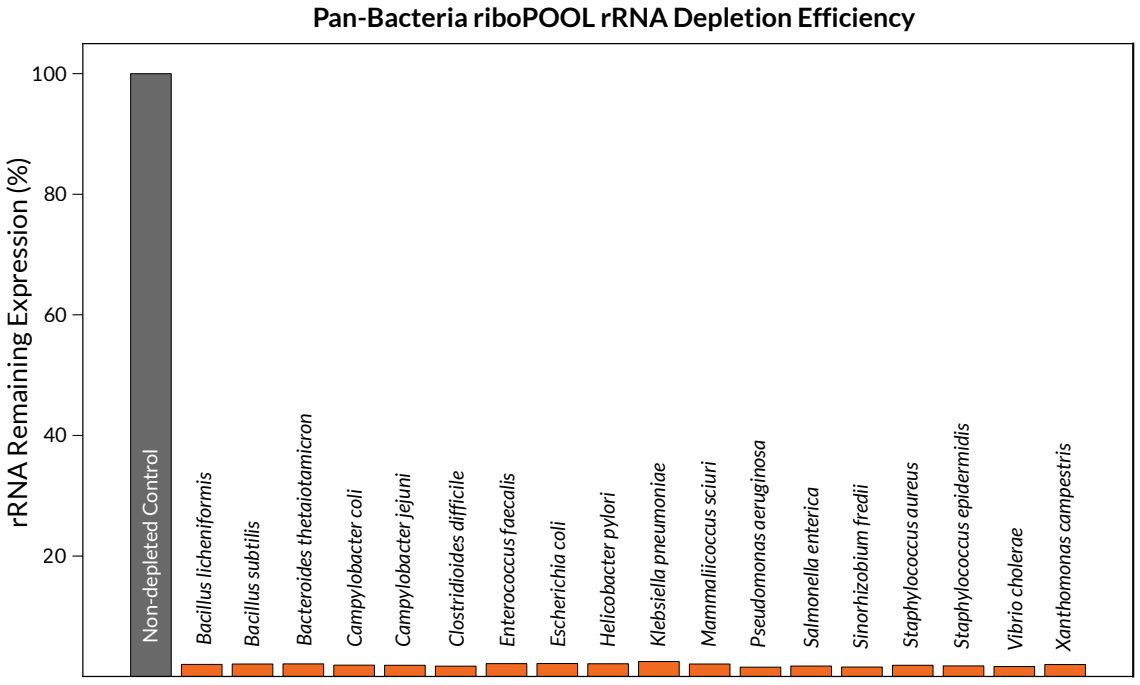


Figure 1 Remaining percentage of rRNA in different bacterial species after depletion with the Pan-Bacteria riboPOOL. Data shown refers to relative percentage of reads mapping to target rRNAs compared to non-depleted sample.

Contents of riboPOOL kit

Abbreviation and volume

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
H_2O	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
LA	Linear acrylamide	1 x 30 µl	1 x 30 µl	1 x 30 µl	1 x 110 µl
SA	Sodium acetate, 3M	1 x 300 µl	1 x 300 µl	1 x 300 µl	1 x 1.1 ml
CB*	Clean-up beads	1 x 1050 µl	1 x 2 ml	1 x 4ml	1x 16 ml

* Use of clean-up beads is not recommended for Ribo-Seq experiments as it can lead to poor recovery of ribosome protected fragments (RPFs).

Storage instructions

The riboPOOL kit is shipped at room temperature.

Upon receipt, please store reagents at the following temperatures:

Reagents	Room temperature	4°C	-20°C	Notes
HB	X			stable for at least 1 year
DB	X			stable for at least 1 year
H_2O	X			stable for at least 1 year
SA	X			stable for at least 1 year
SMB		X		stable for at least 1 year
CB		X		stable for at least 1 year
LA			X	stable for at least 1 year
RP*			X	stable for at least 1 year

* The riboPOOL (RP) when lyophilized, is stable at RT for up to a year, but is best stored at - 20°C upon receipt. Upon resuspension in nuclease-free water (H2O), riboPOOLs are stable for at least one year 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

