JUMPCODE GENOMICS

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USER MANUAL

CRISPRclean™ Plus Stranded Total RNA Prep with rRNA Depletion

(Human, Mouse, Rat, Pan Bacteria)

Contents

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Product overview

The CRISPRclean Plus Stranded Total RNA Prep with rRNA Depletion (Human, Mouse, Rat, Pan Bacteria) is designed to prepare directional, strand specific RNA libraries and deplete human, mouse, rat and bacterial ribosomal RNA (rRNA) sequences in just 9 hours with 3.5 hours of hands-on time. The protocol is suited for total RNA. The final product is a directional, depleted library compatible with sequencing on Illumina® instruments.

This kit contains the reagents necessary to process the user's purified total RNA sample through library preparation, and amplification for sequencing. The NEXTFLEX Unique Dual Index (UDI) Adapter Plate for RNA Prep (Set A) is a required reagent to be used with the CRISPRclean Plus Stranded Total RNA Prep with rRNA Depletion (Human, Mouse, Rat, Pan Bacteria). The 96 unique dual index adapter barcode adapters allows for color-balanced and high-throughput, multiplexed sequencing.

Description	For whole-transcriptome sequencing
Assay time	9 hours
Hands-on time	3.5 hours
Nucleic acid	RNA
Input quantity of total RNA	5 ng to 100 ng
Method	RNA sequencing
Depletion mechanism	CRISPR Cas9 mediated
Strand specificity	> 98% directional
Multiplexing	Up to 96 Unique Dual Indexes using NEXTFLEX Unique Dual Index Adapter Plate for RNA Prep (Set A): NOVA-512920
Species	Human, mouse, rat, 212 bacteria
Designed to deplete	Human 5S, 5.8S, 18S and 28S rRNA genes, 45S rRNA precursor, mitochondrial 12S and 16S rRNA genes 212 bacteria representing all phyla: 5S, 16S, and 23S genes
Compatibility	Short read sequencing instruments such as Illumina® instruments

CRISPRclean Plus workflow

The streamlined workflow for library preparation from total RNA involves seven steps: RNA fragmentation, first strand synthesis, second strand synthesis, adenylation, adapter ligation, depletion, and PCR amplification. The workflow begins with fragmentation of the RNA through incubation at a high temperature and then proceeds to first and second strand synthesis to convert RNA fragments into cDNA libraries. > 98% strand specificity is achieved through incorporation of dUTP during second strand synthesis. Adenylation modifies the 3' ends of the double-stranded cDNA with dATP to prepare the library for adapter ligation. Once unique dual index adapters are ligated onto the library, the library is ready for depletion.

The innovative step in the protocol is the depletion of rRNA sequences of adapter ligated libraries. Depletion is performed in two successive incubations, the first to cleave bacterial rRNAs and the second to cleave mammalian rRNAs. Cas9 protein and the guide RNAs are combined to form ribonucleoprotein complexes specifically programmed to remove rRNA sequences when incubated with the adapter ligated libraries. Cleaved rRNA fragments cannot be amplified, and/or are removed through size selection with magnetic beads. The final step is PCR amplification of the library.

Kit contents, storage, and shelf life

The kit contains enough material to prepare 24 RNA-seq libraries for Illumina® compatible sequencing. The shelf life of all reagents is 12 months from the date of manufacturing when stored properly.

The CRISPRclean Plus Stranded Total RNA Prep with rRNA Depletion (Human, Mouse, Rat, Pan Bacteria) is made up of the following kit contents to be stored at the temperatures indicated in the table below: The NEXTFLEX Unique Dual Index Adapter Plate for RNA Prep (NOVA-512920) is required for library preparation performed with this kit.

Library prep and depletion box: ASY1058-001

Kit contents	Cap color	Storage temp
CRISPRclean Fragmentation Buffer Mix	Brown	-20°C
CRISPRclean First Strand Synthesis Mix	Red	-20°C
CRISPRclean Reverse Transcriptase	Red	-20°C
CRISPRclean Second Strand Synthesis Mix	White or Clear	-20°C
CRISPRclean Adenylation Mix	Yellow	-20°C
CRISPRclean Adenylation Enzyme	Yellow	-20°C
CRISPRclean Ligase Mix	Orange	-20°C
CRISPRclean Ligase Enzyme	Orange	-20°C
CRISPRclean Cas9	Purple	-20°C
CRISPRclean 10X Cas9 Buffer	Purple	-20°C
CRISPRclean RNase Inhibitor	Purple	-20°C
CRISPRclean PCR Master Mix	Green	-20°C
CRISPRclean PCR Primer Mix	Green	-20°C
CRISPRclean Nuclease-free Water *	White or Clear	*4°C
CRISPRclean Resuspension Buffer *	White or Clear	*4°C

* **Note:** Store in 4°C after first use.

Guide RNA box: ASY1056-001

Kit contents	Cap color	Storage temp
CRISPRclean Guide RNA (Human, Mouse, Rat) for rRNA Depletion	Blue	-80°C
CRISPRclean Guide RNA (Pan Bacteria) for rRNA Depletion	Blue	-80°C

Cleanup beads bag: ASY1055-001

Kit contents	Storage temp
CRISPRclean Library Prep Cleanup Beads	4°C

Required materials provided by the user

Reagents

- Total RNA
- NEXTFLEX Unique Dual Index Adapter Plate for RNA Prep (Set A): NOVA-512920
- AMPure® XP beads (stored at 4°C)
- 80% Ethanol (freshly prepared and stored at room temperature)

