# VAHTS Universal V10 RNA-seq Library Prep Kit for Illumina

## NR606


Instruction for Use

Version 24.1

# Contents

## 01/Product Description

VAHTS Universal V10 RNA-seq Library Prep Kit for Illumina is a RNA-seq library preparation kit specifically designed for Illumina high-throughput sequencing platforms. The kit contains two types of 2nd Strand cDNA synthesis buffers for non-strand-specific or strand-specific RNA-seq library preparation.

The kit integrates 2nd Strand cDNA synthesis, End Repair, and dA-Tailing into one step with no need for purification, which greatly simplifies the process of library construction and shortens the operation time. The optimized reaction system improves the library construction efficiency, is compatible with lower Input RNA, and has uniform coverage for different amounts of Input RNA. The kit leverages magnetic bead-based size selection to rapidly obtain customized libraries of specific sizes to accommodate diverse experimental needs. All the components provided in the kit have undergone rigorous quality control and functional testing to ensure the optimal stability and repeatability.

## 02/Components

| Components | NR606-01 (24 rxns) | NR606-02 (96 rxns) |
|---|---|---|
| Frag/Prime Buffer 2 | 480 µl | 2 × 960 µl |
| 1st Strand Buffer 6 | 168 µl | 672 µl |
| 1st Strand Enzyme Mix 4 | 48 µl | 192 µl |
| 2nd Strand Buffer 3 (with dNTP) | 600 µl | 4 × 600 µl |
| 2nd Strand Buffer 3 (with dUTP) | 600 µl | 4 × 600 µl |
| 2nd Strand Enzyme Super Mix 3 | 360 µl | 2 × 720 µl |
| Rapid Ligation Buffer 6 | 600 µl | 4 × 600 µl |
| Rapid DNA Ligase 6 | 120 µl | 480 µl |
| PCR Primer Mix 5 | 120 µl | 480 µl |
| VAHTS HiFi Amplification Mix 3 | 600 µl | 4 × 600 µl |

## 03/Storage

Store at -30 ~ -15°C and transport at ≤0°C.

## 04/Applications

VAHTS Universal V10 RNA-seq Library Prep Kit for Illumina is intended for RNA library preparation from high-integrity total RNA from eukaryotes (eg. animals, plants, and fungi), poly(A) enriched mRNA, and rRNA depleted RNA. As the mRNA level in total RNA varies significantly across different specimens, sufficient total RNA input is required to ensure enough mRNA is obtained for subsequent library preparation.

The amount of Input RNA is related to the RNA enrichment module:
VAHTS mRNA Capture Beads 2.0 (Vazyme #N403): 0.01 - 4 µg
Ribo-off rRNA Depletion Kit (Human/Mouse/Rat) (Vazyme #N406): 0.01 - 1 µg
Ribo-off rRNA Depletion Kit V2 (Bacteria) (Vazyme #N417): 0.01 - 1 µg

Ribo-off Globin & rRNA Depletion Kit (Human/Mouse/Rat) (Vazyme #N408): 0.01 - 1 μg
Ribo-off rRNA Depletion Kit (Plant) (Vazyme #N409): 1 - 5 μg
Ribo-MagOff rRNA Depletion Kit (Human/Mouse/Rat) (Vazyme #N420): 0.01 - 1 μg
Purified mRNA or ribosomal-depleted RNA (for direct library preparation): 0.5 - 100 ng

It is recommended to evaluate the integrity of total RNA using Agilent 2100 Bioanalyzer. High-quality RNA samples (RIN≥7) are required for mRNA enrichment with VAHTS mRNA Capture Beads 2.0 (Vazyme #N403), as degraded samples will lead to 3' bias in RNA-seq. For RNA samples with RIN<7, rRNA removal can be accomplished using the Ribo-off method (Vazyme #N406/417/408/409).

Main fields of RNA-related analysis:

◇ Gene expression analysis
◇ Single nucleotide variation calling
◇ Alternative splicing detection
◇ Gene fusion detection
◇ Target transcriptome analysis

## 05/Self-prepared Materials

◇ RNA Analysis:
Equalbit RNA HS Assay Kit (Vazyme #EQ211)
Equalbit RNA BR Assay Kit (Vazyme #EQ212)
Agilent RNA 6000 Pico Kit (Agilent #5067-1513)

◇ mRNA Enrichment & rRNA Depletion:
VAHTS 2× Frag/Prime Buffer V2 (Vazyme #N405)
VAHTS mRNA Capture Beads 2.0 (Vazyme #N403)
Ribo-off rRNA Depletion Kit (Human/Mouse/Rat) (Vazyme #N406)
Ribo-off rRNA Depletion Kit V2 (Bacteria) (Vazyme #N417)
Ribo-off Globin & rRNA Depletion Kit (Human/Mouse/Rat) (Vazyme #N408)
Ribo-off rRNA Depletion Kit (Plant) (Vazyme #N409)
Ribo-MagOff rRNA Depletion Kit (Human/Mouse/Rat) (Vazyme #N420)

