RNA-seq

This protocol uses the Vazyme Ribo-off rRNA Depletion kit and the QIAseq™ Stranded Total RNA Library Kit to prepare RNA-seq libraries for the Illumina platform. Please look at the manufacturer's instructions and product information for more details.

The following kits are required:

i) Vazyme Ribo-off rRNA Depletion Kit (Human/Mouse/Rat) (Vazyme, cat# N406)
ii) VAHTS RNA Clean Beads (Vazyme, cat# N412)
iii) QIAseq™ Stranded Total RNA Library Kit (QIAGEN, cat# 180743)
iv) KAPA Library Quantification Kit (ROCHE, cat# KK4854)
v) Fragment Analyzer kit DNF-474-0500
vi) Qubit™ dsDNA HS (ThermoFisher, cat# Q32854)

I. Preparation of total RNA sample

1) Extract RNA using a TRIzol protocol or a kit like QIAGEN RNeasy Kits
2) Dilute 1 µg of total RNA with 11 µl of Nuclease-free Water in a Nuclease-free PCR tube and keep on ice.

Vazyme Ribo-off rRNA Depletion Kit

II. rRNA/Probe hybridization

1) Prepare the following reaction solution in a Nuclease-free PCR tube:

Hybridization Mix	
Reagent	**Volume (µL)**
rRNA Probe (H/M/R)	1
Probe Buffer	3
Total RNA	11
TOTAL	**15**

2) Mix by gently pipetting up and down for 10 times.
3) Collect the liquid to the bottom of the tube by a brief centrifugation.
4) Put the sample into a PCR instrument and run the following program (Hot Lid Temperature: 105℃):

Thermocycler Conditions			
Stage 1	95	02:00	1 cycle
	95-22C	0.1C /sec	
	22C	05:00	
	4	∞	

III. Digestion with RNase H

1) Prepare the following reaction solution on ice:

RNase H Digestion Mix	
Reagent	**Volume (µL)**
RNase H Buffer	4
RNase H	1
Hybridized RNA	15
TOTAL	**20**

2) Mix by gently pipetting up and down for 10 times.
3) Put the sample into a PCR instrument and run the following program (Hot Lid Temperature: 105℃):

Thermocycler Conditions			
Stage 1	37	30:00	1 cycle
	4	∞	

IV. Digestion with DNase I

1) Prepare the following reaction solution on ice:

DNase I Digestion Mix	
Reagent	**Volume (µL)**
DNase I Buffer	29
DNase I	1
RNase H Digested Products	20
TOTAL	**50**

2) Mix by gently pipetting up and down for 10 times.
3) Put the sample into a PCR instrument and run the following program (Hot Lid Temperature: 105℃):

Thermocycler Conditions			
Stage 1	37	30:00	1 cycle
	4	∞	

4) Collect the liquid to the bottom of the tube by a brief centrifugation.
5) Put the tube on ice and immediately proceed to the next procedure.

V. Purification of Ribosomal-depleted RNA with VAHTS RNA Clean Beads

1) Suspend the VAHTS RNA Clean Beads thoroughly by vortexing
2) Pipet 110 µl (2.2X) of beads into the RNA sample and mix thoroughly by pipetting up and down for 10 times.
3) Incubate the sample on ice for 15 min to make the RNA bind to the beads.
4) Put the sample onto a magnetic stand until the solution clarifies.
5) Carefully discard the supernatant without disturbing the beads.
6) Keep the sample on the magnetic stand, add 200 µl of freshly prepared 80% ethanol to rinse the beads.
   a. DO NOT re-suspend the beads!
7) Incubate at room temperature for 30 sec
8) Carefully discard the supernatant without disturbing the beads.
   a. Note: It is highly recommended to use a 10 µl pipette to remove the residual supernatant in this step.
9) Repeat the wash step
10) Keep the sample on the magnetic stand, open the tube and air-dry the beads for 5 min-10 min.
11) Take the sample out of magnetic stand
12) Add 30 µl of Nuclease-free Water and mix thoroughly by pipetting for 6 times.
13) Incubate at room temperature without shaking for 2 min.
14) Put the tube back on the magnetic stand and wait until the solution clarifies
15) Transfer 29 µl of the supernatant to a new Nuclease-free PCR tube without disturbing the beads.
16) Use Qubit RNA HS to quantify RNA
17) The eluted Ribosomal-depleted RNA is now ready for reverse transcription or RNA library preparation. It is highly recommended to proceed to the next procedures immediately, rather than to store the RNA at -20℃.