Consumables and hardware

- 10, 20, 200 and 1000 µL pipettes
- RNase-free barrier pipette tips
- Nuclease-free 1.5 mL microcentrifuge tubes
- Thin-walled nuclease-free PCR tubes (Eppendorf™ LoBind) or similar
- 96 well PCR plate non-skirted (Phenix Research™, # MPS-499) or similar
- Adhesive PCR plate seal (BioRad®, # MSB1001)
- Agilent 2100 Bioanalyzer® System RNA 6000 Nano or Pico Kit, and High Sensitivity DNA Kit
- Magnetic stand for bead cleanup (suitable for tube or plate format)
- Microcentrifuge
- Thermal cycler
- Vortex
- Ice

Warnings and precautions

- We strongly recommend reading the following warnings and precautions. Periodically, optimizations and revisions are made to the components and manual. Therefore, it is important to follow the protocol included with the kit.
- Do not use the kit beyond 12 months after the date of manufacturing.
- The CRISPRclean First Strand Synthesis Mix may appear yellow in color.
- The CRISPRclean Plus Stranded Total RNA Prep with rRNA Depletion (Human, Mouse, Rat, Pan Bacteria) is intended to be used with the NEXTFLEX Unique Dual Index Adapter Plate for RNA Prep (Set A): NOVA-512920.
- Try to maintain a laboratory temperature of 20°–25°C (68°–77°F).
- Ensure that all pipette tips, microcentrifuge tubes, and other consumables are RNase-free.
- DTT in buffers may precipitate after freezing. If a precipitate appears, vortex buffer for 1-2 minutes or until the precipitate is in solution. The performance of the buffer is not affected once the precipitate is in solution.

- Use properly calibrated pipettes as library preparation is sensitive to pipetting error.
- Vortex and microcentrifuge each component immediately before use, to collect the contents at the bottom of the tube.
- Do not remove enzymes from -20°C until before use. Return to -20°C immediately after use.
- Do not remove Guide RNA from -80°C until before use. Return to -80°C immediately after use.
- We recommend a maximum of 3 freeze-thaw cycles for Guide RNA.
- Thermal cycling should be performed with a heated lid except where specified.
- Do not heat the CRISPRclean Unique Dual Index Adapter Plate above room temperature.
- For multiplexing options, please use NEXTFLEX Unique Dual Index Adapter Plate for RNA Prep (Set A), NOVA-512920 during STEP E: Adapter Ligation.
  - Once the plate has thawed, spin for one minute in the centrifuge before use to collect the contents at the bottom of the plate.
  - Before use, carefully mix adapters by pipetting up and down several times using a multi-channel pipette with barrier tips.
  - Do not remove the manufacturer provided adhesive film covering the plate. Remove adapters by piercing the seal over the individual wells with a pipette tip. Reseal the plate simply by placing additional pierceable sealing films over the previous seal after each use.

IMPORTANT: NEVER mix plates by vortexing. Mixing samples or barcodes by vortexing results in cross-contamination, even if the plate appears to be securely sealed.

- Allow beads to come to room temperature for 30 minutes before use.
- Vortex beads until they are in a uniform suspension.
- Do not freeze CRISPRclean Library Prep Cleanup Beads and AMPure® XP Beads.
- Keep beads in liquid suspension during storage and handling.
- Ensure beads pellet on the magnet before removing the clear supernatant. Completely remove 80% ethanol before eluting the RNA.

Revision log

Version	Date	Description
V1.0	October 2021	Product launch

Library prep setup

Starting materials

The CRISPRclean Plus Stranded Total RNA Prep with rRNA Depletion (Human, Mouse, Rat, Pan Bacteria) has been optimized and validated for 5 ng to 100 ng of total RNA isolated from samples containing mixtures of mammalian (human, mouse and rat) and bacteria.

Before beginning the CRISPRclean Plus protocol, total RNA is required to be free of contaminating genomic DNA. Treat the samples as recommended in RNA isolation protocols with RNase-free DNase. Resuspend and dilute RNA in RNase-free molecular biology grade water.

Quantify RNA with a fluorometric method for accurate input quantity. The recommended RNA input quantity is between 5 ng and 100 ng to achieve the most efficient rRNA depletion rates. Lower amounts of starting material may result in higher duplication rates, reduced library complexity, and other changes in sequencing data quality.

Analyze RNA integrity on a BioAnalyzer® instrument or equivalent. High quality total RNA are considered to have an RIN of > 7.

The efficiency of rRNA depletion is dependent on accurate quantification, sample quality, and sample type, such as saliva, nasopharyngeal, or fecal samples.

Protocol

Step A: RNA fragmentation

Hands-on time: 10 min | Total time: 25 min

Materials provided

(brown) - CRISPRclean Fragmentation Buffer Mix

(white or clear) - CRISPRclean Nuclease-free Water

Required materials provided by the user

- Total RNA
- Nuclease-free microcentrifuge tube or plate
- Thermal cycler
- Ice

NOTE: This protocol requires prior isolation of RNA through standard methods. Fragmentation times are dependent on the RIN. The RIN of the RNA sample must be determined with an Agilent Bioanalyzer® 2100 instrument or equivalent before starting library preparation.

