JUMPCODE GENOMICS

USER MANUAL

Stranded Total RNA Prep with Depletion

KIT1014 with KIT1022, KIT1024, KIT1025, and KIT1027

Contents

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Product overview

The Stranded Total RNA Prep with rRNA Depletion (Human, Mouse, Rat) is designed to prepare directional, strand specific RNA libraries and deplete human, mouse, and rat ribosomal RNA (rRNA) sequences in just 7.5 hours with ~3 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, depletion, and amplification for sequencing. The KIT1017 Unique Dual Index (UDI) Adapter Plate for RNA Prep (Set A) is a required reagent to be used with the Stranded Total RNA Prep with rRNA Depletion (Human, Mouse, Rat). The 96 unique dual index adapter barcode adapters allow for color-balanced and high-throughput, multiplexed sequencing.

Description	For whole-transcriptome sequencing
Assay time	7.5 hours
Hands-on time	~3 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 Unique Dual Index Adapter Plate for RNA Prep (Set A): KIT1017
Species	Human, mouse, rat
Designed to deplete	Human 5S, 5.8S, 18S and 28S rRNA genes, 45S rRNA precursor, mitochondrial 12S and 16S rRNA genes
Compatible Jumpcode RNA depletion panels	• KIT1022 RNA Depletion Panel (Liver) • KIT1024 RNA Depletion Panel (Globin) • KIT1025 RNA Depletion Panel (Insulin) • KIT1027 RNA Depletion Panel (Ribo, Mito Genes)
Sequencing platform	Short read sequencing instruments such as Illumina® instruments

CRISPRclean 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 or other high expressing genes of adapter ligated libraries. CRISPRclean depletion is performed in one incubation 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 and storage

The Unique Dual Index Adapter Plate for RNA Prep (KIT1017) is required for library preparation performed with this kit. Each kit contains enough material to prepare 24 RNA-seq libraries for Illumina® compatible sequencing. Each kit is made up of the following kit contents to be stored at the temperatures indicated in the table below:

Compatible Jumpcode products for use in this protocol:

- KIT1014 Stranded Total RNA Prep with rRNA Depletion (Human, Mouse, Rat): ASY1052, ASY1053, ASY1055
- KIT1022 RNA Depletion Panel (Liver): ASY1064, ASY1065
- KIT1024 RNA Depletion Panel (Globin): ASY1064, ASY1071
- KIT1025 RNA Depletion Panel (Insulin): ASY1064, ASY1072
- KIT1027 RNA Depletion Panel (Ribo, Mito Genes): ASY1064, ASY1068

Library prep and depletion box: ASY1052-001

Kit contents	Cap color	Part number	Quantity	Storage temperature
Fragmentation Buffer Mix	Brown	REA1010	1	-20°C
First Strand Synthesis Mix	Red	REA1011	1	-20°C
Reverse Transcriptase	Red	REA1012	1	-20°C
Second Strand Synthesis Mix	White or Clear	REA1013	1	-20°C
Adenylation Mix	Yellow	REA1014	1	-20°C
Adenylation Enzyme	Yellow	REA1015	1	-20°C
Ligase Mix	Orange	REA1016	1	-20°C
Ligase Enzyme	Orange	REA1017	1	-20°C
Cas9	Purple	REA1000	1	-20°C
10X Cas9 Buffer	Purple	REA1001	1	-20°C
RNase Inhibitor	Purple	REA1007	1	-20°C
PCR Master Mix	Green	REA1018	1	-20°C
PCR Primer Mix	Green	REA1019	1	-20°C
Nuclease-free Water *	White or Clear	REA1002	1	*4°C
Resuspension Buffer *	White or Clear	REA1020	1	*4°C

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

Guide RNA box: ASY1053-001

Kit contents	Cap color	Part number	Quantity	Storage temperature
Guide RNA (Human, Mouse, Rat) for rRNA Depletion	Blue	BLK1023	1	-80°C

Cleanup beads bag: ASY1055-001

Kit contents	Part number	Quantity	Storage temperature
Library Prep Cleanup Beads	REA1021	1 bottle	4°C

ASY-1064: Depletion reagents

8 samples per box

Kit contents	Part number	Quantity	Storage temperature
Cas9	REA1039	1	-20°C
10x Cas9 Buffer	REA1040	1	-20°C
RNase Inhibitor	REA1041	1	-20°C
Nuclease-Free Water	REA1042	1	-20°C
Tris Buffer	REA1043	1	-20°C
P5 Primer	REA1044	1	-20°C
P7 Primer	REA1045	1	-20°C
2X PCR Mix	REA1046	1	-20°C

ASY-1065 Guide RNA for Liver

24 samples per tube

Kit contents	Part number	Quantity	Storage temperature
Guide RNA for Liver	REA1048	1	-80°C

