JUMPCODE GENOMICS

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USER MANUAL

Post-library bulk ribodepletion (HMR)

NOVA-5229910 : Bulk Ribodepletion Reagents (HMR)

KIT3000s: Species-Specific Ribodepletion Reagents

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Product overview

The Bulk Ribodepletion Reagents remove overabundant human, mouse, or rat rRNA from RNA-Seq libraries to improve sequencing sensitivity and performance. This protocol is designed for high-throughput screening applications by moving the depletion step post-library prep and enabling depletion on multiplexed pools of up to 96 libraries.

Description	
Total assay time	~4 hours
Samples per kit	1,536 samples (16 depletion reactions)
Multiplex	Up to 96 samples per depletion reaction
Designed to deplete	NOVA-5229910 CRISPRclean Bulk Ribodepletion Reagents (HMR) • Human 5S, 5.8S, 18S and 28S nuclear rRNA genes, 45S rRNA precursor, 12S and 16S mitochondrial rRNA genes Upon request, species-specific rRNA depletion guides can be customized and substituted for HMR. See table below for list of available species.

List of species supported for rRNA removal

Catalog	Species name	rRNA species targeted
KIT3000	*Acinetobacter baumannii*	5S, 16S and 23S rRNA
KIT3001	*Campylobacter coli*	
KIT3002	*Campylobacter jejuni*	
KIT3003	*Clostridioides difficile*	
KIT3004	*Enterococcus faecium*	
KIT3005	*Escherichia coli*	
KIT3006	*Klebsiella pneumoniae*	
KIT3007	*Legionella pneumophila*	
KIT3008	*Listeria monocytogenes*	
KIT3009	*Mycobacterium tuberculosis*	
KIT3010	*Mycobacteroides abscessus*	
KIT3011	*Neisseria gonorrhoeae*	
KIT3012	*Neisseria meningitidis*	
KIT3013	*Pseudomonas aeruginosa*	
KIT3014	*Salmonella enterica*	
KIT3015	*Staphylococcus aureus*	
KIT3016	*Streptococcus agalactiae*	
KIT3017	*Streptococcus pneumoniae*	
KIT3018	*Streptococcus pyogenes*	
KIT3019	*Vibrio cholerae*	
KIT3020	*Saccharomyces cerevisiae (yeast)*	

Workflow

1. Library Prep

2. **Ribodepletion**

   Hands-on time: 1 hr 15 min | Total time: 3 hrs 45 min

   A. Ribonucleoprotein Complex Formation
   B. CRISPR Digestion
   C. Size Selection
   D. Amplification
   E. Size Selection
   Safe Stopping Point

3. Sequencing

Kit contents and storage

The Bulk Ribodepletion Reagents (HMR) contains enough material for 16 post-library depletion reactions and each depletion reaction is sufficient to multiplex up to 96 libraries. The kit contents and storage temperatures are indicated in the tables below.

ASY1064 Depletion Reagents (8 depletion reaction per box)

2 boxes
Stored at -20°C

Kit contents	Part number	Quantity per box
Cas9	REA1039	1 tube
10X Cas9 Buffer	REA1040	1 tube
RNase Inhibitor	REA1041	1 tube
Nuclease-Free Water	REA1042	1 tube
Tris Buffer	REA1043	1 tube
P5 Primer	REA1044	1 tube
P7 Primer	REA1045	1 tube
2X PCR Mix	REA1046	1 tube

Guide RNA

1 bag
Stored at -80°C

Kit contents*	Part number	Quantity per bag
Guide RNA for Human, Mouse, Rat rRNA	ASY1053	1 tube
Guide RNA for species-specific rRNA	Variable	1 tube