1. For each reaction, combine the following reagents **on ice** in a nuclease-free PCR tube or 96-well PCR plate:

Component	Volume
Total RNA (in CRISPRclean Nuclease-free Water)	14 µL
CRISPRclean Fragmentation Buffer Mix	6 µL
Total Volume	20 µL

2. Mix thoroughly by pipetting up and down.
3. Program a thermal cycler with the following incubation times and temperatures based on the RIN of individual RNA samples:

RIN: 10 - 7		RIN: 6.99 - 3		RIN: < 3
15 min	94°C	10-12 min	94°C	Fragmentation not recommended
HOLD	4°C	HOLD	4°C	Fragmentation not recommended

4. Proceed to **Step B: First strand synthesis**.

Step B: First strand synthesis

Hands-on time: 10 min | Total time: 45 min

Materials provided

- (red) CRISPRclean First Strand Synthesis Mix
- (red) CRISPRclean Reverse Transcriptase

Required materials provided by the user

- Fragmented RNA (from Step A)
- Thermal cycler
- Ice

NOTE: Due to the viscosity of certain materials, preparing more than the stated number of reactions may result in a shortage of materials. All CRISPRclean enzyme components must be centrifuged at 600 x g for 5 seconds before opening the tube(s). Pipette only the necessary volume. Avoid excess material on the exterior of the pipette tip to ensure sufficient components for the stated number of reactions in the kit.

1. For each reaction combine the following reagents **on ice** in a nuclease-free PCR tube or 96-well PCR plate:

Component	Volume
Fragmented RNA (Step A)	20 µL
CRISPRclean First Strand Synthesis Mix	4 µL
CRISPRclean Reverse Transcriptase	1 µL
Total Volume	25 µL

2. Mix thoroughly by pipetting up and down.
3. Place the tube in a thermal cycler programmed with the cycling parameters below.

Temp	Cycle time
25°C	10 min
50°C	15 min
70°C	10 min
4°C	HOLD

4. Proceed to **Step C: Second strand synthesis.**

Step C: Second strand synthesis

Hands-on time: 40 min | Total time: 100 min

Materials provided

◯ (white or clear) - CRISPRclean Second Strand Synthesis Mix

◯ (white or clear) - CRISPRclean Resuspension Buffer

◯ (white or clear) - CRISPRclean Library Prep Cleanup Beads (room temp)

Required materials provided by the user

- First strand synthesis product (from Step B)
- Thermal cycler
- Ice
- Adhesive PCR plate seal
- 80% Ethanol, freshly prepared (room temp)
- Magnetic stand

1. For each reaction combine the following **on ice** in a nuclease-free PCR tube or plate:

Component	Volume
First strand synthesis product (from Step B)	25 µL
◯ CRISPRclean Second Strand Synthesis Mix	25 µL
Total Volume	50 µL

2. Mix thoroughly by pipetting up and down.
3. Program a thermal cycler as follows:

Temp	Cycle time
16°C	60 min
4°C	HOLD

4. Incubate on the thermal cycler with the above settings with the heated lid **turned off or left open**.
5. Add 90 µL of well mixed CRISPRclean Library Prep Cleanup Beads to each sample and mix thoroughly by pipetting up and down.
6. Incubate for 5 minutes at room temperature.
7. Place the plate on the magnetic stand for 5 minutes or until the solution is clear.
8. Remove and discard the supernatant without disturbing the beads.

9. Keeping the plate on the magnetic stand, add 200 µL of freshly prepared 80% ethanol, incubate for 30 seconds, and remove all of the supernatant. Repeat this step once for a total of 2 ethanol washes.

IMPORTANT: Always use freshly prepared 80% ethanol and do not incubate the bead pellet with 80% ethanol for extended periods.

10. Air dry the sample for 3 minutes. After 1 minute, remove all residual liquid that may have collected at the bottom of the well.
11. Remove the tube or plate from the magnetic stand and resuspend the bead pellet in 34 µL of CRISPRclean Resuspension Buffer by pipetting the volume up and down. Ensure that the beads are completely resuspended.
12. Incubate the sample for 2 minutes at room temperature.
13. Place the tube or plate on the magnetic stand for 3 minutes or until the supernatant appears clear.
14. Transfer 32 µL of supernatant to a new PCR tube or plate.

NOTE: The procedure may be safely stopped at this point and the samples stored at -20°C.

15. Proceed to **Step D: Adenylation.**

Step D: Adenylation

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

Materials provided

(yellow) – CRISPRclean Adenylation Mix

(yellow) - CRISPRclean Adenylation Enzyme

Required materials provided by the user

- Purified second strand synthesis DNA (from Step C)
- Thermal cycler
- Adhesive PCR plate seal
- Ice

1. For each sample, combine the following reagents **on ice** in a nuclease-free PCR tube or 96-well PCR plate:

Component	Volume
Second strand synthesis product (from Step C)	32 µL
CRISPRclean Adenylation Mix	15 µL
CRISPRclean Adenylation Enzyme	3 µL
Total Volume	50 µL

2. Mix thoroughly by pipetting up and down several times.
3. Place the tube or plate on a thermal cycler and perform the reaction with the following cycling parameters:

Temp	Cycle time
65°C	30 min
4°C	HOLD

4. Proceed to **Step E: Adapter Ligation.**

Step E: Adapter ligation

Hands-on time: 45 min | Total time: 65 min

Materials provided

- (orange) – CRISPRclean Ligation Mix
- (orange) - CRISPRclean Ligase Enzyme
- (white or clear) - CRISPRclean Resuspension Buffer
- (white or clear) - CRISPRclean Library Prep Cleanup Beads (room temperature)
- (white or clear) - CRISPRclean Nuclease-free Water

Required materials provided by the user

- 50 µL Adenylated DNA (from Step D)
- NEXTFLEX Unique Dual Index Adapter Plate for RNA Prep (NOVA-512920)
- Thermal cycler
- Adhesive PCR plate seal
- 80% Ethanol, freshly prepared (room temperature)
- Magnetic stand

Total RNA	Desired Adapter Concentration	Adapter Dilution Required
5 ng	0.62 uM	1/10
10 ng	1.56 µM	1/4
25 ng	2.08 uM	1/3
50 ng	3.12 µM	1/2
100 ng	6.25 µM	None

IMPORTANT: The CRISPRclean Ligase Mix is viscous. Mix the following reaction until visibly homogeneous by pipetting or brief vortexing.

