User Manual Version 1.0

Jumpcode™ CRISPRclean™ Ribosomal RNA Depletion from Human RNA-Seq Libraries for Illumina Sequencing

Protocol Notes

Introduction

The Jumpcode CRISPRclean ribosomal RNA depletion kit is designed to remove DNA derived from human ribosomal RNA transcripts in next generation sequencing (NGS) libraries prepared from total cellular RNA. The method utilizes CRISPR technology to selectively target and cleave DNA fragments containing ribosomal RNA sequences. While most ribodepletion methods are performed on samples prior to NGS library preparation, this CRISPR-based ribodepletion method is employed on fully prepared NGS libraries.

An outline of the Jumpcode CRISPRclean ribosomal RNA depletion protocol is provided below (Figure 1).

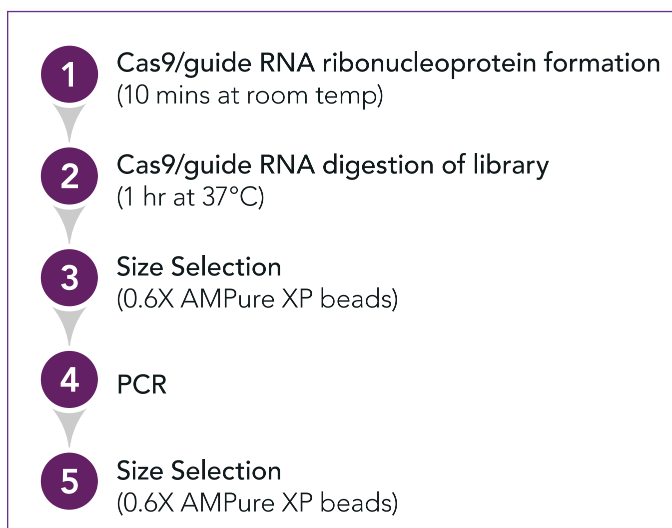

Figure 1: Schematic of the Jumpcode CRISPRclean ribosomal RNA depletion protocol.

NGS library prep requirements:

The CRISPRclean ribosomal 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 Jumpcode 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 pool targets the human nuclear and mitochondrial ribosomal RNA genes. These genes include the human 5S, 5.8S, 18S and 28S nuclear rRNA genes, the 45S rRNA precursor and the 12S and 16S mitochondrial rRNA genes. The pool of CRISPR guide RNAs is complexed to the *Streptococcus pyogenes* Cas9 protein in the first step of the CRISPRclean protocol and employed to cleave specific sites within the human

© 2020 Jumpcode Genomics, Inc.

ribosomal RNA sequences detailed above. Using libraries prepared under conditions optimal for this ribodepletion method, this protocol can be expected to remove >99% of DNA derived from ribosomal RNA in an NGS library.

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.

Library multiplexing and input:

Since the Jumpcode ribodepletion method is employed on fully prepared RNA-Seq libraries, the user has the ability to combine multiple libraries together in a single CRISPR cleavage reaction. Guidelines for multiplexing are provided in Table 1 (below).

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

PCR:

The number of PCR cycles after ribodepletion correlates inversely with DNA input. Recommended post-ribodepletion PCR cycle numbers are listed in Table 1 (below).

Requirements for sequencing:

As shown in Figure 1, the CRISPRclean ribodepletion protocol involves a CRISPR-based DNA cleavage step followed by AMPure XP bead-based size selection, PCR and a second AMPure XP bead-based size selection step. After the second (and final) size selection, the majority of DNA fragments in the CRISPR-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.

Table 1

Approximate total quantity of DNA input for ribodepletion	Suggested library multiplexing for ribodepletion	Suggested PCR cycles after ribodepletion*
1 ng	Not recommended for multiplexing	13
10 ng	8	10
25 ng	8 - 24	9
50 ng	8 - 48	7
100 ng	8 - 96	6

* for final yields between 200 ng and 400 ng

© 2020 Jumpcode Genomics, Inc.

Jumpcode CRISPRclean Protocol for Ribosomal RNA Depletion from Human RNA-Seq Libraries

For use with NGS libraries for Illumina sequencing

Please read the Protocol Notes before attempting this protocol for the first time.

The protocol takes approximately 4½ hours to complete (not including the time for normalization of input DNA concentrations).

Items highlighted in bold throughout the text are provided with the kit. AMPure XP beads are not included and will need to be provided by the user.

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 ribodepletion 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 ribodepletion reaction.
- Preferentially combine libraries pepared 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 7 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. Subtract the volume (of library) calculated in the previous step from 20 µL. This is the volume of ribonucleoprotein complex that you will assemble in the next step.

c. Add the reagents listed below to a 0.5 mL Eppendorf DNA LoBind microcentrifuge tube (or other similar low-DNA binding tube).

y µL *	**Nuclease-Free Water**
2 µL	**10X Cas9 Buffer**
1 µL	**RNase Inhibitor**
2.3 µL	**Cas9**
7.8 µL	**Human rRNA Guide RNA**

Total volume = 20 µL minus the volume of DNA to be added to the reaction

* y = volume of the ribonucleoprotein complex formation reaction calculated in Step 1b minus 13.1 µL (13.1 µL = volume of all reagents listed above, except **Nuclease-Free Water**)

d. 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.

e. Leave the tube on the laboratory bench for 10 minutes at room temperature.

© 2020 Jumpcode Genomics, Inc.

2. CRISPR digestion

a. Obtain the pool of libraries you intend to ribodeplete (from Step 1a). Add the library pool to the ribonucleoprotein complex formation reaction (from Step 1e). The total volume of the reaction after addition of library should be 20 µL. 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.)

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

3. Size Selection

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

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

Input the following parameters into a thermal cycler and perform a PCR (see the Protocol Notes for more information on the number of cycles to be used in the PCR):

1 cycle:

95°C, 2 minutes

Up to 13 cycles:

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 supernatant without disturbing the beads.

© 2020 Jumpcode Genomics, Inc.

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.

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. Please see the Protocol Notes for details of the library requirements for sequencing.

Note: Items highlighted in bold throughout the text are provided with the kit. AMPure XP beads are not included and will need to be provided by the user.

© 2020 Jumpcode Genomics, Inc.

Jumpcode CRISPRclean Human rRNA Depletion Kit

Materials List

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

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

Please note: This kit contains reagents sufficient for three ribodepletion reactions.

© 2020 Jumpcode Genomics, Inc.