CRISPRclean® Plus Ribodepletion Reagents (Human, Mouse, Rat, Pan-Bacterial rRNA)

NOVA-5229920

For post-library depletion of multiplexed Illumina NGS libraries

Contents

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Product Overview

CRISPRclean Plus Ribodepletion Reagents remove abundant ribosomal RNA (rRNA) derived from mammalian species (specifically, human, mouse and rat) and bacterial species in next-generation sequencing (NGS) libraries prepared from total cellular RNA. The depletion method utilizes CRISPR technology to selectively target and cleave DNA fragments containing rRNA sequences. Depletion improves sensitivity for less abundant RNA and reduces the proportion of sequencing wasted on uninformative sequences.

While most ribodepletion methods are performed on samples prior to NGS library preparation, this CRISPR-based method is employed on fully prepared NGS libraries. A consequence of performing depletion downstream of library preparation is that uniquely indexed libraries can be multiplexed during the depletion reaction, which facilitates medium to high-throughput RNA-Seq applications.

Description	
Total Assay Time	~5 hours
Hands-on Time	~1 hour
Samples Per Kit	256 samples (16 depletion reactions)
Sample Type	Human microbiome RNA-Seq libraries
DNA Library Input	10 ng
Average DNA Library Size	≥ 450 bp
Designed to Deplete	• Human 5S, 5.8S, 18S and 28S nuclear rRNA genes, 45S rRNA precursor, 12S and 16S mitochondrial rRNA genes • 5S, 16S, and 23S rRNA genes from 212 bacteria representing most phyla
Method	CRISPR-Cas9 mediated depletion

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Workflow

1 **RNA Library Prep**

2 **CRISPRclean Plus Ribodepletion Reagents (Post-Library Depletion)**

☐ Hands-on time: ~1 hour | Total time: ~5 hours

Step A. Ribonucleoprotein Complex Formation & CRISPR Digestion

Step B. Bead Size Selection

Safe Stopping Point

Step C. PCR Amplification

Step D. Bead Cleanup

Safe Stopping Point

3 **Sequencing**

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Kit Contents and Storage

The CRISPRclean Plus Ribodepletion Reagents contain enough material for 16 post-library depletion reactions and up to 16 libraries can be multiplexed in each depletion reaction. The kit contents and storage temperatures are indicated in the tables below.

ASY1091: Depletion Reagents (8 depletion reactions per box)

2 boxes
Storage 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

ASY1056: Guide RNA

1 bag
Storage at -80°C

Kit contents*	Part number	Quantity per bag
Guide RNA (Human, Mouse, Rat rRNA)	BLK1023	1 tube
Guide RNA (Pan-Bacterial rRNA)	BLK1024	1 tube

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Required Materials and Equipment

Type	Item	Supplier
Plastic Consumables	0.5 mL, 1.5 mL DNA LoBind Tubes	Eppendorf (Cat# 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 (Cat# A63881)
	Absolute Ethanol, 200 Proof	General Lab Supplier
	Qubit dsDNA HS Assay Kit	ThermoFisher Scientific (Cat# 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	General Lab Supplier
	Ice Bucket	General Lab Supplier
	PCR Thermal Cycler	General Lab Supplier
	Qubit Fluorometer	ThermoFisher Scientific (Cat# Q33238)
	Automated electrophoresis Instrument such as Agilent TapeStation or 2100 BioAnalyzer	General Lab Supplier

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Best Practices

General

- Periodically, optimizations and revisions are made to the kit components and user manual. Therefore, following the protocol included with or appropriate for the kit in question is important. This can be done by comparing the name and version number of the product to the name and version number of the protocol.
- This protocol describes the reagents, best practices, workflow and method details for the CRISPRclean Plus Ribodepletion product.
- Use properly calibrated pipettes, as library preparation is sensitive to pipetting error.
- Follow good laboratory practices throughout the protocol, including RNA-safe procedures when handling RNA. Ensure all pipette tips, microcentrifuge tubes, and other consumables are DNase- and RNase-free.
- When undertaking the protocol, always proceed immediately to the next step. If a stop is necessary, safe stopping points are available. Refer to the workflow schematic on page 3.