◇ RNA and DNA Clean Beads:
VAHTS DNA Clean Beads (Vazyme #N411)
or Agencourt AMPure XP Reagent (Beckman #A63880/A63881/A63882)
VAHTS RNA Clean Beads (Vazyme #N412)
or Agencourt RNA Clean XP Beads (Beckman #A63987)

◇ Adapters:
VAHTS DNA Adapters Set 3 - Set 6 for Illumina (Vazyme #N805/N806/N807/N808)
or VAHTS Maxi Unique Dual Index DNA Adapters Set 1 - Set 4 for Illumina (Vazyme #N34201/N34202/N34203/N34204)
or VAHTS Multiplex Oligos Set 4 - Set 5 for Illumina (Vazyme #N321/N322)

◇ Library Quality Control:
Equalbit 1 × dsDNA HS Assay Kit (Vazyme #EQ121)
Agilent DNA 1000 Kit (Agilent #5067 - 1504) or Agilent High Sensitivity DNA Kit (Agilent #5067 - 4626)

◇ Other Materials:
80% ethanol (freshly prepared), Nuclease-free dd$H_2O$; low-adsorption Nuclease-free PCR tubes, pipette tips, and centrifuge tubes; PCR instrument, magnetic rack, Qubit fluorometer, Agilent 2100 Bioanalyzer, or other equivalent products.

## 06/Notes

For research use only. Not for use in diagnostic procedures.

### 06-1/Quality Control of RNA Samples

To ensure the library quality, the RNA samples must undergo quality control before the experiment. The total amount and purity of the RNA samples must meet the following requirements:

1. The initial template total RNA input should be ≥10 ng; otherwise, the mRNA obtained may be insufficient for subsequent library preparation.
2. The OD260/280 ratio should be 1.8 - 2.1; if the ratio>2.1/<1.8, the RNA samples may have been contaminated with genomic DNA/protein. The ratio of OD230/OD260 should be between 0.4 and 0.5; if the ratio>0.5/<0.4, the RNA samples may have been contaminated with salt or small molecular/genomic DNA.

### 06-2/RNA Sample Preparation

1. Pay attention to mixing solution containing RNA by pipetting gently. **Do Not vortex**, avoid unexpected size of library caused by RNA breaking.
2. The mRNA enriched by Poly(A) method or the RNA with rRNA depletion should be performed to subsequent operation as soon as possible to avoid RNA degradation.
3. For the Input RNA with low concentration, it can be concentrated using lyophilization, ethanolprecipitation, column-based or beads-based clean-ups (e.g. VAHTS RNA Clean Beads, Vazyme #N412).

### 06-3/Tips for DNA Purification with Magnetic Beads

1. Equilibrate the beads to room temperature before use to assure capture efficiency.
2. Mix the beads thoroughly every time before pipetting.
3. The sample and the magnetic beads are thoroughly mixed and then placed on a magnetic rack for separation. Please transfer the supernatant after the solution is completely clear, and leave 2 - 3 μl of supernatant to avoid disturbing the beads. If the magnetic beads are accidentally drawn out with the supernatant. The yield will decrease. The effect of size selection will be poor, and even the subsequent enzymatic reaction will be affected. In this

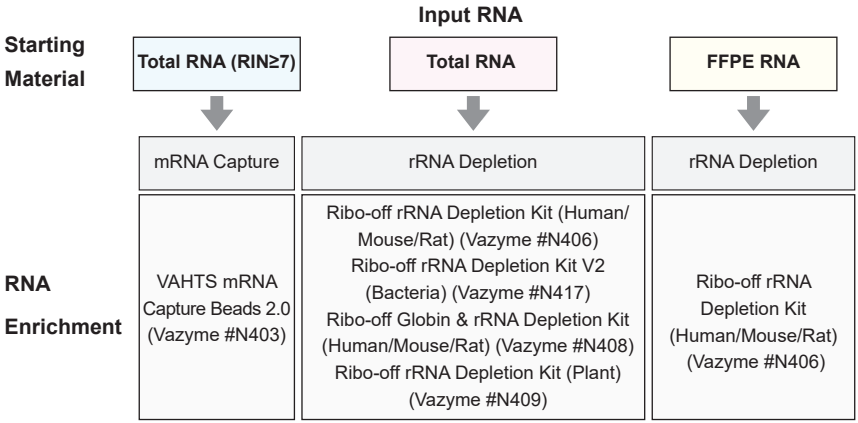
case, the magnetic beads can be mixed and placed on magnetic rack again to separate the beads completely.