IMPORTANT: Adapters in the CRISPRclean Unique Dual Index Adapter Plate for RNA Prep (Set A) are provided at a concentration of 6.25 µM. Use CRISPRclean Nuclease-free Water to dilute the adapters.

1. For each sample, combine the following reagents **on ice** in a nuclease-free PCR tube or 96 well PCR Plate:

Component	Volume
Adenylated second strand synthesis product (from Step D)	50.0 µL
CRISPRclean Ligation Mix*	44.5 µL
NEXTFLEX Unique Dual Index Plate (Set A): one unique barcode per sample, ensure proper concentration	2.5 µL
CRISPRclean Ligase Enzyme*	3.0 µL
Total Volume	100.0 µL

NOTE: *These components can be premixed and added in a single step. Do not premix adapters in order to prevent excess adapter dimer formation.

2. Mix thoroughly by pipetting up and down several times.
3. Place the tube or plate on a thermal cycler programmed with the following cycling parameters:

Temp	Cycle time
20°C	15 min
4°C	HOLD

4. Incubate the reaction on the thermal cycler with above settings with heated **lid turned off or left open**.
5. Add 65 µL of CRISPRclean Nuclease-free Water and 35 µL of well mixed CRISPRclean Library Prep Cleanup Beads to each well containing sample. Mix thoroughly by pipetting.
6. Incubate for 5 minutes at room temperature.
7. Place the tube or plate on the magnetic stand for 5 minutes or until the solution is clear.
8. Remove and discard the supernatant without disturbing the beads.
9. Add 200 µL of freshly prepared 80% ethanol, incubate for 30 seconds, and remove all of the supernatant. Repeat this step once for a total of 2 ethanol washes.

IMPORTANT: Always use freshly prepared 80% ethanol and do not incubate the bead pellet with 80% ethanol for extended periods.

10. Air dry the sample for 3 minutes. After 1 minute, remove all residual liquid that may have collected at the bottom of the well.
11. Remove the tube or plate from the magnetic stand and resuspend the bead pellet in 50 µL of CRISPRclean Resuspension Buffer by pipetting up and down several times. Ensure that the beads are completely resuspended.
12. Add 45 µL of well mixed CRISPRclean Library Prep Cleanup Beads to each well containing sample. Mix thoroughly by pipetting up and down.

13. Incubate for 5 minutes at room temperature.
14. Place the tube or plate on the magnetic stand for 5 minutes or until the solution is clear.
15. Remove and discard the supernatant without disturbing the beads.
16. Add 200 µL of freshly prepared 80% ethanol, incubate for 30 seconds, and remove all of the supernatant. Repeat this step once for a total of 2 ethanol washes.

IMPORTANT: Always use freshly prepared 80% ethanol and do not incubate the bead pellet with 80% ethanol for extended periods.

17. Incubate the sample for 3 minutes. After one minute, remove all residual liquid that may have collected at the bottom of the well.
18. Remove the tube or plate from the magnetic stand and resuspend the bead pellet in 17 µL of CRISPRclean Resuspension Buffer by pipetting up and down. Ensure that the beads are completely resuspended.
19. Incubate the sample for 2 minutes at room temperature.
20. Place the tube or plate on the magnetic stand for 3 minutes or until the supernatant appears clear.
21. Transfer 15 µL of the supernatant (adapter ligated DNA) to a new PCR tube or plate.

NOTE: The procedure may be stopped at this point and the samples stored at -20°C.

22. Proceed to **Step F: Depletion of ribosomal RNA**.

Step F: Depletion of ribosomal RNA

Hands-on time: 60 min | Total time: 210 min

Materials provided

- (blue) - CRISPRclean Guide RNA (Pan Bacteria)
- (blue) - CRISPRclean Guide RNA (Human, Mouse, Rat)
- (purple) - CRISPRclean Cas9
- (purple) - CRISPRclean 10X Cas9 Buffer
- (purple) - CRISPRclean RNase Inhibitor
- (white or clear) - CRISPRclean Nuclease-free water
- (white or clear) - CRISPRclean Resuspension Buffer

Required materials provided by the user

- Ice
- 15 µL Adapter ligated DNA (from Step E)
- AMPure® XP beads (room temp)
- Thermal cycler
- Adhesive PCR plate seal
- 96 well PCR plate or PCR tubes
- 80% Ethanol, freshly prepared (room temp)
- Magnetic stand

F.1. First ribonucleoprotein complex formation (RNP1) for depletion of bacterial rRNA

1. Allow the CRISPRclean Guide RNA (Pan Bacteria) to thaw **on ice**. Each library requires 4.5 µL of the guide RNA. For more than 1 library, multiply the 4.5 µL volume of the guide RNA by the number of libraries to be depleted.
2. Transfer the calculated total guide RNA volume into a new PCR tube.

NOTE: Return the CRISPRclean Guide RNA (Pan Bacteria) stock to the -80°C freezer immediately after use.

3. Place the tube in a pre-heated thermal cycler and incubate at 65°C for 2 minutes.
4. Immediately transfer the tube to ice for 3 minutes.
5. Briefly spin the pre-heated guide RNA to collect the contents at the bottom of the tube.
6. Return the tube to ice.