Reagent Handling

- Do not remove Cas9 and RNase Inhibitor from storage until before use. Maintain on ice during reaction setup. 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. Maintain on ice during reaction setup. Return it to -80°C immediately after use.
- We recommend a maximum of three 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 if more than three freeze-thaw cycles are expected.
- Do not freeze AMPure® XP beads.
- Allow AMPure XP beads to come to room temperature before use. A thirty-minute incubation on the laboratory bench is usually sufficient.
- Vortex AMPure XP beads immediately before use. Ensure that the beads are in a uniform suspension before use.
- Use magnetic stands appropriate for PCR tubes.

Equipment Handling

- Thermal cycling should be performed with a heated lid, except where specified otherwise. Thermal cycler(s) must support uniform heating up to 100 µL sample volume.

Revision Log

Version	Date	Description
V1.0	September 2020	Launch
V1.1	October 2020	Edited for clarity and format
V1.2	April 2024	Edited for clarity and format Step A4.3 added to include post-depletion heat inactivation

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Library Preparation and Pooling Prior to Protocol

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

NGS Library Prep Requirements

CRISPRclean depletion produces best results when the majority of library fragments are >450 bp in length. 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. As an example, if the user employs the NEBNext® Ultra II Directional RNA library kit for library preparation, the following conditions are recommended to achieve efficient depletion results:

RNA-Seq Library Preparation:	Recommended Conditions
Starting Material:	100 ng total RNA
RNA Fragmentation Time:	5 minutes at 94°C
Double-Sided Bead Size-Selection:	DNA Library AVG Size ≥ 420 bp (Target DNA Insert AVG Size: ≥ 300 bp) *Appendix A Step 6.2

Library Pooling for Depletion

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 16 libraries per depletion reaction.
- Preferentially combine libraries prepared by similar methods and originating from similar sample type.
- Use approximately 1 ng of DNA per library with a maximum of 16 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 6.3 µL.

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Protocol

It is highly recommended to read the "Library Preparation and Pooling Prior to Protocol" section on page 8 prior to undertaking this protocol.

To ensure best depletion results, follow guidelines for library pooling (i.e., pooling of uniquely indexed libraries of similar sample type at equal concentrations). Note that the final volume of the multiplexed libraries should not exceed 6.3 µL.

Step A: Ribonucleoprotein Complex Formation & CRISPR Digestion

☐ Hands-on time: 25 min | Total time: ~2.6 hrs

Reagent 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 (Human, Mouse, Rat rRNA)	-80°C	
Guide RNA (Pan-Bacterial rRNA)		

A1: First ribonucleoprotein complex formation (RNP1) for depletion of bacterial rRNA

1. Follow the guidelines on the previous page to pool the libraries together. Ensure that the total volume of the combined libraries is no greater than 6.3 µL. Set aside for CRISPR digestion (Step A2.1).
2. Allow the Guide RNA (Pan-Bacterial rRNA) to thaw on ice.
3. Transfer 4.3 µL of Guide RNA (Pan-Bacterial rRNA) to a new PCR tube. For more than one library, multiply the volume of the guide RNA by the number of libraries to be depleted.

NOTE: 3.9 µL of pre-heated guide RNA is required for each depletion reaction (see Step A1.7). However, 4.3 µL is required in this step because of evaporation during the following step involving heat.

NOTE: Return the guide RNA tube to the -80°C freezer immediately after use.

4. Place the tube in a pre-heated thermal cycler and incubate at 65°C for 2 minutes.
5. Immediately transfer the tube to ice for 3 minutes.
6. Briefly spin the pre-heated guide RNA to collect the contents at the bottom of the tube. Return the tube to ice.
7. At room temperature, combine the following reagents in a new 0.2 mL PCR tube in the order given below.