4. Always use 80% ethanol freshly prepared. Keep tubes on magnet rack without disturbing the beads during elution.
5. Do not leave any 80% ethanol supernatant behind in the second washing step to reduce the residual impurities.
6. It is important to dry all the ethanol at room temperature before proceeding with subsequent reactions. Over-drying the beads may result in reduced yield. Normally, drying of beads for 5 - 10 min at room temperature should be sufficient. Drying of beads at 37°C is not recommended.

### 06-4/Operations Attentions

1. Thaw all kit components on ice before use. After thawing, fully mix the components by inversion several times, centrifuge briefly, and place on ice for later use.
2. It is recommended to use filter pipette tips and change tips for different samples.
3. Please use RNase-free materials before 2nd Strand cDNA synthesis, while use DNase-free materials after that.
4. Be sure to use fresh Nuclease-free dd$H_2O$ during the experiment; it is recommended to dispense it into small tubes for use and discard after use.
5. Be sure to wear gloves; change gloves after touching the equipment outside the RNase-free space or other working areas.
6. Please cover the reagent with lid to avoid contamination whenever finish use.
7. It is recommended to perform the reaction in a PCR instrument with a hot lid, and preheat the PCR instrument to near the reaction temperature before use.
8. Aerosol contamination is easily to be caused by PCR products due to improper operation, affecting the accuracy of the experimental results. Therefore, it is recommended to isolate PCR reaction preparation area and PCR products detection area physically. Use a dedicated pipettor and other equipment, and regularly clean each experimental area to ensure the cleanliness of the experimental environment.
9. If you need to pause during the experiment, please store the samples at the appropriate temperature according to the stop point indicated in protocol. Improper storage may reduce the success rate of the library construction.

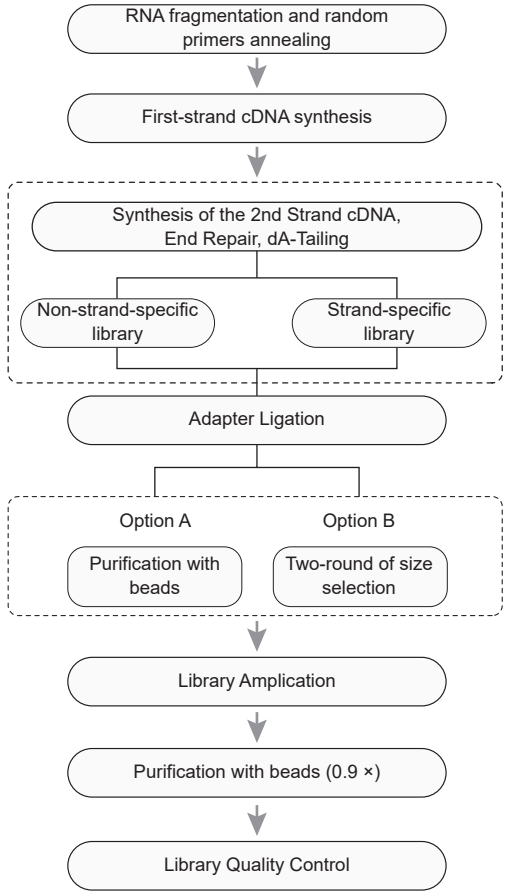
## 07/Mechanism & Workflow




**cDNA Library Preparation**

- 2nd Strand cDNA synthesis, End Repair and dA-Tailing are combined into one step, which greatly shortens the time of library construction. Both stranded specific and non-strand specific transcriptome library construction schemes are provided.

- size selection
  Option A:
  Yields 150 - 200 bp inserts.
  Option B:
  Libraries with inserts of customized sizes can be obtained after two-round of size selection.



## 08/Experiment Process

### 08-1/mRNA Purification and Fragmentation

**Protocol A: Poly(A)-based mRNA enrichment**

Taking VAHTS mRNA Capture Beads 2.0 (Vazyme #N403) for mRNA enrichment as an example, this protocol is applicable for preparing RNA libraries from 10 ng - 4 μg of total RNA of eukaryotes (e.g. animal, plant or fungi) with good integrity.

