USER MANUAL

DepleteX™ Nasopharyngeal Microbial RNA Boost Kit (24 Samples)

For Illumina-compatible sequencing

Contents

Contact Us

If you have any questions, contact Technical Support at support@jumpcodegenomics.com

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Product overview

The DepleteX™ Nasopharyngeal Microbial RNA Boost Kit (KIT1039) used inconjuction with the CRISPRclean™ Plus Stranded Total RNA Prep with rRNA Depletion Kit (KIT1016) is designed to prepare strand-specific RNA libraries depleted of human and bacterial ribosomal RNA (rRNA) and abundant human nasopharyngeal mRNA for sequencing on Illumina® instruments. The protocol is meant for total cellular RNA isolated from nasopharyngeal samples. Protocol time is less than 2 days.

This kit contains the reagents required to process a nasopharyngeal sample from total RNA through to fully depleted NGS library. The CRISPRclean Unique Dual Index (UDI) Adapter Plate for RNA Prep (Set A) is a required component but is sold separately. The 96 unique dual index barcoded adapters allow for high-throughput, multiplexed sequencing.

Description	For whole-transcriptome sequencing
Assay time	13 hours
Hands-on time	4.5 hours
Nucleic acid	RNA
Tissue type	Human nasopharyngeal
Input quantity of total RNA	5 ng to 100 ng
Method	RNA sequencing
Depletion mechanism	CRISPR Cas mediated
Strand specificity	> 98% directional
Multiplexing	Up to 96 Unique Dual Indexes using CRISPRclean Unique Dual Index Adapter Plate for RNA Prep (Set A): KIT1017
Designed to deplete	Human 5S, 5.8S, 18S and 28S rRNA genes, 45S rRNA precursor, mitochondrial 12S and 16S rRNA genes 5S, 16S, and 23S rRNA genes from 212 bacteria representing most bacterial phyla Abundant human nasopharyngeal mRNA
Compatibility	Short read sequencing instruments, such as Illumina® instrumentation
Primary use	High sensitivity detection of respiratory viruses with RNA genomes

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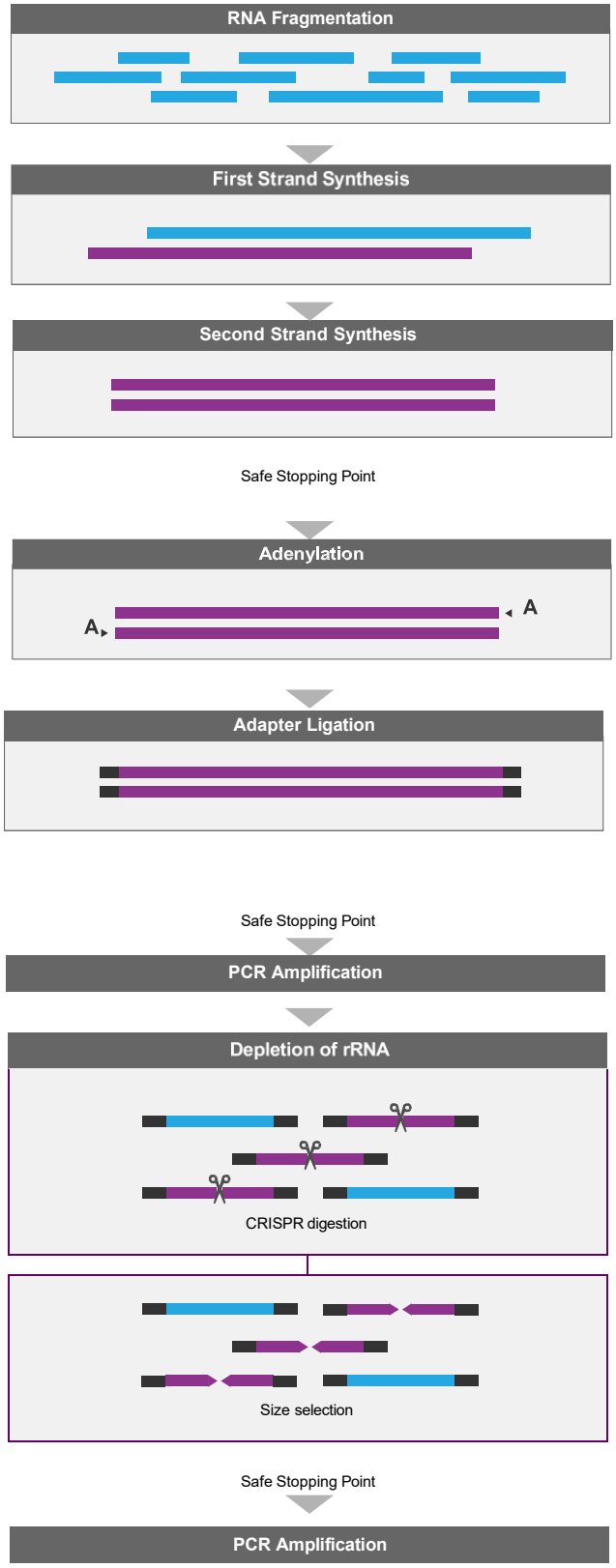