ASY-1068 Guide RNA for (Ribo, Mito Genes)

24 samples per tube

Kit contents	Part number	Quantity	Storage temperature
Guide RNA for RNA (Ribo, Mito Genes)	REA1051	1	-80°C

ASY-1071 Guide RNA for Globin

24 samples per tube

Kit contents	Part number	Quantity	Storage temperature
Guide RNA for Globin	REA1053	1	-80°C

ASY-1072 Guide RNA for Insulin

24 samples per tube

Kit contents	Part number	Quantity	Storage temperature
Guide RNA for Insulin	REA1054	1	-80°C

Required materials and equipment provided by the user

Type	Item	Supplier
Plastics	0.5 mL, 1.5 mL DNA LoBind Tubes	Eppendorf 022431021
	0.2 mL thin wall PCR tubes	General Lab Supplier
	Low-Retention Filtered Sterile Tips(10 µl, 20 µl, 200 µl and 1000 µl)	General Lab Supplier
	96-well PCR plate non-skirted	Phenix Research™, # MPS-499) or similar
	Adhesive PCR plate seal	BioRad®, # MSB1001
Reagents	Total RNA	n/a
	Unique Dual Index Adapter Plate for RNA Prep (Set A)	Jumpcode Genomics KIT1017
	AMPure XP Beads	Beckman Coulter A63881
	Absolute Ethanol, 200 Proof	General Lab Supplier
	Qubit dsDNA HS Assay Kit	ThermoFisher Scientific Q32854
Equipment	Single Channel Pipettes (10 µl, 20 µl, 200 µl, and 1000 µl)	General Lab Supplier
	Multichannel Pipettes (10 µl, 20 µl, and 200 µl)	General Lab Supplier
	Vortex Mixer	General Lab Supplier
	Microcentrifuge	General Lab Supplier
	PCR Magnetic Rack or Stand for use with tubes	General Lab Supplier
	Ice Bucket	General Lab Supplier
	PCR Thermal Cycler	General Lab Supplier
	Qubit Fluorometer	ThermoFisher Scientific Q33238
	Automated electrophoresis such as TapeStation	General Lab Supplier
	DNA analysis instrument, such as the Agilent 2100 Bioanalyzer® System	General Lab Supplier

Best Practices

- 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.
- The First Strand Synthesis Mix may appear yellow in color.
- The Stranded Total RNA Prep with rRNA Depletion (Human, Mouse, Rat) is intended to be used with the Unique Dual Index Adapter Plate for RNA Prep (Set A): KIT1017.
- 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 Unique Dual Index Adapter Plate above room temperature.
- For multiplexing options, please use Unique Dual Index Adapter Plate for RNA Prep (Set A), KIT1017 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 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
V2.0	January 2023	Additional depletion panels

Library prep setup

Starting materials

The Stranded Total RNA Prep with rRNA Depletion (Human, Mouse, Rat) has been optimized and validated for 5 ng to 100 ng of total RNA isolated from human, mouse, or rat samples.

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.

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

Protocol

Step A: RNA fragmentation

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

Materials provided

(brown) - Fragmentation Buffer Mix

(white or clear) - Nuclease-free Water

Required materials provided by the user

- Total RNA
- Nuclease-free microcentrifuge tube or plate
- Thermal cycler
- Adhesive PCR plate seal
- 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 Nuclease-free Water)	14 µL
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
10 min	94°C	8-10 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) First Strand Synthesis Mix
- (red) Reverse Transcriptase

Required materials provided by the user

- Fragmented RNA (from Step A)
- Thermal cycler
- Adhesive PCR plate seal
- 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 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
First Strand Synthesis Mix	4 µL
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) - Second Strand Synthesis Mix

◯ (white or clear) - Resuspension Buffer

◯ (white or clear) - 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 reagents **on ice** in a nuclease-free PCR tube or plate:

Component	Volume
First strand synthesis product (from Step B)	25 µL
◯ 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 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 then 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 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) - Adenylation Mix

(yellow) - 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
Adenylation Mix	15 µL
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) - Ligation Mix
- (orange) - Ligase Enzyme
- (white or clear) - Resuspension Buffer
- (white or clear) - Library Prep Cleanup Beads (room temperature)
- (white or clear) - Nuclease-free Water

Required materials provided by the user

- 50 µL Adenylated DNA (from Step D)
- 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	1/4
25 ng	2.08 uM	1/3
50 ng	3.12 µM	1/2
100 ng	6.25 µM	None

