CRISPRclean™ Human rRNA Depletion Kit

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Introduction

RNA samples frequently contain up to 90% ribosomal RNA. Transcriptome sequencing without removal of these abundant rRNA sequences is an inefficient use of sequencing capacity and obscures detection of lower expressing, but biologically relevant transcripts.

The CRISPRclean Workflow

The CRISPRclean depletion workflow begins with prepared NGS libraries. Individual libraries are treated with Cas9/guide RNA complexes, which cleaves specific sequences for depletion. After targeted sequences are cleaved, they are no longer substrates for PCR amplification and sequencing. Subsequent magnetic bead-based size selection removes cleaved sequences and PCR refines for the uncleaved (i.e., desired) sequences). The output is a sequence-ready NGS library refined for sequences of interest.

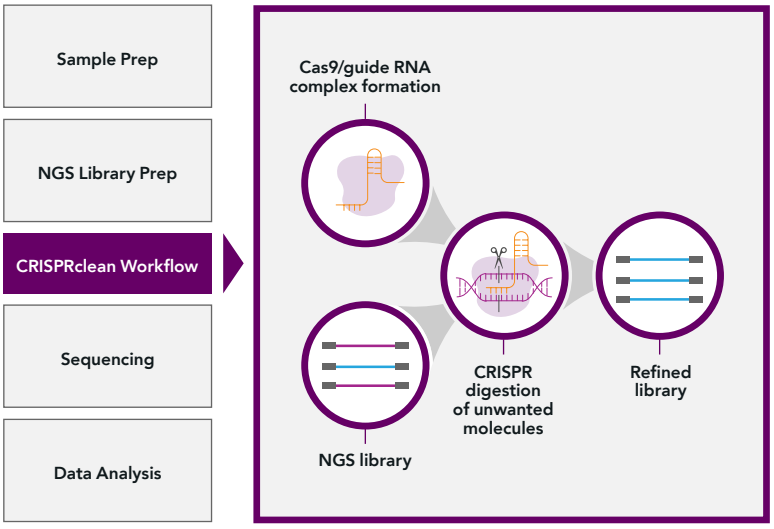

Figure 1: CRISPRclean workflow seamlessly fits into NGS workflows.

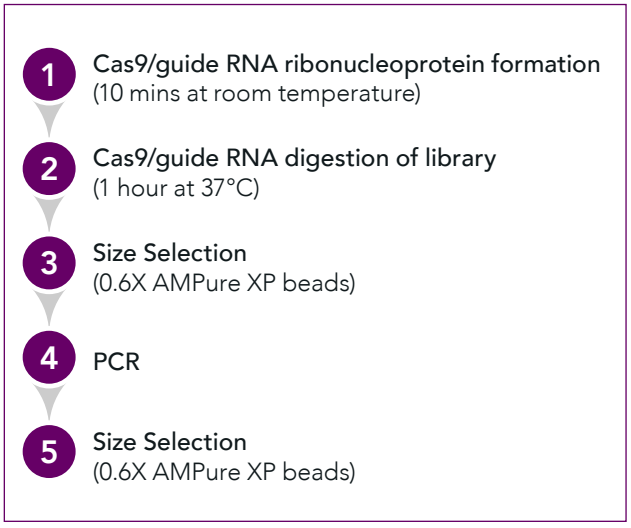

Figure 2: CRISPRclean depletion protocol.

Product

The Jumpcode CRISPRclean Human Ribosomal RNA Depletion Kit utilizes CRISPR technology to selectively target and cleave DNA fragments containing human ribosomal RNA sequences. While most depletion methods are performed on samples prior to NGS library preparation, this CRISPR-based depletion method is employed on fully prepared NGS libraries.