1. Take out mRNA Capture Beads 2.0, Beads Wash Buffer 2.0, Tris Buffer 2.0, and Beads Binding Buffer 2.0 from 2 ~ 8°C and equilibrate to room temperature.
2. Prepare the RNA sample carefully: Dissolve 10 ng - 4 μg of total RNA in Nuclease-free dd$H_2O$ in a Nuclease-free PCR tube, to a total volume of 50 μl. Keep the tube on ice and proceed to the next step as soon as possible.
3. Softly suspend mRNA Capture Beads 2.0 thoroughly by inverting, add 50 μl beads to prepared RNA sample, and mix thoroughly by pipetting up and down 10 times.
   ▲ mRNA Capture Beads 2.0, Beads Wash Buffer 2.0, and Beads Binding Buffer 2.0 contain detergents. DO NOT vortex or oscillate violently when mixing. Avoid foaming when pipetting.
4. Run the following program in the PCR instrument to make the first time binding of mRNA and magnetic beads:

| Temperature | Time |
|---|---|
| 65°C | 5 min |
| 25°C | 5 min |

5. Place the sample on the magnetic rack. After the solution becomes clear (about 5 min), carefully discard the supernatant without disturbing the beads.
6. Take the samples out of the magnetic rack. Add 200 μl of Beads Wash Buffer 2.0, and mix thoroughly by pipetting up and down 10 times. Place the tube back to the magnetic rack. After the solution is clear (about 5 min), carefully discard the supernatant without disturbing the beads.
   ▲ Step 4 - 6 are the first round of mRNA isolation and purification, and Step 7 - 12 are the second round of mRNA isolation and purification to ensure the removal efficiency of rRNA.
   ▲ For some special samples, please repeat step 6 and wash again to ensure the removal efficiency of rRNA.
7. Take the samples out of the magnetic rack. Add 50 μl of Tris Buffer 2.0 and mix thoroughly by gently pipetting up and down 10 times.
8. Run the following program in the PCR instrument to release mRNA:

| Temperature | Time |
|---|---|
| 80°C | 2 min |
| 25°C | Hold |

9. Add 50 μl of Beads Binding Buffer 2.0 and mix thoroughly by gently pipetting up and down 10 times.

10. Incubate at room temperature for 5 min to make the mRNA to bind to the beads.
11. Place the sample on the magnetic rack to isolate mRNA from total RNA. After the solution becomes clear (about 5 min), carefully discard the supernatant without disturbing the mRNA Capture Beads.
12. Take the samples out of the magnetic rack, add 200 µl of Beads Wash Buffer 2.0, and mix thoroughly by pipetting up and down 10 times. Place the tube on the magnetic rack. After the solution is clear (about 5 min), carefully discard the supernatant without disturbing the mRNA Capture Beads.
    ▲It is highly recommended to completely remove the residual supernatant in this step. The residual of Beads Wash Buffer 2.0 will affect the fragmentation of mRNA.
13. Take the samples out of the magnetic rack. Add 18 µl of Frag/Prime Buffer 2 and mix thoroughly by gently pipetting up and down 10 times. Place the sample into the PCR instrument, and set fragmentation conditions based on the desired insert size:

| Insert Size (bp) | Fragmentation Condition |
|---|---|
| 150 - 200 | 94°C 8 min, 4°C hold |
| 200 - 300 | 94°C 5 min, 4°C hold |
| 250 - 450 | 85°C 6 min, 4°C hold |
| 450 - 550 | 85°C 5 min, 4°C hold |

    ▲ Do not stop or pause between steps from the fragmentation to the 1st Strand cDNA synthesis, as mRNA is easy to degrade under this system.
    ▲ The reagents for 08-2/Synthesis of Double Strand cDNA/Step 1 can be taken out from -30 ~ -15°C in advance and placed on ice for use.
14. Place the sample on the magnetic rack. After the solution is clear (about 5 min), carefully transfer 16 µl of the supernatant to a fresh Nuclease-free PCR tube. Then immediately proceed to synthesis of 1st Strand cDNA.

**Protocol B: rRNA depletion**

Taking Ribo-off rRNA Depletion Kit (Human/Mouse/Rat) (Vazyme #N406) as an example, this protocol is applicable to the generation of total RNA transcriptome library of human, mouse, rat and other species with a Input RNA of 10 ng - 1 µg. To remove rRNA from other species, please refer to the corresponding rRNA depletion kit instructions.

1. Prepare the total RNA sample carefully: Dilute 10 ng - 1 µg of total RNA with 11 µl Nuclease-free dd$H_2O$ in a Nuclease-free centrifuge tube, and keep on ice for later use.
2. rRNA/probe hybridization:
   A. Prepare the following reaction mix in a Nuclease-free microcentrifuge tube:

| Components | Volume |
|---|---|
| rRNA Probe (H/M/R) | 1 µl |
| Probe Buffer | 3 µl |
| Total RNA | 11 µl |
| Total | 15 µl |

Mix the solution thoroughly by gently pipetting up and down 10 times.

▲ For multiple samples, it is recommended to prepare a mixture of the rRNA Probe (H/M/R) and the Probe Buffer in a suitable size tube first, and then dispense into each PCR tube. It is recommended to prepare 1.1 × volumes of the actual volume to compensate for the loss.