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RNP1 Complex Formation Reaction	Volume
10X Cas9 Buffer	1.5 µL
Cas9	2.3 µL
RNase Inhibitor	1.0 µL
Guide RNA (Pan-Bacterial rRNA), pre-heated	3.9 µL
Total Volume	8.7 µL

8. 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.

9. Leave the tube on the laboratory bench for 10 minutes at room temperature. This mixture contains the first ribonucleoprotein complex (RNP1) for depleting the bacterial rRNA.

A2: First CRISPR digestion for depletion of bacterial rRNA

1. To the tube containing the first ribonucleoprotein complex (RNP1) from Step A1.9, add the pooled library (from Step A1.1) at room temperature:

Component	Volume
First ribonucleoprotein complex (RNP1 from Step A1.9)	8.7 µL
Pooled libraries (prepared before start of protocol)	6.3 µL
Total Volume	15 µL

2. Mix the contents gently by flicking the tube or carefully pipetting up and down several times.

3. Centrifuge briefly to collect the contents at the bottom of the tube.

4. Incubate the tube at 37°C for 60 minutes with heated lid turned on (≥50°C). After the incubation is complete, transfer the tube to ice for ~ 2 minutes. This tube contains the product of the first CRISPR digestion, which is the library depleted of bacterial rRNA.

NOTE: Proceed to Step A3 15 – 20 minutes before the end of this incubation.

A3: Second ribonucleoprotein complex formation (RNP2) for depletion of human, mouse or rat rRNA

1. At room temperature, combine the following reagents in a new 0.2 mL PCR tube in the order given below.

RNP2 Complex Formation Reaction	Volume
Nuclease Free Water	2.8 µL
10X Cas9 Buffer	1.0 µL
Cas9	2.3 µL
Guide RNA (Human, Mouse, Rat rRNA)	3.9 µL
Total Volume	10 µL

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NOTE: Return the Guide RNA (Human, Mouse, Rat) stock to the -80°C freezer immediately after use.

2. 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.

3. Leave the tube on the laboratory bench for 10 minutes at room temperature. This mixture contains the second ribonucleoprotein complex (RNP2) for depleting the human, mouse or rat rRNA.

A4: Second CRISPR digestion for depletion of human, mouse or rat rRNA

1. Obtain the product of first CRISPR digestion (from Step A2.4). Add the contents of the tube to the second ribonucleoprotein complex (RNP2) reaction (from Step A3.3). The total volume of the reaction should be ~25 µL.

2. 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.

3. Place the tube in a thermal cycler with a heated lid set to ≥80°C. Run the following program:

Temperature	Cycle Time
37°C	1 hour
65°C	5 min
4°C	HOLD

4. Centrifuge briefly to collect the contents at the bottom of the tube. This tube contains the rRNA-depleted library. Proceed to Step B. Bead Size Selection.

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Step B: Bead Size Selection

☐ Hands-on time: 10 min | Total time: 40 min

Reagent 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	Bring to room temperature. Vortex and invert mix.
80% Ethanol	Room temperature	Prepare fresh.

1. Add 25 µL of Nuclease-Free Water to the CRISPR digestion reaction from Step A4.4.
2. Add 30 µL (0.6X) of resuspended AMPure XP beads to the reaction. Mix well by pipetting up and down. Collect the contents at the bottom of the tube by briefly spinning the tube in a microcentrifuge.
3. Incubate at room temperature for 10 minutes. Mix the sample halfway through the incubation by pipetting up and down.
4. Place the tube on a magnetic stand until the solution is clear (3-5 minutes). Discard the supernatant without disturbing the beads.
5. Add 200 µL of freshly prepared 80% ethanol to the tube while still on the magnetic stand. Wait 30 seconds, then remove and discard the ethanol. Do not remove the tube from the magnetic stand while performing the ethanol wash.
6. Repeat the previous wash step.
7. Briefly spin the tube, place it back on the magnetic stand. Ensure that all residual ethanol is removed at this step.
8. Open the cap of the tube while it is on the magnetic stand and allow the beads to air dry for up to 5 minutes.
9. Remove the tube from the magnetic stand and add 40 µL of Nuclease-Free Water to the beads. Mix well to resuspend the beads by pipetting up and down. Collect the contents at the bottom of the tube by briefly spinning the tube in a microcentrifuge.
10. Incubate at room temperature for 10 minutes. Mix the sample halfway through the incubation by pipetting up and down. Collect the contents at the bottom of the tube by briefly spinning the tube in a microcentrifuge.
11. Place the tube on the magnetic stand. Allow the solution to clear and transfer the supernatant containing the eluted DNA to a new 0.2 mL PCR tube.
12. Proceed to the next step (PCR Amplification).