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

Important: Adapters in the KIT1017 Unique Dual Index Adapter Plate for RNA Prep (Set A) are provided at a concentration of 6.25 µM. Use 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
Ligation Mix*	44.5 µL
KIT1017 Unique Dual Index Plate (Set A): one unique barcode per sample, ensure proper concentration	2.5 µL
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 Nuclease-free Water and 35 µL of well mixed 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 then 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 Resuspension Buffer by pipetting up and down several times. Ensure that the beads are completely resuspended.
12. Add 45 µL of well mixed 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 then 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 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

Hands-on time: 50 min | Total time: 130 min

Materials provided

Varies - Guide RNA

(purple) - Cas9

(purple) - 10X Cas9 Buffer

(purple) - RNase Inhibitor

(white or clear) - Nuclease-free water

(white or clear) - 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. Ribonucleoprotein complex formation (RNP) for depletion

1. Remove the tube of Guide RNA from the -80°C freezer and allow it to thaw on ice.
2. Centrifuge briefly to collect the contents at the bottom of the tube. Return the tube to ice.

   Step F.1.3 of this section is split into sub-sections based on what guide RNA set you intend to use. Follow the appropriate sub-sections below:

   - Depletion of human, mouse, and rat rRNA (KIT1014)
   - Depletion of human, mouse, and rat rRNA (KIT1014) with either globin (KIT1024) or insulin (KIT1025)
   - Depletion of human, mouse, and rat rRNA (KIT1014) with either ribo-mito genes (KIT1027) or liver (KIT1022)

> **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.

> **Important:** Place the Cas9 on ice or in a benchtop cooler and return the stock to the -20°C freezer immediately after use. Thaw the Guide RNA on ice and return the stock to the -80°C freezer immediately after use.

Depletion of human, mouse, and rat rRNA (KIT1014)

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

Component	Volume
10X Cas9 Buffer	1.0 µL
Cas9	2.4 µL
RNase Inhibitor	1.0 µL
Guide RNA (Human, Mouse, Rat)	4.0 µL
Total Volume	**8.4 µL**

Depletion of human, mouse, and rat rRNA (KIT1014) with either globin (KIT1024) or insulin (KIT1025)

3. Dilute guide RNA for globin/insulin 1:5 in Nuclease-Free Water. Assemble the following reagents in a new PCR tube at room temperature in the order given below for each library:

Component	Volume
10X Cas9 Buffer	1.0 µL
Cas9	2.55 µL
RNase Inhibitor	1.0 µL
Guide RNA (Human, Mouse, Rat)	4.0 µL
Diluted Guide RNA for either globin (KIT1024) or insulin (KIT1025)	1.3 µL
Total Volume	**9.85 µL**

Depletion of human, mouse, and rat rRNA (KIT1014) with either ribo-mito genes (KIT1027) or liver (KIT1022)

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

Component	Volume
10X Cas9 Buffer	1.0 µL
Cas9	3.0 µL
RNase Inhibitor	1.0 µL
Guide RNA (Human, Mouse, Rat)	4.0 µL
Guide RNA for either “ribo-mito genes (KIT1027) or liver (KIT1022)	1.1 µL
Total Volume	**10.1 µL**

4. Mix the contents gently by flicking the tube or carefully pipetting up and down several times.
5. Centrifuge briefly to collect the contents at the bottom of the tube.
6. Leave the tube on the laboratory bench for 10 minutes at room temperature. This mixture contains the ribonucleoprotein complex (RNP).

F.2. CRISPR digestion

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

7. To the tube containing the ribonucleoprotein complex (RNP) from Step **F.1.6**, add the following reagents at room temperature:

Component	Depletion of human, mouse, and rat rRNA (KIT1014)	Depletion of human, mouse, and rat rRNA (KIT1014) with either globin (KIT1024) or insulin (KIT1025)	Depletion of human, mouse, and rat rRNA (KIT1014) with either ribo-mito genes (KIT1027) or liver (KIT1022)
Ribonucleoprotein complex (RNP from F.1.6)	8.4 µL	9.85 µL	10.1 µL
Adapter-ligated DNA (from Step E)	15.0 µL	15.0 µL	15.0 µL
10X Cas9 Buffer	1.5 µL	1.6 µL	1.6 µL
Total Volume	**~25.0 µL**	**26.45 µL**	**26.7 µL**

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. 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 CRISPR digestion, which is the depleted library.

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.

11. After the incubation is complete, transfer the tube or plate to ice for ~2 minutes.
12. Centrifuge briefly at room temperature to collect the contents at the bottom of the well.