General

- RNA input should be DNA-free.
- RNA input amount may range from 10 ng to 3 µg. For larger amounts, split into aliquots ≤ 3 µg and follow protocol accordingly.
- Take necessary precautions to avoid RNase contamination i.e. keep work area clean, wear gloves, leave tubes open for prolonged times.
- Equilibrate all reagents to room temperature before use.
- To agitate tubes containing streptavidin magnetic 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 – 3 µg as modifying volumes may result in decreased efficiency. For input amount < 100 ng, reduce probe amount to 30 pmol/reaction.
- Be sure to resuspend clean-up beads completely before using them. The beads should appear homogenous and consistent in color.
- When purifying the RNA via ethanol precipitation, avoid touching the RNA pellet during the last wash step.
- Make sure all the supernatant is removed after the last wash step.

riboPOOL Kit 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.

Preparation and Depletion

1. Resuspension of riboPOOL

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

Kit size	H_2O to add (µl)
6 reaction (1 nmol)	10
12 reaction (1.5 nmol)	15
24 reaction (3 nmol)	30
96 reaction (11 nmol)	110

b. For respective kit size, add the following amount of nuclease-free water (● H_2O) provided into RP tube (●):

c. Vortex well.

d. Spin down contents of tube before using.

1.1 (Special case) Combination riboPOOL

a. Pre-mixed

- If the Combination riboPOOL was provided pre-mixed, follow instructions provided in Step 1 to resuspend the riboPOOL.
- Proceed to Step 2.

b. Separate tubes

- If each component of the Combination riboPOOL was provided in a separate tube, resuspend each riboPOOL as described in Step 1.
- Prepare the Combination riboPOOL by adding the required amount of each riboPOOL to a new tube based on the desired final ratio.
- Vortex well and proceed to section 2.

2. Hybridization of riboPOOL to sample

a. To 14 µl of RNA sample (containing 10 ng - 3 µg of total RNA), add:

- 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.
- If sample volume is > 14 µl, adjust HB volume accordingly to 0.25X total volume. Total volume however should not exceed 40 µl.

b. Vortex well and spin down droplets.

c. Incubate at 68°C for 10 minutes 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/minutes.

3. Preparation of beads

a. Resuspend the streptavidin-coated magnetic beads (SMB ●) by carefully vortexing the 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 minutes. 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 minutes**, followed by a **50°C incubation for 5 minutes**.

d. Briefly spin down droplets.

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

f. Place tube on magnet for 1 minute to get rid of trace amounts of beads.

g. Carefully transfer the supernatant to a new tube. 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 purification with clean-up beads (option A) or ethanol precipitation (option B)

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 sequencing results. The riboPOOL kit includes RNA clean-up beads for SPRI bead-based RNA purification and may recover RNA fragments > 200 nt (which excludes tRNAs and small RNAs) dependent on specific buffer conditions used. It also includes reagents for ethanol precipitation which will recover RNA fragments < 200 nt. This method is recommended for Ribo-Seq samples.

Option A: Clean-up beads purification (total RNA-Seq)

The SPRI bead-based RNA purification workflow can be completed in ~30 minutes, is enzyme-free, and compatible with high-throughput automation.

a. Add 90 µl of your RNA sample (e.g. rRNA depleted RNA) in an appropriate tube.

b. Add 162 µl **Clean-up Beads (CB ●)** to each sample. Be sure to resuspend the **beads** completely before adding them by flicking the tube or by pipetting. Note: The volume of Clean-up Beads for a given reaction can be calculated from the following equation: Volume of Clean-up Beads per reaction = 1.8 x reaction volume.

c. Mix by pipetting up and down 6-8 times and let the tube incubate at room temperature for 5 minutes. This step binds RNA products to the magnetic beads. Vortexing is not recommended.

d. Separate the **Clean-up Beads** by placing the tube on a magnet for 5 minutes. The separation time is dependent on size. Larger reactions will take longer to separate. Wait for the solution to clear before proceeding to the next step.

e. With the tube still on the magnet, slowly remove and discard the supernatant without disturbing the beads.

f. With the tube still on the magnet, add 500 - 1000 µl of 70% ethanol (70% ethanol and 30% H_2O) to each sample and incubate for 30 seconds at room temperature. Carefully remove and discard the ethanol without disturbing the pellet. **Repeat for a total of three washes.** Note: Be sure to remove all ethanol from the tube as it may contain residual contaminants. The wash solution should completely cover the pellet in the tube.