B. Collect the liquid to the bottom of the tube by a brief centrifugation, and place the tube into a PCR instrument and run the following program:

| Temperature | Time |
|---|---|
| 105°C | On |
| 95°C | 2 min |
| 95 ~ 22°C | 0.1°C/sec |
| 22°C | 5 min |

▲This step takes about 15 - 20 min. The duration may vary for different PCR instrument models.

▲The reagents for step 3 can be taken out from -30 ~ -15°C in advance and placed on ice for use.

3. RNase H digestion:

A. Prepare the following reaction solution on ice:

| Components | Volume |
|---|---|
| RNase H Buffer | 4 µl |
| RNase H | 1 µl |
| Product from previous step | 15 µl |
| Total | 20 µl |

Mix the solution thoroughly by gently pipetting up and down 10 times.

▲ For multiple samples, it is recommended to prepare a mixture of the RNase H Buffer and the RNase H in a suitable size tube first, and then dispense into each PCR tube. It is recommended to prepare 1.1 × volumes of the actual volume to compensate for the loss.

B. Put the sample into the PCR instrument and run the following program for RNase H digestion:

| Temperature | Time |
|---|---|
| 37°C | 30 min |
| 4°C | Hold |

▲The reagents for step 4 can be taken out from -30 ~ -15°C in advance and placed on ice for use.

4. Digestion with DNase I:

A. Prepare the following reaction solution on ice:

| Components | Volume |
|---|---|
| DNase I Buffer | 29 µl |
| DNase I | 1 µl |
| RNase H digestion product | 20 µl |
| Total | 50 µl |

Mix the solution thoroughly by gently pipetting up and down 10 times.

▲ For multiple samples, it is recommended to prepare a mixture of the DNase I Buffer and the DNase I in a suitable size tube first, and then dispense into each PCR tube. It is recommended to prepare 1.1 × volumes of the actual volume to compensate for the loss.

B. Put the sample into the PCR instrument and run the following program for DNase I digestion:

| Temperature | Time |
|---|---|
| 37°C | 30 min |
| 4°C | Hold |

Collect the liquid to the bottom of the tube by a brief centrifugation. Place the tube on ice and immediately proceed to the next program.

5. Purification of ribosomal-depleted RNA with VAHTS RNA Clean Beads:
   a. Suspend the VAHTS RNA Clean Beads thoroughly by inverting or vortexing, pipet 110 μl (2.2 ×) of beads into the RNA sample of last step. Mix thoroughly by gently pipetting up and down 10 times.
   b. Incubate the sample on ice for 15 min to bind RNA to the beads.
   c. Place the tube onto a magnetic rack. After the solution is clear (about 5 min), carefully discard the supernatant without disturbing the beads.
   d. Keep the tube on the magnetic rack. Add 200 μl of 80% ethanol (freshly prepared with Nuclease-free dd$H_2O$) to rinse the beads. Incubate at room temperature for 30 sec and carefully discard the supernatant without disturbing the beads.
   e. Repeat Step d.
   f. Keep the tube on the magnetic rack, open the lid and air-dry the beads for 5 - 10 min.
   ▲Do not disturb the beads when adding 80% ethanol.
   ▲It is highly recommended to use a 10 μl pipettor to remove the residual supernatant in this step.
   ▲Avoid over-drying the beads, which resulting in the reduction of recovery efficiency.

6. Take the sample out the magnetic rack, Add 18 μl of Frag/Prime Buffer 2 and mix thoroughly by pipetting up and down 6 times. Incubate at room temperature for 2 min. Put the tube back on the magnetic rack, after the solution is clear (about 5 min), and carefully transfer 16 μl of the supernatant to a new Nuclease-free PCR tube without disturbing the beads.
   Put the sample into the PCR instrument, and set programs according to the fragment size:

| Insert Size (bp) | Fragmentation Condition |
|---|---|
| 150 - 200 | 94°C 8 min, 4°C hold |
| 200 - 300 | 94°C 5 min, 4°C hold |
| 250 - 450 | 85°C 6 min, 4°C hold |
| 450 - 550 | 85°C 5 min, 4°C hold |

▲ Do not stop or pause between steps from the fragmentation to the 1st Strand cDNA synthesis, as mRNA is easy to degrade under this system.

▲ The reagents for 08-2/Synthesis of Double Strand cDNA/Step 1 can be taken out from -30 ~ -15°C in advance and placed on ice for use.

**Protocol C: Use purified mRNA or Ribosomal-depleted RNA as templates.**

This protocol is applicable for  for using 0.5 - 100 ng of purified mRNA or Ribosomal-depleted RNA as templates for library preparation, and need to be paired with VAHTS 2 × Frag/Prime Buffer V2 (Vazyme #N405).