* Each kit contains one type of guide RNA from the above table

Required materials and equipment provided by the user

Type	Item	Supplier
Plastics	0.5 mL, 1.5 mL DNA LoBind Tubes	Eppendorf 022431021
	0.2 mL thin wall PCR tubes	General Lab Supplier
	Low-Retention Filtered Sterile Tips (10 µl, 20 µl, 200 µl and 1000 µl)	General Lab Supplier
Reagents	AMPure XP Beads	Beckman Coulter A63881
	Absolute Ethanol, 200 Proof	General Lab Supplier
	Qubit dsDNA HS Assay Kit	ThermoFisher Scientific Q32854
Equipment	Single Channel Pipettes (10 µl, 20 µl, 200 µl, and 1000 µl)	General Lab Supplier
	Multichannel Pipettes (10 µl, 20 µl, and 200 µl)	General Lab Supplier
	Vortex Mixer	General Lab Supplier
	Microcentrifuge	General Lab Supplier
	PCR Magnetic Rack or Stand for use with tubes	General Lab Supplier
	Ice Bucket	General Lab Supplier
	PCR Thermal Cycler	General Lab Supplier
	Qubit Fluorometer	ThermoFisher Scientific Q33238
	Automated electrophoresis such as TapeStation	General Lab Supplier
	DNA analysis instrument, such as the Agilent 2100 Bioanalyzer® System	General Lab Supplier

Best practice

General

- Periodically, optimizations and revisions are made to the kit components and user manual. Therefore, it is important to follow the protocol included with or appropriate for the kit in question. This can be done by comparing the name and version number of the CRISPRclean product to the name and version number of the protocol.
- This protocol describes the reagents, best practices, workflow and method details for CRISPRclean bulk ribodepletion.
- Use properly calibrated pipettes as library preparation is sensitive to pipetting error.
- Ensure that all pipette tips, microcentrifuge tubes, and other consumables are DNase- and RNase-free.
- Follow good laboratory practices throughout the protocol, including RNA-safe procedures when handling RNA.

Reagent Handling

- Do not remove Cas9 and RNase Inhibitor from -20°C until before use. Return to -20°C immediately after use.
- Store the Guide RNA at -80°C. Do not remove the reagent from -80°C until time of use. Return it to -80°C immediately after use.
- We recommend a maximum of 3 freeze-thaw cycles for the Guide RNA. It is strongly recommended that multiple smaller aliquots of the Guide RNA be prepared when the reagent is first thawed in order to reduce the number of freeze-thaw cycles affecting the Guide RNA.
- Do not freeze AMPure® XP beads.
- Allow AMPure XP beads to come to room temperature for 30 minutes before use.
- Vortex AMPure XP beads immediately before use. Ensure that they are in a uniform suspension before use.
- Use magnetic stands appropriate for PCR tubes.

Revision log

Version	Date	Description
V1.0	December 2022	Launch release
V1.5	February 2023	Clarity edits

Before starting protocol

Please review and follow the guidelines below for preparing RNA libraries that are optimal for depletion:

NGS library prep requirements

The Jumpcode RNA depletion protocol works most effectively with RNA libraries with ~450 bp fragment sizes. **The kit produces best results with libraries size selected for a relatively narrow fragment size range in which most fragments are >450 bp**. For many library preparation methods involving RNA fragmentation by heat, this can be achieved with a combination of short RNA fragmentation times and dual SPRI bead size selection or agarose gel-based size selection. For example, if your RNA library preparation method is NEBNext® Ultra II Directional RNA libraries then the following conditions are recommended to achieve efficient depletion results:

1. 100 ng total RNA input
2. RNA fragmentation time of 5 minutes
3. Dual AMPure XP bead size selection to select fragments with an Agilent Bioanalyzer peak of 500 bp or greater (referred to as libraries with 300 bp, 400 bp and 450 bp approximate insert sizes or 420 bp, 520 bp and 570 bp approximate final library sizes in Appendix A of the NEBNext Ultra II Directional RNA Library Prep User Manual)
4. 8 PCR cycles

Combining multiple RNA libraries before proceeding to Step A.

Make sure to measure the final volume of the **pooled DNA library** and ensure that the total volume of the combined libraries is no greater than 10.8 µL.

This protocol is designed to be performed on multiplexed NGS libraries. When combining multiple libraries for a single depletion treatment, please follow the guidelines below:

- Review the library preparation method(s). The libraries should have been prepared and stored under nuclease-free conditions.
- Ensure that the libraries are uniquely indexed.
- Combine no more than 96 libraries per depletion reaction.
- Preferentially combine libraries prepared by similar methods.
- Use approximately 1 ng of DNA per library with a maximum of 100 ng of total DNA per reaction.
- Combine the libraries together in equal quantities. This will likely require normalizing individual library DNA concentrations by diluting or concentrating some or all the libraries.
- Ensure that the total volume of the combined libraries is no greater than 10.8 µL.