Human RNA Depletion workflow

The streamlined workflow for library preparation from total RNA involves eight main steps: RNA fragmentation, first strand synthesis, second strand synthesis, adenylation, adapter ligation, PCR amplification, depletion and final PCR amplification. The workflow begins with optional fragmentation of the RNA at high temperature. This is followed by first and second strand synthesis, which convert the RNA fragments to double-stranded cDNA. > 98% strand specificity is achieved through incorporation of dUTP during second strand synthesis. Adenylation modifies the 3' ends of the stranded cDNA to prepare the library for adapter ligation. Once unique dual indexed adapters are ligated to the ends of the DNA fragments and a few cycles of amplification are performed, the library is ready for depletion.

The depletion of rRNA and abundant mRNA occurs with the use of the CRISPR Cas9 double-stranded endonuclease and associated, programmable CRISPR guide RNA. Depletion is performed in two successive steps: bacterial and human rRNA are cleaved in the first step and abundant human mRNA is cleaved in the second step. Subsequent bead-based size selection removes fragmented material. Post-depletion PCR amplification ensures that only uncleaved fragments, i.e., those containing sequences of interest, are amplified further. A final bead-based cleanup step ensures that the library is ready for sequencing.

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Kit contents, storage, and shelf life

The The DepleteX™ Nasopharyngeal Microbial RNA Boost Kit (KIT1039) used inconjuction with the CRISPRclean™ Plus Stranded Total RNA Prep with rRNA Depletion Kit (KIT1016) contains enough material to prepare twenty-four RNA-Seq libraries for Illumina® compatible sequencing. The shelf life of all reagents is six months from the date of manufacturing when stored properly.

The kit contains the following reagents to be stored at the temperatures indicated in the table below: The CRISPRclean Unique Dual Index Adapter Plate for RNA Prep (KIT1017) (sold separately) is also required for library preparation 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 bag: 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

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Cleanup beads bag: ASY1055-001

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

Add On: Depletion box: ASY1088-001

Kit contents	Cap color	Storage temp
CRISPRclean Cas9	Purple	-20°C
CRISPRclean 10X Cas9 Buffer	Purple	-20°C
CRISPRclean RNase Inhibitor	Purple	-20°C
CRISPRclean Nuclease-free Water *	White or Clear	*4°C

* Note: Store in 4°C after first use.