1. Prepare the reaction system as follows:

| Components | Volume |
|---|---|
| 2 × Frag/Prime Buffer V2 | 8 µl |
| RNA | 8 µl |
| Total | 16 µl |

2. Mix thoroughly by pipetting up and down 10 times. Incubate the tube in the PCR instrument and set programs according to fragment sizes:

| Insert Size (bp) | Fragmentation Condition |
|---|---|
| 150 - 200 | 94°C 8 min, 4°C hold |
| 200 - 300 | 94°C 5 min, 4°C hold |
| 250 - 450 | 85°C 6 min, 4°C hold |
| 450 - 550 | 85°C 5 min, 4°C hold |

▲ Do not stop or pause between steps from the fragmentation to the 1st Strand cDNA synthesis, as mRNA is easy to degrade under this system.

▲ The reagents for 08-2/Synthesis of Double Strand cDNA/Step 1 can be taken out from -30 ~ -15°C in advance and placed on ice for use.

## 08-2/Synthesis of Double Strand cDNA

1. The components for synthesis of double Strand cDNA should be dissolved on ice, and mixed by inverting the tubes, briefly centrifuged to the bottom of the tube, and placed on ice for use. Prepare the reaction solution to synthesize the 1st Strand of cDNA as follows:

| Components | Volume | |
|---|---|---|
| Fragmented mRNA from previous step | 16 µl | |
| 1st Strand Buffer 6 | 7 µl | |
| 1st Strand Enzyme Mix 4 | 2 µl | |
| Total | 25 µl | |

2. Adjust the pipettor to a 20 µl range and mix thoroughly by gently pipetting up and down 10 times.

▲ For multiple samples, it is recommended to prepare a mixture of the 1st Strand Buffer 6 and the 1st Strand Enzyme Mix 4 in a tube first, and then dispense into each PCR tube. It is recommended to prepare 1.1 × volumes of the actual volume to compensate for the loss.

▲ Before add the mixture to the reaction system, keep it away from light.

3. Run the following program in a PCR instrument for the synthesis of 1st Strand cDNA:

| Temperature | Time |
|---|---|
| Heating lid 105°C | On |
| 25°C | 10 min |
| 42°C | 15 min |
| 70°C | 15 min |
| 4°C | Hold |

▲ The synthesis of 2nd Strand cDNA should be performed immediately after the synthesis of the 1st Strand of cDNA.

▲ The reagents for step 4 can be taken out from -30 ~ -15°C in advance and placed on ice for use.

4. Prepare the reaction solution to synthesize the 2nd Strand of cDNA as follows:

| Components | Volume | |
|---|---|---|
| 1st Strand cDNA from previous step | 25 µl | |
| 2nd Strand Buffer 3 (with dNTP or dUTP)* | 25 µl | / |
| 2nd Strand Enzyme Super Mix 3 | 15 µl | |
| Total | 65 µl | |

*For **non-strand-specific RNA-seq**, use 2nd Strand Buffer 3 (with dNTP);
For **strand-specific RNA-seq**, use 2nd Strand Buffer 3 (with dUTP).

▲For multiple samples, it is recommended to prepare a mixture of the 2nd Strand Buffer 3 (with dNTP or dUTP) and the 2nd Strand Enzyme Super Mix 3 in PCR tube first, and then dispense into each PCR tube. It is recommended to prepare 1.1 × volumes of the actual volume to compensate for the loss.

5. Adjust the pipettor to a 50 µl range and mix thoroughly by gently pipetting up and down 10 times.

6. Run the following program in a PCR instrument for the synthesis of 2nd Strand cDNA:

| Temperature | Time |
|---|---|
| Heating lid 105°C | On |
| 16°C | 30 min |
| 65°C | 15 min |
| 4°C | Hold |

▲The reagents for 08-3/Adapter Ligation/Step 1 can be taken out from -30 ~ -15°C in advance and placed on ice for use.

**The second-strand cDNA synthesis products can be stored at -30 ~ -15°C for 24 h.**

## 08-3/Adapter Ligation

1. Prepare the reaction solution of adapter ligation as follows:

| Components | Volume | |
|---|---|---|
| ds cDNA from previous step | 65 µl | |
| Rapid Ligation Buffer 6 | 25 µl | |
| Rapid DNA Ligase 6 | 5 µl | |
| Adapter* | 5 µl | |
| Total | 100 µl | |

▲The mixture of Rapid Ligation Buffer 6 and Rapid DNA Ligase 6 can be stored at 2 ~ 8°C for no more than 24 h.

▲It is recommended to add the adapter to the ds cDNA first and mix thoroughly, then add the mixture of Rapid Ligation Buffer 6 and Rapid DNA Ligase 6.