g. Let the reaction tube air-dry for 10 minutes on the magnet. The tube should air-dry until the last visible traces of ethanol evaporate. Be careful and do not over dry the pellet as it may lead to lower recovery.

h. Remove the tube from the magnet. Elute the purified RNA from the beads by adding at least 30 µL of nuclease-free water. Resuspend the beads by pipetting up and down several times. Elution volume can be modified if higher/lower RNA concentration is needed, but it's not recommended to eluate in less than 10 µl.

i. Incubate for 2 minutes at room temperature.

j. Place the tube back on the magnet and wait for 5 minutes or until the **Clean-up Beads** clear from solution.

k. Transfer the eluate containing your purified RNA sample to a clean tube.

Option B: Ethanol Precipitation (total RNA-Seq and Ribo-Seq)

a. Add 90 µl of your RNA sample (e.g. rRNA depleted RNA) in an appropriate tube.

b. Add 10 µl of 3M sodium acetate (SA ○) , 1 µl of Linear acrylamide (LA ○) and 333 µl of 100% ethanol.

c. Vortex well.

d. Incubate tube at -80°C for 30 minutes OR -20°C overnight.

e. Centrifuge at 11 000 x g (or max speed) for 30 minutes at 4°C

f. Carefully remove and discard all supernatant, making sure not to disrupt pellet.

g. Add 200 µl of 70% ethanol to wash pellet.

h. Centrifuge at 11 000 x g for 5 minutes at 4°C.

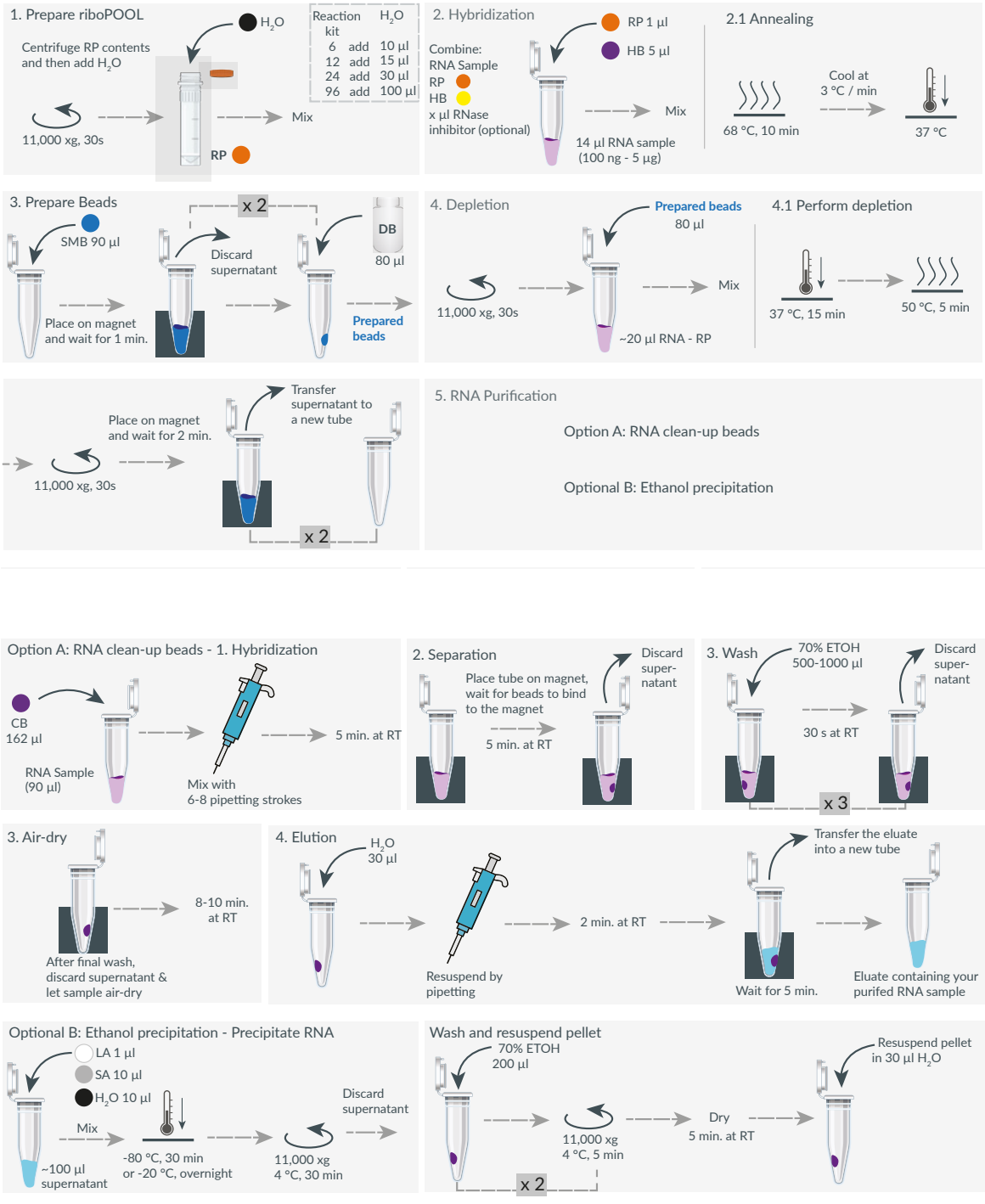
i. Carefully remove and discard all supernatant, making sure not to disrupt pellet.