Add On: Guide RNA bag: ASY1073-001

Kit contents	Cap color	Storage temp
CRISPRclean Guide RNA (NP Microbial Boost Guide Set 1)	White or Clear	-80°C
CRISPRclean Guide RNA (NP Microbial Boost Guide Set 2)	White or Clear	-80°C

Required materials provided by the user

Reagents

- Total RNA
- CRISPRclean Unique Dual Index Adapter Plate for RNA Prep (Set A): KIT1017
- 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

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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. If you need further assistance, contact support@jumpcodegenomics.com.
- Do not use the kit beyond 6 months from the date of manufacturing.
- The CRISPRclean First Strand Synthesis Mix may appear yellow in color.
- The CRISPRclean® Plus Stranded Total RNA Prep with Microbial RNA Boost (Nasopharyngeal) Kit is intended to be used with the CRISPRclean Unique Dual Index Adapter Plate for RNA Prep (Set A): KIT1017.
- Maintain a laboratory temperature of 20°–25°C (68°–77°F) for optimum use of the kit.
- 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 goes back into solution. The performance of the buffer is not affected by this occurrence.
- Use properly calibrated pipettes as library preparation is sensitive to pipetting error.
- Vortex and briefly 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 the 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 the guide RNA.
- Thermal cycling should be performed with a heated lid except where specified.
- Do not allow the temperature of the CRISPRclean Unique Dual Index Adapter Plate to rise above room temperature.
- For multiplexing options, please use CRISPRclean Unique Dual Index Adapter Plate for RNA Prep (Set A), KIT1017 during STEP E: Adapter Ligation.
  - Once the plate has thawed, spin it for one minute in the centrifuge 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 by placing them on the laboratory bench for ~30 minutes before use.
- Vortex beads immediately before use to ensure they are in a uniform suspension.
- Do not freeze CRISPRclean Library Prep Cleanup Beads and AMPure® XP Beads.
- Maintain beads in liquid suspension during storage and handling.
- Ensure that beads pellet on the magnet before attempting to remove the clear supernatant.
- Completely remove 80% ethanol before eluting the RNA. This might require a fine pipet tip.

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Revision log

Version	Date	Description
V1.0	December 2023	Product launch

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Library prep setup

Starting materials

The CRISPRclean® Plus Stranded Total RNA Prep with DepleteX™ Nasopharyngeal Microbial RNA Boost has been optimized and validated for 5 ng to 100 ng of total RNA isolated from human nasopharyngeal samples that contain a mix of human and bacterial content.

Before beginning the 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.

Measure RNA concentration with a fluorometric method for accurate input quantification. The recommended RNA input quantity is between 5 ng and 100 ng and the highest rRNA depletion rates are achieved within this input range. Lower amounts of starting material may result in higher duplication rates, reduced library complexity, and other negative impacts on data quality.

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

Efficiency of depletion is dependent on accurate quantification, sample quality and sample type.

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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. Place the tube or plate in the thermal cycler and run the program.

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.

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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 or plate in a thermal cycler programmed with the cycling parameters below. Run the program.

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.

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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 96-well 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 switched off.
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.

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9. Keeping the tube or 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 tube or 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.

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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 incubate with the following parameters:

Temp	Cycle time
65°C	30 min
4°C	HOLD

4. Proceed to Step E: Adapter Ligation.

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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)
- CRISPRclean Unique Dual Index Adapter Plate for RNA Prep (KIT1017)
- 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	¼
25 ng	2.08 uM	1/3
50 ng	3.12 µM	½
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.

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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
CRISPRclean Unique Dual Index Plate (Set A): (two unique barcodes per sample; ensure appropriate 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. However, to prevent excess adapter dimer formation, do not premix the adapters.

2. Mix thoroughly by pipetting up and down several times.
3. Place the tube or plate in a thermal cycler programmed with the following cycling parameters (heated lid not necessary):

Temp	Cycle time
20°C	15 min
4°C	HOLD

4. Run the thermal cycler.
5. Add 65 µL of CRISPRclean Nuclease-free Water and 35 µL of well mixed CRISPRclean Library Prep Cleanup Beads to each tube or 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 the supernatant. Repeat this step once more 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 tube or 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 tube or well containing sample. Mix thoroughly by pipetting up and down.

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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 more 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. Air dry the sample for 3 minutes. After 1 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 in the liquid.
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 96-well plate.