*Please refer to the following table for the amount of Adapter:

| Input RNA | | Concentration of Adapter |
|---|---|---|
| Protocol A/B | Protocol C | |
| ≥1 µg | 100 ng | 5 µM |
| 500 ng | 10 ng | 3 µM |
| 100 ng | 1 ng | 2 µM |
| 10 ng | 0.5 ng | 1.5 µM |

2. Adjust the pipettor to an 80 μl range and mix thoroughly by gently pipetting up and down 10 times.
3. Run the program of ligation reaction in the PCR instrument:

| Temperature | Time |
|---|---|
| Heating lid 105°C | On |
| 20°C | 15 min |
| 4°C | Hold |

▲VAHTS DNA Clean Beads for 08-4/Purification/Size-Selection Protocol can be taken out from 2 ~ 8°C in advance and placed at room temperature.

**The adapter ligation products can be temporarily stored at 2 ~ 8°C for 1 h.**

### 08-4/Purification/Size-Selection Protocol

Two options are provided in this step, please select a suitable solution according to the actual situation.

Option A: The purification protocol comprises one round of purification and no size selection. This protocol yields 150 - 200 bp inserts without residual adapters (suitable for mRNA fragmented at 94°C for 8 min).

Option B: The size selection protocol comprises purification and two rounds of size selection. This protocol yields different inserts >200 bp (based on size selection conditions) without residual adapters.

**Option A: Purification: For libraries with 150 - 200 bp inserts (suitable for mRNA fragmented by incubation at 94°C for 8 min)**

1. Equilibrate the VAHTS DNA Clean Beads to room temperature.
2. Suspend the VAHTS DNA Clean Beads thoroughly by inverting or vortexing. Pipet 60 μl (0.6 ×) of beads into the above adapter ligation products. Mix thoroughly by pipetting up and down 10 times.
3. Incubate at room temperature for 10 min to make the DNA to bind to the beads.
4. Place the tube on a magnetic rack. After the solution is clear (about 5 min), keep it on magnetic rack, and carefully discard the supernatant without disturbing the beads.
5. Keep the tube on the magnetic rack and add 200 μl of freshly prepared 80% ethanol to rinse the beads. Incubate for 30 sec at room temperature and carefully discard the supernatant without disturbing the beads.
6. Repeat Step 5.
7. Keep the tube on the magnetic rack, open the lid and air-dry the beads for 5 - 10 min.
   ▲Do not disturb the beads when adding 80% ethanol.
   ▲Use a 10 μl pipette to remove all the residual liquid.
   ▲Over-drying of beads will result in the reduction of recovery efficiency. Please avoid over-drying.

8. Take the tube out of the magnetic rack. Add 22 μl of Nuclease-free dd$H_2O$ to elute DNA. Mix thoroughly by vortexing or pipetting and incubate for 2 min at room temperature. Place the tube back on the magnetic rack, after the solution is clear (about 5 min), and carefully transfer 20 μl of the supernatant to a new Nuclease-free PCR tube without disturbing the beads.

**Option B: Size selection: For libraries with >200 bp inserts (suitable for mRNA fragmented by incubation at 94°C for 5 min, 85°C for 6 min, or 85°C for 5 min)**

**Purify the ligation products using 0.6 × VAHTS DNA Clean Beads.**

1. Equilibrate the VAHTS DNA Clean Beads to room temperature.
2. Suspend the VAHTS DNA Clean Beads thoroughly by inverting or vortexing. Pipet 60 μl (0.6 ×) of beads into the above adapter ligation products. Mix thoroughly by pipetting up and down 10 times.
3. Incubate at room temperature for 10 min to make the DNA to bind to the beads.
4. Place the tube on the magnetic rack. After the solution is clear (about 5 min), keep it on magnetic rack, and carefully discard the supernatant without disturbing the beads.
5. Keep the tube on the magnetic rack, add 200 μl of freshly prepared 80% ethanol to rinse the beads. Incubate for 30 sec at room temperature and carefully discard the supernatant without disturbing the beads.
6. Repeat Step 5.
7. Keep the tube on the magnetic rack. open the lid and air-dry the beads for 5 - 10 min.
   ▲ Do not disturb the beads when adding 80% ethanol.
   ▲ Use a 10 μl pipette to remove all the residual liquid.
   ▲ Over-drying of beads will result in the reduction of recovery efficiency. Please avoid over-drying.
8. Take the tube out of the magnetic rack. Add 102 μl of Nuclease-free dd$H_2O$ to elute DNA. Mix thoroughly by vortexing or pipetting and incubate for 2 min at room temperature. Place the tube back on the magnetic rack, after the solution is clear (about 5 min), and carefully transfer 100 μl of the supernatant to a new Nuclease-free PCR tube without disturbing the beads.