7. Assemble the following reagents in a new PCR tube at room temperature in the order given below for each library:

Component	Volume
CRISPRclean 10X Cas9 Buffer	1.0 µL
CRISPRclean Cas9	2.4 µL
CRISPRclean RNase Inhibitor	1.0 µL
CRISPRclean Guide RNA (Pan Bacteria) – pre-heated	4.0 µL
Total Volume	8.4 µL

IMPORTANT: For each library, prepare a separate tube for RNP complex formation of Cas9 with the guide RNA. Do not prepare a master mix of the components to process multiple libraries simultaneously.

NOTE: Place the CRISPRclean Cas9 and RNase Inhibitor reagents **on ice** or in a benchtop cooler and return the stocks to -20°C immediately after use. Allow the CRISPRclean 10X Cas9 Buffer to thaw **on ice**. Keep **on ice** for subsequent preparation of RNP2.

8. Mix the contents gently by flicking the tube or carefully pipetting up and down several times.
9. Centrifuge briefly to collect the contents at the bottom of the tube.
10. Leave the tube on the laboratory bench for 10 minutes at room temperature. This mixture contains the first ribonucleoprotein complex (RNP1) for depleting of the bacterial rRNA.

F.2. First CRISPR digestion for depletion of bacterial rRNA

IMPORTANT: If safe stopping point after Step E was used, thaw the Adapter-ligated DNA (from Step E) before the first CRISPR digestion.

11. To the tube containing the first ribonucleoprotein complex (RNP1) from Step **F.1.10**, add the following reagents at room temperature:

Component	Volume
First ribonucleoprotein complex (RNP1 from F.1.10)	8.4 µL
Adapter-ligated DNA (from Step E)	15.0 µL
CRISPRclean 10X Cas9 Buffer	1.5 µL
Total Volume	~ 25.0 µL

12. Mix the contents gently by flicking the tube or carefully pipetting up and down several times.
13. Centrifuge briefly to collect the contents at the bottom of the tube.

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

NOTE: Proceed to **Step F.3.** about 15 – 20 minutes before the end of this incubation.

F.3. Second ribonucleoprotein complex formation (RNP2) to deplete Human, Mouse, and Rat rRNA

15. Remove the tube of CRISPRclean Guide RNA from the -80°C freezer and allow it to thaw **on ice**.

16. Assemble the following reagents in a new tube at room temperature in the order given below:

Component	Volume
CRISPRclean Nuclease-free water	3.0 µL
CRISPRclean 10X Cas9 buffer	1.0 µL
CRISPRclean Cas9	2.4 µL
CRISPRclean Guide RNA (Human, Mouse, Rat)	3.6 µL
Total Volume	10.0 µL

IMPORTANT: For each library, prepare a separate tube for the RNP complex formation of Cas9 with the guide RNA. Do not prepare a master mix for processing multiple libraries simultaneously.

IMPORTANT: Place the CRISPRclean Cas9 **on ice** or in a benchtop cooler and return the stock to the -20°C freezer immediately after use. Thaw the CRISPRclean Guide RNA (Human, Mouse, Rat) **on ice** and return the stock to the -80°C freezer immediately after use.

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

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

19. Leave the tube on the laboratory bench for 10 minutes at room temperature. This mixture contains the second ribonucleoprotein complex (RNP2) for depletion of the human, mouse, and rat rRNA.

F.4. Second CRISPR digestion for the depletion of human, mouse, and rat rRNA

20. Add the product of the first CRISPR digestion (25 µL from F.2.14) to a tube containing the RNP2 generated in the previous step (F.3.19) at room temperature.

Component	Volume
Product of first CRISPR digestion (from F.2.14)	25 µL
Second ribonucleoprotein complex RNP2 (from F.3.19)	10 µL
Total Volume	~35 µL

21. Mix the contents gently by flicking the tube or pipetting up and down.
22. Centrifuge briefly at room temperature to collect the contents at the bottom of the well.
23. Incubate the tube or plate at 37°C for 60 minutes with heated lid turned on (≥50°C).

IMPORTANT: While the reaction incubates, remove the AMPure® XP beads from the refrigerator and place them on the laboratory bench to bring them to room temperature. A minimum of 30 minutes at room temperature is recommended before use.

24. After the incubation is complete, transfer the tube or plate to ice for ~ 2 minutes.
25. Centrifuge briefly at room temperature to collect the contents at the bottom of the well and **proceed to F.5. Size Selection**.

F.5. Size Selection

26. Add 15 µL of CRISPRclean Nuclease-Free Water to the product from the previous step F.4.25.
27. Mix gently by pipetting the solution up and down several times.
28. Place the tube on the laboratory bench at room temperature.
29. Add 30 µL of well-resuspended, room-temperature AMPure® XP beads, pipette up and down several times to mix and incubate the tube at room temperature for 10 minutes. Mix the sample after 5 minutes by pipetting up and down several times.
30. Place the tube on the magnetic stand.
31. Allow the solution to clear (3 – 5 minutes) and remove and discard the supernatant without disturbing the beads.
32. Add 200 µL of freshly prepared 80% ethanol to the tube.
33. After 30 seconds, remove and discard the ethanol. Leave the tube on the magnetic stand during this step.
34. Repeat the wash step with 200 µL of 80% ethanol. Remove as much ethanol as possible after the second wash.
35. Open the cap of the tube while it is on the magnetic stand and allow the beads to air dry for 5 minutes.
36. Add 23 µL of CRISPRclean Resuspension Buffer to the beads.
37. Remove the tube from the magnetic stand.
38. Mix the contents by pipetting up and down several times to fully resuspend the beads in the liquid.
39. Incubate the tube at room temperature for 10 minutes. Mix the sample again after 5 minutes by pipetting up and down several times.
40. Place the tube on the magnetic stand. Allow the solution to clear.
41. Transfer the supernatant which contains the eluted DNA to a new PCR tube. This product is the rRNA depleted and adapter ligated DNA sample.