j. Repeat ethanol wash (steps f to i).

k. Dry pellet at room temperature for 5 minutes.

l. Resuspend the pellet in 30 µl of nuclease-free water (H_2O ●) or appropriate elution buffer for library preparation. If required elution volume can be decreased down to 10 µl.

Snapshot Protocol



List of Available riboPOOLs

Single Species riboPOOLs

Aedes albopictus
Aedes albopictus rPseudogenes
Amphimedon queenslandica
Anaeramoeba flamelloides
Anopheles gambiae
Apis mellifera
Arabidopsis thaliana
Argiope bruennichi
Azolla filiculoides
Bacillus subtilis
Bemisia tabaci
Blood Parasite
Caenorhabditis elegans
Caulobacter crescentus
Chinchilla lanigera
Chlamydomonas reinhardtii
Clostridium perfringens
Crassostrea gigas
Cryptococcus neoformans
Cupriavidus necator
Cyanidioschyzon merolae
Danio rerio
Drosophila melanogaster
Emiliania huxleyi
Escherichia coli
Eubacterium limosum
Filamentous-Fungi
Gallus gallus domesticus
Haloferax volcanii
Human Blood
Human Globin mRNA
Human - cytoplasmatic rRNAs only
Human+hGlobin+7SL [10:1:1]
Ixodes scapularis
Leptinotarsa decemlineata
Loripes orbiculatus and Lucinoma aequizonata
Nematostella vectensis
Olavius algarvensis
Oryza sativa
Pan-Actinobacteria
Pan-Archaea
Pan-Bacteria
Pan-Bird
Pan-Chlamydia
Pan-Dictyostelia
Pan-Fungi
Pan-Mammal
Pan-Mussel
Pan-Plant
Pan-Prokaryote
Pan-Sponge
Pichia pastoris
Pinus sylvestris
Plautia stali
Poly A (Poly-Adenylated RNAs)
Pseudomonas aeruginosa
Saccharomyces cerevisiae
Salmonella enterica
SARS-CoV-2 RNA
Schistosoma mansoni
Schizosaccharomyces pombe
Schmidtea mediterranea
Seawater
Spodoptera exigua
Staphylococcus aureus
Stenotrophomonas sp.
Strongyloides Ratti
Thalassiosira pseudonana
Ustilago maydis
Varroa destructor
Willaertia-Naegleria
Wolbachia pipientis

Human-Mouse-Rat riboPOOLs

Human-Mouse-Rat riboPOOL
Human riboPOOL
Mouse-Rat riboPOOL

Add-ons (can be added to any human or mouse riboPOOL)

Human tRNAs
Mouse tRNAs
Signal Recognition Particle (7SL)

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 RiboSeq_riboPOOLKitManual_v2, was first created on 30th September 2019 and last revised in March 2024. It may be subject to future revisions. Please refer to our website for latest updates.

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