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

22. Proceed to Step F: PCR Amplification.

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Step F: 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 PCR tube or 96-well PCR plate:

Component	Volume
Adapter ligated DNA (from Step E.21.)	15 µL
CRISPRclean PCR Master Mix	14 µL
CRISPRclean PCR Primer Mix	1 µL
Total Volume	30 µL

2. Mix thoroughly by pipetting the solution up and down several times.

3. Place the tube or plate in a thermal cycler with the cycling parameters below.

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Temp	Cycle time	
98°C	30 sec	
98°C	15 sec	5 cycles
65°C	30 sec	
72°C	30 sec	
72°C	2 min	
4°C	HOLD	

4. After the reaction is complete, add 20 µL of CRISPRclean Nuclease-Free Water. Mix thoroughly by pipetting up and down several times.
5. Add 45 µL of well mixed CRISPRclean Library Prep Cleanup Beads to the sample. Mix thoroughly by pipetting up and down several times.
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 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.

10. Air dry the samples for 3 minutes. After 1 minute, remove all residual liquid that may have collected at the bottom of the tube or 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 the volume up and down several times. Ensure that the beads are completely resuspended.
12. Add a second volume of 45 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.
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.
16. 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.

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

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18. To elute the DNA, 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 complete suspension of the beads.
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 to a new tube or PCR plate.

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

22. Proceed to Step G: Depletion

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Step G: Depletion

☐ **Hands-on time: 60 min | Total time: 510 min**

Materials provided

(blue) - CRISPRclean Guide RNA (Pan Bacteria)

(blue) - CRISPRclean Guide RNA (Human, Mouse, Rat)

(white or clear) CRISPRclean Guide RNA (Microbial Boost Guide Set 1)

(white or clear) CRISPRclean Guide RNA (Microbial Boost Guide Set 2)

(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

G.1. First ribonucleoprotein complex formation (RNP1) for depletion of bacterial and human rRNA

1. Allow the CRISPRclean Guide RNA (Pan Bacteria) and CRISPRclean Guide RNA (Human, Mouse, Rat) to thaw on ice.
2. Each library requires 4.0 µL of CRISPRclean Guide RNA (Pan Bacteria) and 3.6 µL of CRISPRclean Guide RNA (Human, Mouse, Rat) For more than one library, multiply the volume of each guide RNA by the number of libraries to be depleted.
3. Transfer the calculated total Pan-Bacteria guide RNA volume into a new PCR tube. Make sure to pipette 1 extra reaction from the calculated number to account for potential evaporation/pipetting error.

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

4. Transfer the CRISPRclean Guide RNA (Pan Bacteria) to a pre-heated thermal cycler and incubate at 65°C for 2 minutes. Immediately transfer the tube to ice for 3 minutes.

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NOTE: Leave the tube containing CRISPRclean Guide RNA (Human, Mouse, Rat) on ice.

5. Briefly spin the tubes in a microcentrifuge to collect the contents at the bottom of each tube. Return the tube containing the CRISPRclean guide to ice.
6. Assemble the following reagents in a new PCR tube at room temperature in the order given below. The volumes in the table are for one library.

IMPORTANT: The components below can be assembled as a Master Mix, however, ***do not*** perform the room temperature incubation as Master Mix. Add 1-1.5 extra reaction worth of reagents if making master mix for RNP.

Component	Volume
CRISPRclean 10X Cas9 Buffer	1.5 µL
CRISPRclean Cas9	4.8 µL
CRISPRclean RNase Inhibitor	1.0 µL
CRISPRclean Guide RNA (Pan Bacteria) – pre-heated	4.0 µL
CRISPRclean Guide RNA (Human, mouse, rat)	3.6 µL
Total Volume	14.9 µL

NOTE: Place the Cas9 and RNase Inhibitor reagents on ice or in a benchtop cooler and return the stocks to -20°C immediately after use. Allow the 10X Cas9 Buffer to thaw on ice. Keep on ice for subsequent preparation of RNP2. Return the Guide RNA stock (Human, Mouse, Rat) to the -80°C freezer immediately after use.