Protocol

Step A: Ribonucleoprotein complex formation

Hands-on time: 5 min | Total time: 15 min

Reagents preparation

Item	Storage	Handling
10X Cas9 Buffer	-20°C	If frozen, thaw on ice. Finger-tap mix and spin down. Keep on ice. Return to freezer immediately after use.
Cas9		
RNase Inhibitor		
Guide RNA for Human, Mouse, Rat or Species-specific rRNA	-80°C	

1. Follow the guidelines on the previous page to pool the libraries together before proceeding to the next step. Measure the final volume of the pooled DNA library. Ensure that the total volume of the combined libraries is no greater than 10.8 µL. This is your **pooled DNA library**.
2. Follow the table below and add the reagents listed accordingly into a low-nucleic acid binding and nuclease-free microcentrifuge tube:

"RNP Complex Formation" Reaction Mix	Volume
10X Cas9 Buffer	2.0 µL
Guide RNA for Human, Mouse, Rat rRNA or Species-specific rRNA	3.9 µL
Cas9	2.3 µL
RNase Inhibitor	1.0 µL
Total Volume	9.2 µL

3. Mix the contents gently by carefully flicking the tube or pipetting up and down. Collect the contents at the bottom of the tube by briefly spinning the tube in a microcentrifuge.
4. Leave the tube on the laboratory bench for 10 minutes at room temperature. This is the **ribonucleoprotein complex (RNP)**. Proceed to Step B: CRISPR digestion.

Step B: CRISPR digestion

Hands-on time: 5 min | Total time: 65 min

Reagents preparation

Item	Storage	Handling
Nuclease-Free Water	-20°C	If frozen, thaw on ice. Finger-tap mix and spin down. Keep on ice. Return to freezer immediately after use.
AMPure XP beads	4°C	During incubation, the AMPure XP beads required for subsequent size selection steps may be removed from the refrigerator and placed on the laboratory bench to bring them to room temperature. A minimum of 20 minutes at room temperature is recommended before use.

1. Obtain the **pooled DNA library** you intend to deplete. Add Nuclease-Free Water to bring the volume to ~10 µL.
2. Add the **pooled DNA library** from Step A-1 to the **ribonucleoprotein complex (RNP)** from Step A. The total volume of the reaction should be ~20 µL.
3. Mix the CRISPR digestion reaction gently by carefully flicking the tube or pipetting up and down. If necessary, briefly spin the tube in the microcentrifuge to collect the contents at the bottom of the tube. Incubate the tube at 37°C for 60 minutes.
4. After the 60 minute incubation at 37°C is complete, transfer the tube to ice for ~2 minutes.
5. Spin the tube briefly in the microcentrifuge. Transfer the tube to the laboratory bench. This is the **CRISPR digestion reaction**. Proceed to Step C: Size selection.

Step C: Size selection

Hands-on time: 30 min | Total time: 40 min

Item	Storage	Handling
Nuclease-Free Water	-20°C	If frozen, thaw on ice. Finger-tap mix and spin down. Keep on ice. Return to freezer immediately after use.
AMPure XP beads	4°C	Bring to room temperature. Vortex and invert mix.
80% Ethanol	Room temperature	Prepare fresh.

1. Add 30 µL of Nuclease-Free Water to the CRISPR digestion reaction from Step B. Mix gently.
2. Add 30 µL (0.6 volumes) of well-resuspended, room-temperature AMPure XP beads, pipette several times to mix and incubate the tube at room temperature for 10 minutes. Mix the sample halfway through the incubation by pipetting up and down a few times.
3. Place the tube on the magnetic stand. Allow the solution to clear (3 – 5 minutes) and discard the supernatant without disturbing the beads.
4. Add 200 µL of freshly prepared 80% ethanol to the tube. Wait 30 seconds, then remove and discard the ethanol. It is unnecessary to remove the tube from the magnetic stand during this step.
5. Repeat the wash step with another 200 µL of 80% ethanol. Ensure that all residual ethanol is removed after this wash.
6. Open the cap of the tube while it is on the magnetic stand and allow the beads to air dry for 5 minutes.
7. Add 40 µL of Nuclease-Free Water to the beads. Remove the tube from the magnetic stand. Mix the contents by pipetting up and down to fully resuspend the beads in the liquid. Allow the tube to sit at room temperature for 10 minutes. Mix the sample halfway through the incubation by pipetting up and down a few times.
8. Place the tube on the magnetic stand. Allow the solution to clear and transfer the supernatant containing the eluted DNA to a thin-walled PCR tube. Place the tube on ice. This is the **eluted DNA**. Proceed to Step D: Amplification.