NOTE: The procedure may be stopped at this point and the samples stored at -20°C.

42. Proceed to **Step G: PCR amplification**.

Step G: PCR amplification

Hands-on time: 40 min | Total time: 60 min

Materials provided

- (green) - CRISPRclean PCR Primer Mix
- (green) - CRISPRclean PCR Master Mix
- (white or clear) - CRISPRclean Resuspension Buffer
- (white or clear) - CRISPRclean Library Prep Cleanup Beads (room temp)

Required materials provided by the user

- rRNA depleted and adapter ligated DNA (from Step F)
- Thermal cycler
- Adhesive PCR plate seal
- 96 well PCR plate
- 80% ethanol, freshly prepared (room temp)
- Magnetic stand

1. For each sample, combine the following reagents **on ice** in a tube or 96 well PCR plate:

Component	Volume
rRNA depleted and adapter ligated DNA (from Step F.5.42.)	23 µL
CRISPRclean PCR Master Mix	25 µL
CRISPRclean PCR Primer Mix	2 µL
Total Volume	50 µL

2. Mix thoroughly by pipetting the solution up and down several times.
3. Place the tube in a thermal cycler with the cycling parameters below.

NOTE: The number of cycles will vary depending on the amount of the sample. Choose the number of cycles from the table based on the amount of input RNA. Always use the least number of cycles possible. Further optimization may be necessary.

Total RNA Input	PCR Cycles
5 ng	19
10 ng	17
25 ng	16
50 ng	15
100 ng	14

Temp	Cycle time	
98°C	30 sec	
98°C	15 sec	Use the table on the left to determine the number of PCR cycles based on the total RNA input amount used.
65°C	30 sec	
72°C	30 sec	
72°C	2 min	
4°C	HOLD	

4. After the reaction is completed, add 40 µL of well mixed CRISPRclean Library Prep Cleanup Beads to each sample. Mix thoroughly by pipetting up and down several times.
5. Incubate for 5 minutes at room temperature.
6. Place the tube or plate on the magnetic stand for 5 minutes or until the solution is clear.
7. Remove and discard the supernatant without disturbing the beads.
8. Add 200 µL of freshly prepared 80% ethanol, incubate for 30 seconds, and remove the supernatant. Repeat this step once for a total of 2 ethanol washes.

IMPORTANT: Use freshly prepared 80% ethanol. Do not incubate the bead pellet with 80% ethanol for extended periods.

9. Air dry the samples for 3 minutes. After 1 minute, remove all residual liquid that may have collected at the bottom of the well.
10. Repeat the bead clean-up once. Remove the tube or plate from the magnetic stand and resuspend the bead pellet in 50 µL of CRISPRclean Resuspension Buffer by pipetting the volume up and down several times. Ensure that the beads are completely resuspended.
11. Add a second volume of 40 µL of well mixed CRISPRclean Library Prep Cleanup Beads to the tube or each well containing sample. Mix thoroughly by pipetting up and down several times.
12. Incubate for 5 minutes at room temperature.
13. Place the tube or plate on the magnetic stand for 5 minutes or until the solution is clear.
14. Remove and discard the supernatant.
15. Add 200 µL of freshly prepared 80% ethanol, incubate for 30 seconds, and remove all the supernatant. Repeat this step once for a total of 2 ethanol washes.

IMPORTANT: Use only freshly prepared 80% ethanol. Do not incubate the bead pellet with 80% ethanol for extended periods.

16. Air dry the samples for 3 minutes. After 1 minute, remove all residual liquid that may have collected at the bottom of the well.

17. To elute the DNA, remove the tube or plate from the magnetic stand and resuspend the bead pellet in 15 µL of CRISPRclean Resuspension Buffer by pipetting up and down. Ensure complete suspension of the beads.
18. Incubate the sample for 2 minutes at room temperature.
19. Place the plate on the magnetic stand for 3 minutes or until the supernatant appears clear.
20. Transfer the full volume of the supernatant which contains the depleted library to a new tube or PCR plate.
21. Examine libraries on an Agilent BioAnalyzer® 2100 instrument or equivalent to ensure proper library sizing and to verify exclusion of contaminating small and large fragments. Examples of Bioanalyzer® graphs with expected library size distributions are provided in the Library Validation section.

The library is now ready for cluster generation according to standard Illumina® protocols. Proceed to cluster generation or store the library at -20°C. qPCR quantification is recommended to quantify DNA library templates for optimal cluster density.

Library validation

Jumpcode Genomics recommends assessing the quality, quantity, and size distribution of the library on an Agilent Bioanalyzer® 2100 instrument or equivalent before sequencing for best results.

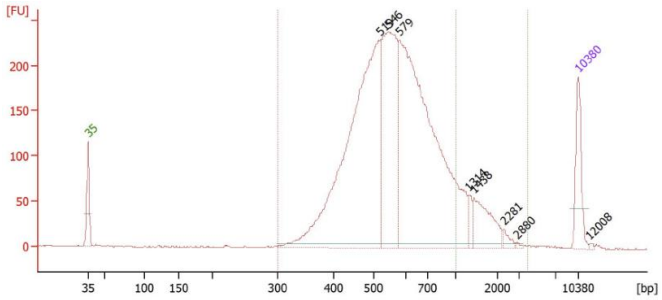

Figure 1: Example of an expected library size distribution using 10 ng of total RNA from nasopharyngeal sample. An aliquot of the library was loaded on the Agilent BioAnalyzer® using the Agilent High Sensitivity DNA Kit.