<u>QIAseq™ Stranded Total RNA Library Kit</u>

VI. RNA fragmentation

1) Place 29 µL of rRNA-depleted RNA into a PCR tube
   a. RNA should be depleted of riboRNA using the Vazyme kit
   b. Ribo-depleted RNA should have quantified with Qubit RNA HS and total amount of RNA calculated (necessary for adaptor addition)
2) Add 8 µl 5x RT Buffer to each sample
3) Incubate in a PCR machine as described:

Thermocycler Conditions for 150–250 bp Fragmentation				
	RNA Quality (use one based on score)			
Stage 1	RIN > 9	95C	15:00	1 cycle
	RIN 5-6	95C	10:00	
	RIN <3	None	None	
Stage 2		4	∞	1 cycle

VII. Reverse Transcription

1) Make 0.4M DTT by diluting 1 µL of 1M DTT with 1.5 µl with RNase-free water.
   a. Discard after 48 hrs
2) Add first strand synthesis reagents to each tube as follows:

1st Strand Synthesis Mix	
Reagent	**Volume (µL)**
RNase Inhibitor	1
0.4M DTT	1
RT Enzyme	1
fragmented RNA	37
TOTAL	**40**

3) Pipette to mix and spin down briefly.
4) Incubate in a thermocycler programmed as outlined below:

Thermocycler Conditions			
Stage 1	25	10:00	1 cycle
	42	15:00	
	70	15:00	
	4	∞	

5) Clean up reverse transcription samples using QIAseq beads

VIII. Reverse Transcription Cleanup

1) Equilibrate QIAseq beads to room temperature (30min)
2) In the same plate/tube(s), perform a 1.4X bead based cleanup by adding 56 µL QIAseq beads to your 40 µL reaction (total vol = 96 µL)
3) Mix thoroughly by vortexing and/or pipetting up and down multiple times.
4) Incubate the plate/tube(s) at room temperature for 5 min to bind DNA to the beads.
5) Place the plate/tube(s) on a magnet to capture the beads. Incubate until the liquid is clear.
6) Carefully remove and discard the supernatant.
7) Keeping the plate/tube(s) on the magnet, add 200 µL of 80% ethanol.
8) Incubate the plate/tube(s) on the magnet at room temperature for ≥30 sec.
9) Carefully remove and discard the ethanol.
10) Keeping the plate/tube(s) on the magnet, add 200 µL of 80% ethanol.
11) Incubate the plate/tube(s) on the magnet at room temperature for ≥30 sec.
12) Carefully remove and discard the ethanol. Try to remove all residual ethanol without disturbing the beads.
13) Dry the beads at room temperature for 3-5 min, or until all of the ethanol has evaporated. Do not over-dry.
14) Remove the plate/tube(s) from the magnet.
15) Thoroughly resuspend the beads in 40 µL of elution buffer or 10 mM Tris-HCl, pH 8.0 or H2O
16) Incubate the plate/tube(s) at room temperature for 5 min to elute DNA off the beads.
17) Place the plate/tube(s) on a magnet to capture the beads. Incubate until the liquid is clear.
18) Transfer 38.5 µL of the clear supernatant to a new plate/tube(s)
19) Proceed to second strand synthesis or store samples in -20C

IX. Second strand synthesis / end-repair / A-addition

1) Assemble each second strand synthesis reaction to each tube as follows:

2nd Strand Synthesis Mix	
Reagent	**Volume (µL)**
Second Strand Buffer	5
Second Strand Enzyme Mix	6.5
cDNA	38.5
TOTAL	**50**

2) Mix thoroughly and centrifuge briefly.
3) Incubate in a thermocycler programmed as outlined below:

Thermocycler Conditions			
Stage 1	25	30:00	1 cycle
	65	15:00	
	4	∞	

4) Clean up second strand synthesis reactions using QIAseq beads

X. Second Strand Synthesis Cleanup

1) In the same plate/tube(s), perform a 1.4X bead based cleanup by adding 70 µL QIAseq beads to your 50 µL reaction (total vol = 120 µL)
2) Mix thoroughly by vortexing and/or pipetting up and down multiple times.
3) Incubate the plate/tube(s) at room temperature for 5 min to bind DNA to the beads.
4) Place the plate/tube(s) on a magnet to capture the beads. Incubate until the liquid is clear.
5) Carefully remove and discard the supernatant.
6) Keeping the plate/tube(s) on the magnet, add 200 µL of 80% ethanol.
7) Incubate the plate/tube(s) on the magnet at room temperature for ≥30 sec.
8) Carefully remove and discard the ethanol.
9) Keeping the plate/tube(s) on the magnet, add 200 µL of 80% ethanol.
10) Incubate the plate/tube(s) on the magnet at room temperature for ≥30 sec.
11) Carefully remove and discard the ethanol. Try to remove all residual ethanol without disturbing the beads.
12) Dry the beads at room temperature for 3-5 min, or until all of the ethanol has evaporated. Do not over-dry.
13) Remove the plate/tube(s) from the magnet.
14) Thoroughly resuspend the beads in 52 µL of elution buffer or 10 mM Tris-HCl, pH 8.0 or H2O
15) Incubate the plate/tube(s) at room temperature for 5 min to elute DNA off the beads.
16) Place the plate/tube(s) on a magnet to capture the beads. Incubate until the liquid is clear.
17) Transfer 50 µL of the clear supernatant to a new plate/tube(s)
18) Proceed to adaptor ligation or freeze overnight at -20C

XI. Strand-Specific Adaptor Ligation

1) Thaw and keep Ligation Initiator at room temperature until the ligation mix is prepared.
2) Vortex briefly and centrifuge the adapter plate.
3) Remove the protective lid on the adapter plate, pierce the foil seal and dilute the QIAseq Adapters as described below:

Adaptor Dilution for total RNA input	
RNA starting amt (ng)	**Adaptor Dilution**
100	1:100
500	1:25
1000	1:12.5
5000	1:5

Adaptor Dilution for ribo-depleted RNA input	
RNA starting amt (ng)	**Adaptor Dilution**
1	1:100
10	1:25
50	1:5
100	1:2.5

4) For each sample, add 2 µl of diluted adapter.
   a. Important: To avoid cross-contamination, do not reuse diluted adapters.

5) Assemble each ligation reaction as follows on ice:
    a. Note: Mix reaction slowly and thoroughly, the reagents are very viscous

Ligation Mix	
Reagent	**Volume (µL)**
Pre-mixed sample	50
Ultralow Input Ligation Buffer, 4X	25
Ultralow Input Ligase	5
Ligation Initiator	6.5
H2O	11.5
TOTAL	**23**

6) Incubate at 25°C for 10 min.
    a. Important: Do not use a heated lid.
7) Clean up ligation reactions using QIAseq beads

XII. Adaptor Ligation Cleanup 1

1) In the same plate/tube(s), perform a 0.8X bead based cleanup by adding 80 µL QIAseq beads to your 100 µL ligation reaction (total vol = 180 µL)
2) Mix thoroughly by vortexing and/or pipetting up and down multiple times.
3) Incubate the plate/tube(s) at room temperature for 5 min to bind DNA to the beads.
4) Place the plate/tube(s) on a magnet to capture the beads. Incubate until the liquid is clear.
5) Carefully remove and discard the supernatant.
6) Keeping the plate/tube(s) on the magnet, add 200 µL of 80% ethanol.
7) Incubate the plate/tube(s) on the magnet at room temperature for ≥30 sec.
8) Carefully remove and discard the ethanol.
9) Keeping the plate/tube(s) on the magnet, add 200 µL of 80% ethanol.
10) Incubate the plate/tube(s) on the magnet at room temperature for ≥30 sec.
11) Carefully remove and discard the ethanol. Try to remove all residual ethanol without disturbing the beads.
12) Dry the beads at room temperature for 3-5 min, or until all of the ethanol has evaporated. Do not over-dry.
13) Remove the plate/tube(s) from the magnet.
14) Thoroughly resuspend the beads in 92 µL of elution buffer or 10 mM Tris-HCl, pH 8.0 or H2O
15) Incubate the plate/tube(s) at room temperature for 5 min to elute DNA off the beads.
16) Place the plate/tube(s) on a magnet to capture the beads. Incubate until the liquid is clear.
17) Transfer 90 µL of the clear supernatant to a new plate/tube(s) for the 2nd bead clean up