SAFE STOPPING POINT: If stopping at this point in the protocol, store the sample at -20ºC.

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Step C: Amplification

☐ Hands-on time: 10 min | Total time: 50 min

Reagent Preparation

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

1. On ice, combine the following reagents in the order listed below in a 0.2 mL PCR tube.

Amplification Reaction	Volume
Depleted sample (Step B.11)	40 µL
2X PCR Mix	50 µL
P7 Primer	5 µL
P5 Primer	5 µL
Total Volume	100 µL

2. Place the reaction in a thermal cycler with a heated lid set to 105°C. Run the following program:

Temperature	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	

NOTE: The number of PCR cycles after depletion correlates inversely with DNA input. Library yield of 100-200 ng is expected with the recommended input and PCR cycles as shown below.

Total quantity of DNA input from multiplexed libraries	Number of libraries per depletion reaction	PCR Cycles
8 ng	8	13
16 ng	16	12

3. Briefly spin the PCR tube in a microcentrifuge before proceeding with the next Step D: Bead Cleanup.

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Step D: Bead Cleanup

☐ Hands-on time: 10 min | Total time: 40 min

Reagent 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 (0.6X) of resuspended AMPure XP beads to the reaction. Mix well by pipetting up and down. Collect the contents at the bottom of the tube by briefly spinning the tube in a microcentrifuge.
2. Incubate the sample at room temperature for 10 minutes. Mix the sample halfway through the incubation by pipetting up and down.
3. Place sample tube on a magnetic stand until the solution is clear (3-5 minutes). Discard the supernatant without disturbing the beads.
4. Add 200 µL of freshly prepared 80% ethanol to the tube while still on the magnetic stand. Wait 30 seconds, then remove and discard the ethanol. Do not remove the tube from the magnetic stand while performing the ethanol wash.
5. Repeat the previous wash step.
6. Briefly spin the tube, place it back on the magnetic stand. Ensure that all residual ethanol is removed at this step.
7. Open the cap of the tube while it is on the magnetic stand and allow the beads to air dry for up to 5 minutes.
8. Remove the tube from the magnetic stand and add 30 µL of Tris Buffer to the beads. Mix well to resuspend the beads by pipetting up and down. Collect the contents at the bottom of the tube by briefly spinning the tube in a microcentrifuge.
9. Incubate the sample at room temperature for 10 minutes. Mix the sample halfway through the incubation by pipetting up and down. Collect the contents at the bottom of the tube by briefly spinning the tube in a microcentrifuge.
10. Place the tube on the magnetic stand. Allow the solution to clear and transfer the supernatant containing the eluted DNA to a new DNA LoBind tube. This constitutes the rRNA-depleted DNA library.

SAFE STOPPING POINT: If stopping, store the library at -20°C.

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Subsequent Steps

Jumpcode Genomics recommends assessing the library yield using a dsDNA-specific fluorescence-based method (such as a Qubit fluorometer) and library size on an Agilent Bioanalyzer 2100 or equivalent instrument before sequencing for best results.

After the second (and final) bead cleanup, most DNA fragments in the depleted library should be larger than 450 bp. If so, the library can be loaded directly on an Illumina sequencer. If a large proportion of fragments are less than 450 bp, it is recommended that a gel-based size selection step be performed to isolate DNA between 450 bp and 800 bp in size. The DNA can be loaded on a sequencing instrument after gel purification.

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