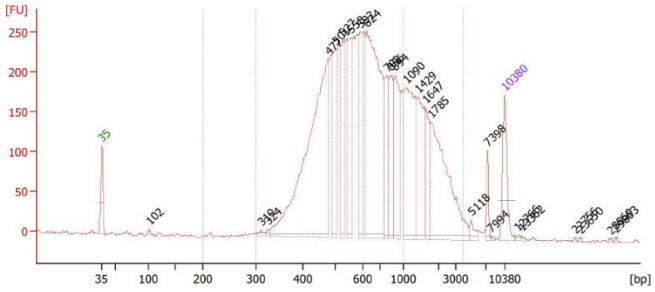

Figure 2: Example of a large library size distribution using 10 ng of total RNA from nasopharyngeal sample. An aliquot of the library was loaded on the Agilent BioAnalyzer® using the Agilent High Sensitivity DNA Kit.

UDI Barcoded Primer Plate Format

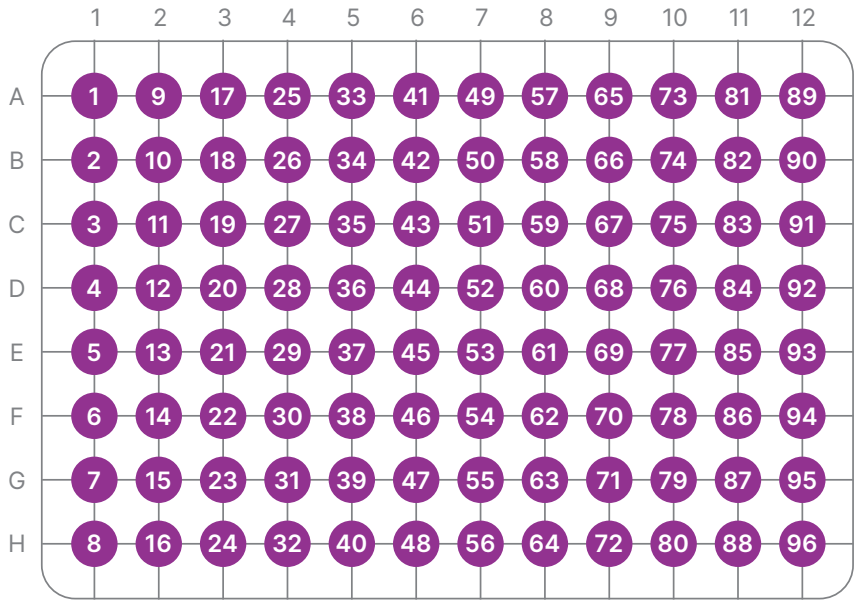

Figure 3: Representative plate layout of UDI Barcoded Primers 1-96.

Low level multiplexing guidelines

Barcodes 1 and 2, 13 and 14, 25 and 26, 37 and 38, 49 and 50, 61 and 62, 73 and 74, and 85 and 86 are fully color balanced and are suitable to be used in a pool of two libraries. When designing low-plexity index pools (< 4 libraries), always include two libraries barcoded with a set of two unique and fully color balanced barcodes to avoid laser color complexity issues during sequencing. Additional libraries may be safely multiplexed with one set of fully color balanced barcodes in a pool.

Instructions for entering index sequences for Illumina® platforms

Visit the Illumina® website for the latest guidelines, software, and training recommendations for the use of compatible Illumina® instruments.

IMPORTANT: The adapter plate contains only the CRISPRclean UDI barcoded adapters. PCR primers for amplification of the library are provided in the kits for library preparation:

- NOVA-5229950 CRISPRclean Stranded Total RNA Prep with rRNA Depletion (Human, Mouse, Rat)
- NOVA-5229960 CRISPRclean Plus Stranded Total RNA Prep with rRNA Depletion (Human, Mouse, Rat, Pan Bacteria).

	P7 index	P5 Index	P5 Index Reverse Complement
UDI0001	AATCGTTA	AATAACGT	ACGTTATT
UDI0002	GTCTACAT	TTCTTGAA	TTCAAGAA
UDI0003	CGCTGCTC	GGCAGATC	GATCTGCC
UDI0004	GATCAACA	CTATGTTA	TAACATAG
UDI0005	CGAAGGAC	GTTGACGC	GCGTCAAC
UDI0006	GATGCCGG	ATCTACGA	TCGTAGAT
UDI0007	CTACGAAG	CTCGACAG	CTGTCGAG
UDI0008	GATGCGTC	GAGGCTGC	GCAGCCTC
UDI0009	CTACGGCA	CCTCGTAG	CTACGAGG
UDI0010	GATTCCTT	CATAGGCA	TGCCTATG
UDI0011	CTACTCGA	AGATGAAC	GTTCATCT
UDI0012	GATTCGAG	CCGAGTAT	ATACTCGG
UDI0013	AATCGGCG	AATATTGA	TCAATATT
UDI0014	TTCGCCGA	GTATACCG	CGGTATAC
UDI0015	CTGGCCTC	GATCCAAC	GTTGGATC
UDI0016	GAACTTAT	AGATACGC	GCGTATCT
UDI0017	CGTATTGG	GGTATCTT	AAGATACC
UDI0018	GAAGCACA	CCTCTGGC	GCCAGAGG
UDI0019	CTTAATAC	CCATTGTG	CACAATGG
UDI0020	GAAGTCTT	ACTACGGT	ACCGTAGT
UDI0021	GAAGAGGC	AAGTGCTA	TAGCACTT
UDI0022	CGGATAAC	GCCGAACG	CGTTCGGC
UDI0023	GAATCTGG	TGTCCACG	CGTGGACA
UDI0024	CTGATTGA	GACACACT	AGTGTGTC
UDI0025	AATCCGTT	AATATGCT	AGCATATT
UDI0026	TGCGTACA	TTCTCATA	TATGAGAA
UDI0027	GAATCAAT	TCTGTGAT	ATCACAGA
UDI0028	TGAGTCAG	CCGAACTT	AAGTTCGG
UDI0029	GAATGCTC	GTCTAACA	TGTTAGAC
UDI0030	GAATATCC	GACGCCAT	ATGGCGTC
UDI0031	CTTATGAA	GCCAATGT	ACATTGGC
UDI0032	TCGGCACC	CCAACGTC	GACGTTGG
UDI0033	AAGAAGCG	GTAGATAA	TTATCTAC
UDI0034	CTCACGAT	CTTACGGC	GCCGTAAG
UDI0035	TCGGTCGA	CCAAGTGC	GCACTTGG
UDI0036	TCGGTAAG	CTAACTCA	TGAGTTAG
UDI0037	AAGATACA	AATATCTG	CAGATATT
UDI0038	GTCGCTGT	TTATATCA	TGATATAA
UDI0039	TCGGATGT	CTGCGGAT	ATCCGCAG