XIII. Adaptor Ligation Cleanup 2

18) In the same plate/tube(s), perform a 1.2X bead based cleanup by adding 108 µL QIAseq beads to your 90 µL sample (total vol = 198 µL)
19) Mix thoroughly by vortexing and/or pipetting up and down multiple times.
20) Incubate the plate/tube(s) at room temperature for 5 min to bind DNA to the beads.
21) Place the plate/tube(s) on a magnet to capture the beads. Incubate until the liquid is clear.
22) Carefully remove and discard the supernatant.
23) Keeping the plate/tube(s) on the magnet, add 200 µL of 80% ethanol.
24) Incubate the plate/tube(s) on the magnet at room temperature for ≥30 sec.
25) Carefully remove and discard the ethanol.
26) Keeping the plate/tube(s) on the magnet, add 200 µL of 80% ethanol.
27) Incubate the plate/tube(s) on the magnet at room temperature for ≥30 sec.
28) Carefully remove and discard the ethanol. Try to remove all residual ethanol without disturbing the beads.
29) Dry the beads at room temperature for 3-5 min, or until all of the ethanol has evaporated. Do not over-dry.
30) Remove the plate/tube(s) from the magnet.
31) Thoroughly resuspend the beads in 25 µL of elution buffer or 10 mM Tris-HCl, pH 8.0 or H2O
32) Incubate the plate/tube(s) at room temperature for 5 min to elute DNA off the beads.
33) Place the plate/tube(s) on a magnet to capture the beads. Incubate until the liquid is clear.

34) Transfer 23.5 µL of the clear supernatant to a new plate/tube(s) for the 2nd bead clean up
35) Proceed to cycle determination and library amplification or store the samples at -20C

XIV. Cycle Determination for Library Amplification

1) Dilute each library to 1:10, 1:100, and 1:1000 in water or dilution buffer
2) Make enough qPCR master mix for each sample at 3 different concentrations in triplicate and 6 standards in triplicate (#samples x 3 x 3 + 6 standards x 3):

KAPA qPCR Mix	
Reagent	**Volume (µL)**
KAPA Sybr Fast Mix (2X)	5
KAPA Library Amplification Primer Mix (10X)	1
H2O	2
diluted Adapter-ligated library	2
TOTAL	**10**

3) Place 8 µL of master mix into each well of a 384 well qPCR plate
4) Add in 2 µL of standard or diluted sample in each well
5) Cover the top securely with clear film
6) Spin down in a centrifuge
7) Run on the Roche Lightcycler 480 with the following program

Program Name	Cycles	Analysis Mode	Target (°C)	Acquisition Mode	Hold (hh:mm:ss)	Ramp Rate (°C/s)	Acquisition (per °C)	Sec Target (°C)	Step Size (°C)	Step Delay (cycles)
Pre-Incubation	1	None	95	None	00:05:00	4.8	---	0	0	0
Amplification	35	Quantification	95	None	00:00:30	4.8	---	0	0	0
			60	Single	00:00:45	2.5	---	0	0	0
Melting Curve	1	Melting Curves	95	None	00:00:05	4.8	---	---	---	---
			65	None	00:01:00	2.5	---	---	---	---
			95	Continuous	---	0.11	5	---	---	---
Cooling	1	None	40	None	00:00:30	2.5	---	0	0	0

8) Use Ct values to calculate optimal number of cycles

XV. CleanStart library amplification

1) Assemble each library amplification reaction as follows:

PCR Mix	
Reagent	**Volume (µL)**
CleanStart PCR Mix 2X	25
CleanStart PCR Primer Mix	1.5
Adapter-ligated library	23.5
TOTAL	**50**

2) Mix thoroughly and centrifuge briefly.
3) Amplify using the following cycling protocol:

Thermocycler Conditions			
Stage 1	37	15:00	
	98	02:00	
Stage 2	98	00:20	Calculated # of cycles
	60	00:30	
	72	00:30	
Stage 3	72	01:00	
	4	∞	