7. Mix the contents gently by carefully pipetting up and down several times. Centrifuge briefly to collect the contents at the bottom of the tube.
8. Aliquot 14.9 µL of the master mix into a new PCR tube for each reaction. Repeat for the number of reactions the master mix was originally assembled for.
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). Proceed immediately to Step G.2: First CRISPR digestion.

G.2. First CRISPR digestion for depletion of bacterial and human rRNA

IMPORTANT: If the adapter-ligated DNA was frozen at the safe stopping point at the end of Step F, thaw the DNA before undertaking this step

11. To the plate or tube containing the RNP1, add the adapter-ligated DNA (from Step F), add 1.5 µL of 10X Cas9 Buffer as detailed below:

Component	Volume
First ribonucleoprotein complex (RNP1 from G.1.10)	14.9 µL
Adapter-ligated amplified DNA (from Step F)	15.0 µL

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CRISPRclean 10X Cas9 Buffer	1.5 µL
Total Volume	31.4 µL

12. Mix the contents gently by pipetting up and down several times.
13. Centrifuge briefly to collect the contents at the bottom of the tube or plate.
14. Incubate the tube or plate in a thermal cycler at 42°C for 60 minutes, followed by 65°C for 5 minutes, hold at 4°C (heated lid should be set to ≥80°C). After the incubation is complete, transfer the tube or plate to ice. This contains the product of the first CRISPR digestion.

NOTE: Proceed to Step G.3. about 15 – 20 minutes before the end of this step.

G.3. Second ribonucleoprotein complex formation (RNP2) to deplete abundant human mRNA and less abundant human and bacterial rRNA

15. Remove the tubes of CRISPRclean Guide RNA (Microbial Boost Guide Set 1 and Guide Set 2) from the -80°C freezer and allow the contents to thaw on ice. **Dilute 1 µL of CRISPRclean Guide RNA (Guide Set 2) with an equal amount of Nuclease-Free Water.**
16. Each library requires 2.41 µL of CRISPRclean Guide RNA (Microbial Boost Guide Set 1) and 1.44 µL of diluted CRISPRclean Guide RNA (Microbial Boost guide set 2). For more than one library, multiply the volume of each guide RNA by the number of libraries to be depleted. Add an additional one reaction to compensate for pipetting error.
17. Place the tube containing Microbial Boost Guide Set 2 in a pre-heated thermal cycler and incubate at 65°C for 2 minutes. Immediately transfer the tube to ice for 3 minutes. Include 2 extra reactions worth of GS2 guides while pre-heating to account for any potential evaporation/pipetting error.

NOTE: Leave the tube containing CRISPRclean Guide RNA (Microbial Boost Guide Set 1) on ice.

18. Briefly spin the tubes in a microcentrifuge to collect the contents at the bottom of each tube. Return the tube to ice.
19. Assemble the following reagents in a new tube at room temperature in the order given below:

IMPORTANT: the components below can be assembled as a Master Mix, however ***do not*** perform the room temperature incubation as Master Mix.

Component	Volume
CRISPRclean Nuclease-Free water	2.0 µL
CRISPRclean 10X Cas9 buffer	1.0 µL
CRISPRclean Cas9	1.82 µL
CRISPRclean RNase Inhibitor	1.0 µL
CRISPRclean Guide RNA (Microbial Boost Guide Set 1)	2.41 µL
CRISPRclean Guide RNA (Microbial Boost Guide Set 2)	1.44 µL
Total Volume	9.67 µL

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

IMPORTANT: Keep Cas9 on ice or in a benchtop cooler when in use. Return the enzyme to the -20°C freezer immediately after use. Thaw the guide RNA on ice and return it to the -80°C freezer immediately after use.

21. Aliquot 9.67 µL of the master mix into a new PCR tube for each reaction. Repeat for the number of reactions the master mix was originally assembled for.