	P7 index	P5 Index	P5 Index Reverse Complement
UDI0040	CGAGCCGG	GCGGCTTG	CAAGCCGC
UDI0041	CGATTATC	GAGTTGAT	ATCAACTC
UDI0042	TCGAAGCT	GCACTGAG	CTCAGTGC
UDI0043	CTATCATT	GACCACCT	AGGTGGTC
UDI0044	CGCGCCAA	TGGCTAGG	CCTAGCCA
UDI0045	CGAACGGA	CCTACCGG	CCGGTAGG
UDI0046	CTACTGAC	GGAGGATG	CATCCTCC
UDI0047	TCTTAAGT	CGCTGAAT	ATTCAGCG
UDI0048	TTAGAGTC	TGTGACGA	TCGTCACA
UDI0049	AAGACGAA	AATAGATT	AATCTATT
UDI0050	TTATTATG	TTAGCGCA	TGCGCTAA
UDI0051	CGCTATTA	GCGGCCGT	ACGGCCGC
UDI0052	TCTATCAG	CAGTAACC	GGTTACTG
UDI0053	CGGTGGTA	GCCTAGTA	TACTAGGC
UDI0054	TCACCAAT	CACGGCGC	GCGCCGTG
UDI0055	CTGGAAGC	GGTGCAGA	TCTGCACC
UDI0056	TCCTCGAT	GTAACTGC	CGAGTTAC
UDI0057	AAGAGAGC	CAGCCAGT	ACTGGCTG
UDI0058	TCAACGAG	CGTCAACC	GGTTGACG
UDI0059	TGCGAGAC	GCCGGCGA	TCGCCGGC
UDI0060	CCTGGTGT	GCCTCCGG	CCGGAGGC
UDI0061	AAGTAAGT	AATAGTCC	GGACTATT
UDI0062	TGACTGAA	TTAGACGT	ACGTCTAA
UDI0063	AAGACTGT	GTGGACTA	TAGTCCAC
UDI0064	CAATGATG	CACGGACG	CGTCCGTG
UDI0065	CACAGTAA	CACTAGAG	CTCTAGTG
UDI0066	TGGTCATT	GCAGATGG	CCATCTGC
UDI0067	CAACCGTG	CTCTCACG	CGTGAGAG
UDI0068	TGGTGCAC	GGAATCAC	GTGATTCC
UDI0069	CCACAATG	CGTTGACG	CGTCAACG
UDI0070	TGTGTGCC	CATCAGGT	ACCTGATG
UDI0071	CACCACGG	CGTTGTAA	TTACAACG
UDI0072	TGTGTTAA	GGCACGGT	ACCGTGCC
UDI0073	AAGTTATC	AATAGCAA	TTGCTATT
UDI0074	GTACAGCT	TGATCGGT	ACCGATCA
UDI0075	CAACTGCT	AGTAGTAT	ATACTACT
UDI0076	CATGATGA	GTTAGAGG	CCTCTAAC
UDI0077	TGACTACT	CCTTACAG	CTGTAAGG
UDI0078	CAGAAGAT	GTACATTG	CAATGTAC

	P7 index	P5 Index	P5 Index Reverse Complement
UDI0079	TGAGGCGC	GGAGACCA	TGGTCTCC
UDI0080	CAGGTTCC	CGAACACC	GGTGTTCG
UDI0081	TGAACAGG	GAGAACAA	TTGTTCTC
UDI0082	CAGTGTGG	TGTGAATC	GATTCACA
UDI0083	TTCCACCA	GGTTAAGG	CCTTAACC
UDI0084	CCGCTGTT	AGACCGCA	TGCGGTCT
UDI0085	AAGTTGGA	AATACAGG	CCTGTATT
UDI0086	GGACAACG	TGATGGCC	GGCCATCA
UDI0087	TTCGAACC	TGTCACCT	AGGTGACA
UDI0088	CAGACCAC	GCTTCGGC	GCCGAAGC
UDI0089	TTCTGGTG	CCAGTGGT	ACCACTGG
UDI0090	CAATCGAA	GCACACGC	GCGTGTGC
UDI0091	AAGTACAG	GTCACGTC	GACGTGAC
UDI0092	CCGTGCCA	GCAGCTCC	GGAGCTGC
UDI0093	CATTGCAC	CATGCAGC	GCTGCATG
UDI0094	TTACCTGG	ACGATTGC	GCAATCGT
UDI0095	CTGCAACG	GACATTCG	CGAATGTC
UDI0096	TACTGTTA	GCGAATAC	GTATTCGC