4) Clean up PCR samples using QIAseq beads

XVI. Post-amplification Cleanup

1) Add 5 µL H2O to each of your samples for a total vol of 55 µL
2) In the same plate/tube(s), perform a 1.1X bead based cleanup by adding 60 µL QIAseq beads to your 55 µL PCR reaction (total vol = 115 µL)
3) Mix thoroughly by vortexing and/or pipetting up and down multiple times.
4) Incubate the plate/tube(s) at room temperature for 5 min to bind DNA to the beads.
5) Place the plate/tube(s) on a magnet to capture the beads. Incubate until the liquid is clear.
6) Carefully remove and discard the supernatant.
7) Keeping the plate/tube(s) on the magnet, add 200 µL of 80% ethanol.
8) Incubate the plate/tube(s) on the magnet at room temperature for ≥30 sec.
9) Carefully remove and discard the ethanol.
10) Keeping the plate/tube(s) on the magnet, add 200 µL of 80% ethanol.
11) Incubate the plate/tube(s) on the magnet at room temperature for ≥30 sec.
12) Carefully remove and discard the ethanol. Try to remove all residual ethanol without disturbing the beads.
13) Dry the beads at room temperature for 3-5 min, or until all of the ethanol has evaporated. Do not over-dry.
14) Remove the plate/tube(s) from the magnet.
15) Thoroughly resuspend the beads in 25 µL of elution buffer or 10 mM Tris-HCl, pH 8.0 or H2O
16) Incubate the plate/tube(s) at room temperature for 5 min to elute DNA off the beads.
17) Place the plate/tube(s) on a magnet to capture the beads. Incubate until the liquid is clear.
18) Transfer 24 µl of the clear supernatant to a new plate/tube(s)
19) Store libraries at -20C

XVII. Library Quantification: Qubit (dsDNA HS)

1) Mix enough components A and B (1:200) together for all your samples and 2 standards
2) For standards, add 190 µL mix with 10 µL of standard in a Qubit tube
3) For samples, add 199 µL mix with 1 µL sample in a Qubit tube
4) Vortex to mix and incubate in the dark at room temp for 2min
5) Quantify using Qubit machine

XVIII. Average Size Determination: Fragment Analyzer

1) From the 5200 Fragment Analyzer DNF-474 High Sensitivity NGS 1-6000bp kit, mix gel and intercalating dye together (1 µL dye to 10 mL gel)
2) Pipette 22 µL diluent marker to the wells of the 96 well plate that will contain samples or ladder
    a. FA runs row by row
    b. The 12th well always contains the ladder
    c. Do not go over 5ng of sample
3) Add in 2 µL of sample or ladder
4) Add Blank Solution to the unused wells
5) Cover the row securely with a strip of clear film
6) Spin down in a centrifuge
7) Take gel and sample plate and run on FA

XIX. Library Quantification: qPCR

1) Dilute each library to 1:1000, 1:10,000, and 1:100,000 in water or dilution buffer
2) Make enough qPCR master mix for each sample at 3 different concentrations in triplicate and 6 standards in triplicate (#samples x 3 x 3 + 6 standards x 3):

KAPA qPCR Mix	
Reagent	**Volume (µL)**
KAPA Sybr Fast Mix (2X)	5
KAPA Library Amplification Primer Mix (10X)	1
H2O	2
diluted Adapter-ligated library	2
TOTAL	**10**

3) Place 8 µL of master mix into each well of a 384 well qPCR plate
4) Add in 2 µL of standard or diluted sample in each well
5) Cover the top securely with clear film
6) Spin down in a centrifuge
7) Run on the Roche Lightcycler 480 with the following program

Program Name	Cycles	Analysis Mode	Target (°C)	Acquisition Mode	Hold (hh:mm:ss)	Ramp Rate (°C/s)	Acquisition (per °C)	Sec Target (°C)	Step Size (°C)	Step Delay (cycles)
Pre-Incubation	1	None	95	None	00:05:00	4.8	---	0	0	0
Amplification	35	Quantification	95	None	00:00:30	4.8	---	0	0	0
			60	Single	00:00:45	2.5	---	0	0	0
Melting Curve	1	Melting Curves	95	None	00:00:05	4.8	---	---	---	---
			65	None	00:01:00	2.5	---	---	---	---
			95	Continuous	---	0.11	5	---	---	---
Cooling	1	None	40	None	00:00:30	2.5	---	0	0	0

8) Use Ct values to determine sample concentration
9) Pool together samples that need to be sequenced
10) Qubit (optional) and qPCR to quantify the pooled library