CRISPRclean Human rRNA Depletion Kit	
Designed to deplete	Human 5S, 5.8S, 18S and 28S nuclear rRNA genes, 45S rRNA precursor, 12S and 16S mitochondrial rRNA genes
Reactions	This kit contains reagents sufficient for three depletion reactions
Methods	Ribosomal RNA depletion for RNA-Seq
Recommended library prep kit	NEBNext Ultra II Directional RNA Library Prep Kit
Sequencing platform	Illumina instruments

Kit Components

CRISPRclean Human rRNA Depletion Kit

Box 1

Store at -20°C
8 reagents

- 1 vial: Cas9
- 1 vial: 10X Cas9 Buffer
- 1 vial: Nuclease-Free Water
- 1 vial: RNase Inhibitor
- 1 vial: 2X PCR Mix
- 1 vial: P5 Primer
- 1 vial: P7 Primer
- 1 vial: Tris Buffer

Box 2

Lot #6587958

Store at -80°C
1 reagent

- 1 vial: Human rRNA Guide RNA

User supplied items

AMPure XP beads

80% ethanol

Eppendorf LoBind tubes

PCR tubes

Magnetic stand

Best Practices

NGS library prep requirements:

The CRISPRclean RNA depletion protocol is recommended for and works most effectively with large fragment NGS libraries meant for Illumina sequencing. The kit produces best results with libraries size selected for a relatively narrow fragment size range in which the majority of 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, the CRISPRclean ribosomal RNA depletion protocol is most effective with NEBNext® Ultra™ II Directional RNA libraries when the following conditions are employed during library preparation:

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. Eight PCR cyles

CRISPR guide RNA:

The CRISPR guide RNA should be stored at -80°C as soon as it is received and should be handled with RNA-safe sterile laboratory techniques. We recommend a maximum of 3 freeze-thaw cycles for CRISPR guide RNA.

Guidelines for library multiplexing, input, and PCR cycles:

Since the CRISPRclean depletion protocol is employed on fully prepared RNA-Seq libraries, the user has the ability to combine multiple libraries together in a single CRISPR cleavage reaction.

It is recommended that when setting up a multiplexed CRISPRclean reaction, the user work with no less than 1 ng of each library, while ensuring that the total DNA input in the CRISPRclean reaction is restricted to no more than 100 ng.

The number of PCR cycles after depletion correlates inversely with DNA input. Guidelines for multiplexing and recommended post-depletion PCR cycle numbers are listed in Table 1.

Table 1: Guidelines for library multiplexing, input, and PCR cycles

Total quantity of DNA input from multiplexed libraries	Suggested library number for multiplexed depletion	Suggested PCR cycles after depletion*
8 ng	8	10
25 ng	8-24	9
50 ng	8-48	7
100 ng	8-96	6

* For final library yields between 200 ng and 400 ng, post-depletion.

Protocol

CRISPRclean Human rRNA Depletion Kit for Illumina Sequencing

Please read **Best Practices** before attempting this protocol for the first time.

Follow good laboratory practices, including RNA-safe procedures when handling RNA.

The protocol takes approximately 4 hours to complete.

Items highlighted in bold throughout the text are provided with the kit. See **Kit Components** for user supplied items such as AMPure XP beads.

1. Ribonucleoprotein complex formation

a. The Jumpcode CRISPRclean rRNA depletion 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 no more than 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 of the libraries.
- Ensure that the total volume of the combined libraries is no greater than 10.8 µL.

Follow the guidelines to pool the libraries together before proceeding to the next step. Measure the final volume of the **pooled DNA library**.

b. Add the reagents listed below to a 0.5 mL Eppendorf DNA LoBind microcentrifuge tube (or other similar low-nucleic acid binding tube). Add them to the tube in the order given below:

2 µL	**10X Cas9 Buffer**
3.9 µL	**Human rRNA Guide RNA**
2.3 µL	**Cas9**
1 µL	**RNase Inhibitor**

Total volume = 9.2 µL

Note: The **Cas9** and **RNase Inhibitor** reagents should be maintained on ice or in a benchtop cooler during this step and transferred back to the -20°C freezer immediately thereafter. The **Guide RNA** should be completely thawed on ice before use and transferred back to the -80°C freezer immediately after use.

c. 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.

d. Leave the tube on the laboratory bench for 10 minutes at room temperature. This is the **ribonucleoprotein complex (RNP)**.