Step D: Amplification

Hands-on time: 5 min | Total time: 65 min

Reagents preparation

Item	Storage	Handling
P5 Primer	-20°C	If frozen, thaw on ice. Finger-tap mix and spin down. Keep on ice.
P7 Primer		
2X PCR Mix		

1. Add the following components to the **eluted DNA** in the thin-walled PCR tube from Step C.

Amplification Reaction Mix	Volume
P5 Primer	5.0 µL
P7 Primer	5.0 µL
2X PCR Mix	50 µL
Total volume	100 µL

2. Input the following parameters into a thermal cycler and perform a PCR amplification.

Temp	Cycle time	Number of cycles
95°C	2 min	1 cycle
98°C	20 sec	See below table for the recommended number of cycles according to the total QTY of DNA input from multiplexed libraries
55°C	30 sec	
72°C	30 sec	
72°C	2 min	1 cycle
4°C	HOLD	

Guidelines for multiplexing and recommended post-depletion PCR cycle numbers are listed in the below table

Note: The number of PCR cycles after depletion correlates inversely with DNA input.

Total QTY of DNA input from multiplexed libraries	Suggested number of libraries for each multiplexed depletion reaction	Suggested PCR cycles after depletion for final library yields between 200-400 ng.
8 ng	8 libraries	10 cycles
25 ng	8-24 libraries	9 cycles
50 ng	8-48 libraries	7 cycles
100 ng	8-96 libraries	6 cycles

3. Briefly spin the PCR tube in a microcentrifuge before proceeding with the next step E: Size selection.

> **Note:** At this time, the AMPure XP beads required for subsequent size selection steps may be removed from the refrigerator and placed on the laboratory bench to bring them to room temperature. A minimum of 20 minutes at room temperature is recommended before use.

Step E: Size selection

Hands-on time: 30 min | Total time: 40 min

Reagents preparation

Item	Storage	Handling
Nuclease-Free Water	-20°C	If frozen, thaw on ice. Finger-tap mix and spin down. Keep on ice. Return to freezer immediately after use.
Tris Buffer		
AMPure XP beads	4°C	Bring to room temperature. Vortex and invert mix.
80% Ethanol	Room temperature	Prepare fresh.

1. Add 60 µL (0.6 volumes) of well-resuspended, room-temperature AMPure XP beads to the supernatant from Step D, pipette several times to mix and incubate the tube at room temperature for 10 minutes. Mix the sample halfway through the incubation by pipetting up and down a few times.
2. Place the tube on the magnetic stand. Allow the solution to clear (3 – 5 minutes) and discard the PCR product without disturbing the beads.
3. Add 200 µL of freshly prepared 80% ethanol to the tube. Wait 30 seconds, then remove and discard the ethanol. It is unnecessary to remove the tube from the magnetic stand during this step.
4. Repeat the wash step with another 200 µL of 80% ethanol. Ensure that all residual ethanol is removed after this wash.
5. Open the cap of the tube while it is on the magnetic stand and allow the beads to air dry for ~5 minutes. Do not allow the beads to dry for longer than 10 minutes. Over drying the beads could result in lower yields.
6. Add 30 µL of room-temperature Tris Buffer to the beads. Remove the tube from the magnetic stand. Mix the contents by pipetting up and down to fully resuspend the beads in the liquid. Allow the tube to sit at room temperature for 10 minutes.
7. Place the tube on the magnetic stand. Allow the solution to clear and transfer the supernatant containing the eluted DNA to a new Eppendorf DNA LoBind microcentrifuge tube (or other low-DNA binding tube). Avoid transferring any beads along with the supernatant.

Safe Stopping Point

Store the samples at -20°C

Subsequent steps

It is recommended that the library be quantitated on an Agilent Bioanalyzer or TapeStation instrument prior to sequencing.

After the second (and final) size selection, most DNA fragments in the CRISPRclean-treated library should be larger than 500 bp. If so, the library can be loaded directly on an Illumina sequencer. If a large proportion of fragments are less than 500 bp, it is recommended that a gel-based size selection step be performed to isolate DNA between 500 bp and 800 bp in size. The DNA can be loaded on a sequencing instrument after gel purification.