F.3. Size Selection

13. Step F.3.13 of this section is split into sub-sections based on what guide RNA set you intend to use. Follow the appropriate sub-sections below:
    a. Depletion of human, mouse, and rat rRNA (KIT1014):
        i. Add 25 µL of Nuclease-Free Water to the product from the previous step F.2.12.
    b. Depletion of human, mouse, and rat rRNA (KIT1014) with either globin (KIT1024) or insulin (KIT1025):
        i. Add 23.55 µL of Nuclease-Free Water to the product from the previous step F.2.12.
    c. Depletion of human, mouse, and rat rRNA (KIT1014) with either ribo-mito genes (KIT1027) or liver (KIT1022):
        i. Add 23.3 µL of Nuclease-Free Water to the product from the previous step F.2.12.
4. Mix gently by pipetting the solution up and down several times.
5. Place the tube on the laboratory bench at room temperature.
6. 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.
7. Place the tube on the magnetic stand.
8. Allow the solution to clear (3 – 5 minutes) and remove and discard the supernatant without disturbing the beads.
9. Add 200 µL of freshly prepared 80% ethanol to the tube.
10. After 30 seconds, remove and discard the ethanol. Leave the tube on the magnetic stand during this step.
11. Repeat the wash step with 200 µL of 80% ethanol. Remove as much ethanol as possible after the second wash.
12. Open the cap of the tube while it is on the magnetic stand and allow the beads to air dry for 5 minutes.
13. Add 23 µL of Resuspension Buffer to the beads.
14. Remove the tube from the magnetic stand.
15. Mix the contents by pipetting up and down several times to fully resuspend the beads in the liquid.
16. Incubate the tube at room temperature for 10 minutes. Mix the sample again after 5 minutes by pipetting up and down several times.
17. Place the tube on the magnetic stand. Allow the solution to clear.
18. 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.

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

Step G: PCR amplification

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

Materials provided

- (green) - PCR Primer Mix
- (green) - PCR Master Mix
- (white or clear) - Resuspension Buffer
- (white or clear) - 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.3.)	23 µL
PCR Master Mix	25 µL
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	18
10 ng	16
25 ng	15
50 ng	14
100 ng	13

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 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 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 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 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.

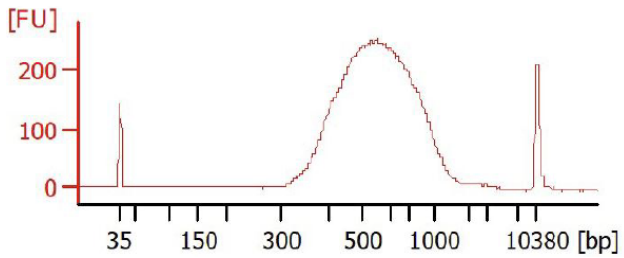

Figure 1: Example of an expected library size distribution using 5-100 ng of total RNA from Universal Human Reference RNA sample. An aliquot of the library was loaded on the Agilent BioAnalyzer® using the Agilent High Sensitivity DNA Kit.

UDI Barcoded Primer Plate Format

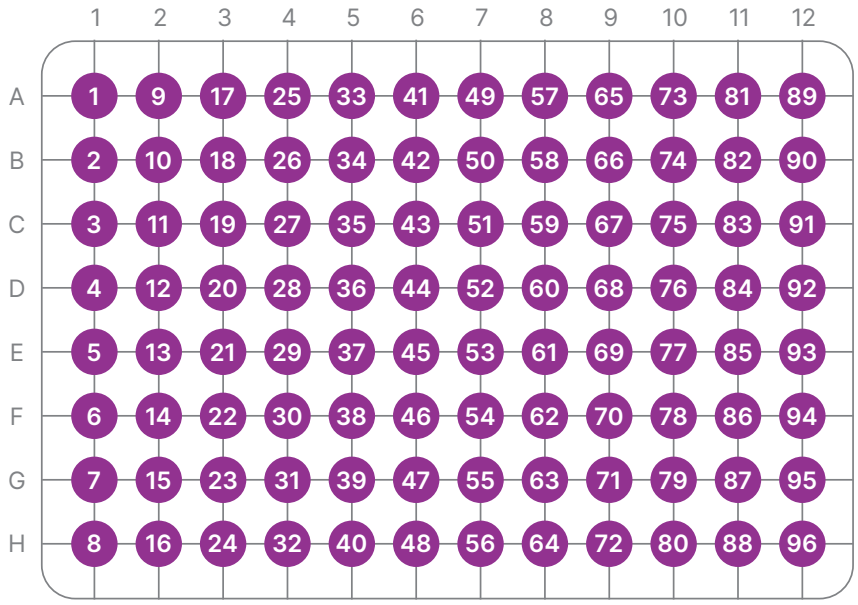

Figure 3: Representative plate layout of UDI Barcoded Primers 1-96.

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:

- KIT1014 Stranded Total RNA Prep with rRNA Depletion (Human, Mouse, Rat)
- KIT1016 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