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

23. Leave the tube on the laboratory bench for 10 minutes at room temperature. This mixture contains the second ribonucleoprotein complex (RNP2).

G.4. Second CRISPR digestion for the depletion of human mNA and less abundant human and bacterial rRNA

24. Add the product of the first CRISPR digestion (~31 µL from G.2.14) to the RNP2 (from step G.4.23):

Component	Volume
Product of first CRISPR digestion (from G.2.14)	31.4 µL
Second ribonucleoprotein complex RNP2 (from G.3.23)	9.67 µL
Total Volume	41.07 µL

25. Mix the contents gently by pipetting up and down.

26. Centrifuge briefly at room temperature to collect the contents at the bottom of the tube or well.

27. Incubate the tube or plate in a thermal cycler at 42°C for 4 hours, followed by 5 min at 65°C, hold at 4°C. Heated lid should be set to a temperature ≥ 80°C.

28. After the incubation is complete, transfer the tube or plate to ice for ~ 2 minutes.

29. Centrifuge briefly at room temperature to collect the contents at the bottom of the tube or well(s). Proceed to step G.5: Size Selection.

G.5. Size Selection

30. Add 9 µL of CRISPRclean Nuclease-Free Water to the product from the previous step (G.4.29).

31. Mix gently by pipetting the solution up and down several times.

32. Place the tube or plate on the laboratory bench at room temperature.

33. 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.

34. Place the tube or plate on the magnetic stand.

35. Allow the solution to clear (3 – 5 minutes) and remove and discard the supernatant without disturbing the beads.

36. Add 200 µL of freshly prepared 80% ethanol to the tube or wells of the plate.

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37. After 30 seconds, remove and discard the ethanol. Leave the tube or plate on the magnetic stand during this step.
38. Repeat the wash step with 200 µL of 80% ethanol. Remove as much ethanol as possible after the second wash.
39. Open the cap of the tube while it is on the magnetic stand and allow the beads to air dry for 5 minutes.
40. Add 23 µL of CRISPRclean Resuspension Buffer to the beads.
41. Remove the tube or plate from the magnetic stand.
42. Mix the contents by pipetting up and down several times to fully resuspend the beads in the liquid.
43. Incubate the tube or plate at room temperature for 10 minutes. Mix the sample again after 5 minutes by pipetting up and down several times.
44. Place the tube or plate on the magnetic stand. Allow the solution to clear.
45. Transfer the supernatant containing the eluted DNA to a new PCR tube or 96-well plate. This product is the depleted, adapter-ligated DNA sample.

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

46. Proceed to Step H: PCR amplification.

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Step H: 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 G)
- 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 in a tube or 96-well PCR plate on ice:

Component	Volume
rRNA-depleted and adapter-ligated DNA (from Step G.5.45)	23 µL
● CRISPRclean PCR Master Mix	25 µL
● CRISPRclean PCR Primer Mix	2 µL
Total Volume	50 µL

2. Mix thoroughly by pipetting the contents up and down several times.
3. Place the tube or plate in a thermal cycler with the cycling parameters below. Run the PCR.

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Temp	Cycle time	
98°C	30 sec	
98°C	15 sec	21 cycles
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. Perform this step once more 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. 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 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. Perform this step once more 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 tube or plate.

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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 or tube on the magnetic stand for 3 minutes or until the supernatant appears clear.
20. Transfer the full volume of the supernatant to a new tube or 96-well plate. The supernatant contains the final, depleted library.
21. Examine libraries on an Agilent BioAnalyzer® 2100 instrument or equivalent instrument to ensure that the fragment profile is consistent with expectation. Examples of typical Bioanalyzer® graphs are provided in the Library Validation section (below).

The library is now ready for cluster generation according to standard Illumina® sequencing protocols. Proceed to cluster generation or store the library at -20°C until sequencing is undertaken. 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 or similar instrument for best results when sequencing.