2. CRISPR digestion

a. Obtain the **pooled DNA library** you intend to deplete (from Step 1a). Add **Nuclease-Free Water** to bring the volume to 10.8 µL.

b. Add the **pooled DNA library to the ribonucleoprotein complex (RNP)** reaction (from Step 1d). The total volume of the reaction after addition of library should be 20 µL.

c. 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.

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.

d. After the 60 minute incubation at 37°C is complete, transfer the tube to ice for ~2 minutes.

e. Spin the tube briefly in the microcentrifuge. Transfer the tube to the laboratory bench. Proceed with Step 3a.

3. Size Selection

a. Add 30 µL of **Nuclease-Free Water** to the CRISPR digestion reaction (from Step 2e). Mix gently.

b. 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 (take the beads out of the refrigerator 20 - 30 minutes prior to use to bring them to room temperature). Mix the sample halfway through the incubation by pipetting up and down a few times.

c. Place the tube on the magnetic stand. Allow the solution to clear (3 – 5 minutes) and discard the supernatant without disturbing the beads.

d. 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.

e. Repeat the wash step with another 200 µL of 80% ethanol. Ensure that all residual ethanol is removed after this wash.

f. Open the cap of the tube while it is on the magnetic stand and allow the beads to air dry for 5 minutes.

g. 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.

h. 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.

4. Amplification

a. Add the following components to the eluted DNA (in the thin-walled PCR tube):

5 µL	**P5 Primer**
5 µL	**P7 Primer**
50 µL	**2X PCR Mix**
Total = 100 µL	

Note: The **2X PCR Mix** should be thawed on ice and returned to the freezer immediately after use.

Input the following parameters into a thermal cycler and perform a PCR (see the **Best Practices** section Table 1 for information on the number of cycles to be used in the PCR):

1 cycle:

95°C, 2 minutes

Up to 13 cycles (see Table 1 in Best Practices):

98°C, 20 seconds
55°C, 30 seconds
72°C, 30 seconds

1 cycle:

72°C, 2 minutes
4°C, hold

b. Briefly spin the PCR tube in a microcentrifuge before proceeding with the next step.

5. Size Selection

a. Add 60 µL (0.6 volumes) of well-resuspended, room-temperature AMPure XP beads to the supernatant from Step 4b (the previous step), 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.

b. Place the tube on the magnetic stand. Allow the solution to clear (3 – 5 minutes) and discard the PCR product without disturbing the beads.

c. 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.

d. Repeat the wash step with another 200 µL of 80% ethanol. Ensure that all residual ethanol is removed after this wash.

e. Open the cap of the tube while it is on the magnetic stand and allow the beads to air dry for 10 minutes. Do not allow the beads to dry for longer than 10 minutes. Overdrying the beads could result in lower yields.

f. 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.

g. 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.

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, the majority of 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.

Ordering Information

Contact your sales representative for more information.

Catalog	Product	Designed to deplete
KIT1000	CRISPRclean Human rRNA Depletion Kit	Human 5S, 5.8S, 18S, 28S, 45S (precursor), mitochondrial 12S and 16S rRNA
KIT1001	CRISPRclean Pan Bacterial rRNA Depletion Kit	Genes from over 200 bacteria representing all phyla: 5S, 16S, and 23S rRNA
KIT1005	CRISPRclean Metatranscriptomic rRNA Depletion Kit	Human and over 200 bacteria rRNA
KIT1006	CRISPRclean Housekeeping Genes 100 Depletion Kit	10 mitochondrial and 90 ribosomal protein coding transcripts (mRNA)
KIT1012	CRISPRclean Globin Depletion Kit	*HBA1*, *HBA2*, *HBB*, *HBD* genes

Revision Log

Version:	Distribution Date:	Key Updates:
1.0	09-01-2020	Initial release
1.1	10-30-2020	**Protocol changes:** Update process by which ribonucleoprotein complex is prepared (affects Steps 1b - 2b). Include warning not to dry beads for longer than 10 minutes.
1.2	03-18-2021	**Protocol changes:** Concentration of Human rRNA Guide RNA has changed.
1.3	06-11-2021	**Update to format and Best Practices Table 1**