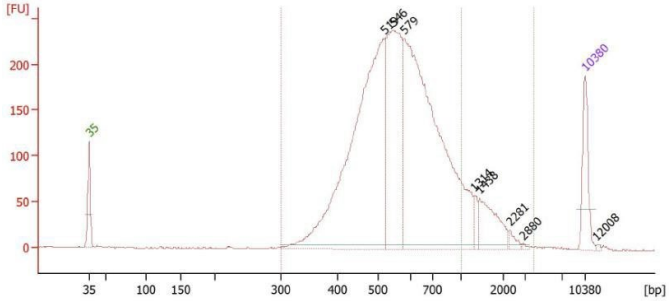

Figure 1: Example of an expected library size distribution using 10 ng of total RNA from nasopharyngeal sample. An aliquot of the library was loaded on the Agilent BioAnalyzer® using the Agilent High Sensitivity DNA Kit.

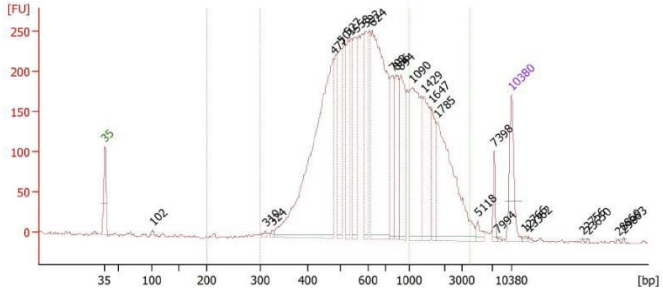

Figure 2: Example of a large library size distribution using 10 ng of total RNA from nasopharyngeal sample. An aliquot of the library was loaded on the Agilent BioAnalyzer® using the Agilent High Sensitivity DNA Kit.

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UDI Barcoded Primer Plate Format

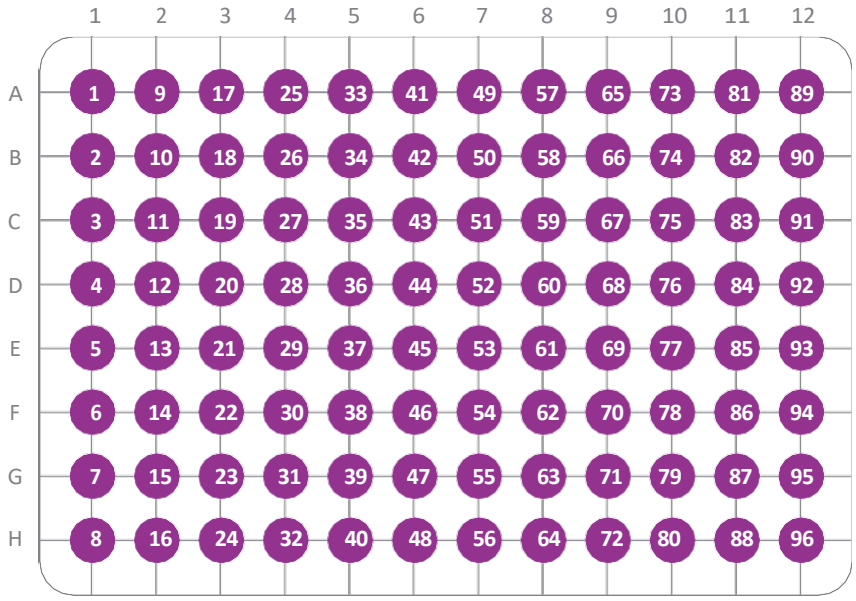

Figure 3: Representative plate layout of UDI Barcoded Primers 1-96.

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:

- KIT1014 CRISPRclean Stranded Total RNA Prep with rRNA Depletion (Human, Mouse, Rat)
- KIT1016 CRISPRclean Plus Stranded Total RNA Prep with rRNA Depletion (Human, Mouse, Rat, Pan Bacteria).

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	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

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	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

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	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

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