**The purified product can be temporarily stored at -30 ~ -15°C for 24 h.**

**Conduct two rounds of size selection with VAHTS DNA Clean Beads. (The following is an illustration with a fragmentation condition of 85°C for 6 min, and an insert size of 350 - 450 bp. For other insert sizes, choose the volume of beads according to the tables below).**

For full length adapters, such as VAHTS DNA Adapters Set 3 - Set 6 for Illumina (Vazyme #N805/N806/N807/N808), or VAHTS Maxi Unique Dual Index DNA Adapters Set 1 - Set 4 for Illumina (Vazyme #N34201/N34202/N34203/N34204), please refer to Table 1 for size selection.

Table 1. Size Selection for Full Length Adapter Ligation Products

| | | | | |
|---|---|---|---|---|
| Insert size (bp) | 200 - 300 | 250 - 350 | 350 - 450 | 450 - 550 |
| Library size (bp) | 320 - 420 | 370 - 470 | 470 - 570 | 570 - 670 |
| Fragmentation condition | 94°C 5 min | 85°C 6 min | 85°C 6 min | 85°C 5 min |
| Volume of beads for 1st round (μl) | 70 (0.7 ×) | 65 (0.65 ×) | 60 (0.6 ×) | 55 (0.55 ×) |
| Volume of beads for 2nd round (μl) | 10 (0.1 ×) | 10 (0.1 ×) | 10 (0.1 ×) | 10 (0.1 ×) |

For Stubby adapters, such as VAHTS Multiplex Oligos Set 4 - Set 5 for Illumina (Vazyme #N321/N322), please refer to Table 2 for size selection.

Table 2. Size Selection for Stubby Adapter Ligation Products

| | | | | |
|---|---|---|---|---|
| Insert size (bp) | 200 - 300 | 250 - 350 | 350 - 450 | 450 - 550 |
| Library size (bp) | 320 - 420 | 370 - 470 | 470 - 570 | 570 - 670 |
| Fragmentation condition | 94°C 5 min | 85°C 6 min | 85°C 6 min | 85°C 5 min |
| Volume of beads for 1st round (μl) | 80 (0.8 ×) | 65 (0.65 ×) | 60 (0.6 ×) | 55 (0.55 ×) |
| Volume of beads for 2nd round (μl) | 20 (0.2 ×) | 20 (0.2 ×) | 10 (0.1 ×) | 10 (0.1 ×) |

▲ The size of the library here is the final size. The volume of magnetic beads will affect the final library size. The volume ratio used in size selection is the ratio to the Input DNA. For example: For 100 μl of Input DNA, the first-round bead volume 60 μl is 60% of 100 μl and is hence noted as 0.6 ×; the second-round bead volume 10 μl is 10% of 100 μl (rather than 10% of 155 μl of supernatant) and is hence noted as 0.1 ×.

9. Suspend the VAHTS DNA Clean Beads thoroughly by inverting or vortexing. Transfer 60 μl (0.6 ×) of the beads into the samples above. Mix thoroughly by pipetting up and down 10 times.
10. Incubate at room temperature for 10 min to make the DNA to bind to the beads.
11. Place the tube on the magnetic rack, After the solution is clear (about 5 min), keep the tube on magnetic rack, and carefully transfer 155 μl of the supernatant (**DO NOT discard**) to a new Nuclease-free PCR tube.
    ▲ Do not disturb the beads when transferring the supernatant. If beads are carried over, the large DNA fragments on the beads will be introduced into the next step and remain in the final library.
12. Add 10 μl (0.1 ×) of VAHTS DNA Clean Beads and mix thoroughly by gently pipetting up and down 10 times.
13. Incubate at room temperature for 10 min to make the DNA to bind to the beads.
14. Place the tube on the magnetic rack. After the solution is clear (about 5 min), keep the tube on magnetic rack, and carefully discard the supernatant without disturbing the beads.
15. Keep the tube on the magnetic rack, add 200 μl of freshly prepared 80% ethanol to rinse the beads. Incubate for 30 sec at room temperature and carefully discard the supernatant without disturbing the beads.
16. Repeat Step 15.

17. Keep the tube on the magnetic rack. open the lid and air-dry the beads for 5 - 10 min.
    ▲Do not disturb the beads when adding 80% ethanol.
    ▲Use a 10 µl pipette to remove all the residual liquid.
    ▲Over-drying of beads will result in the reduction of recovery efficiency. Please avoid over-drying.
18. Take the tube out of the magnetic rack. Add 22 µl of Nuclease-free dd$H_2O$ to elute DNA. Mix thoroughly by vortexing or pipetting and incubate for 2 min at room temperature. Place the tube back on the magnetic rack, after the solution is clear (about 5 min), and carefully transfer 20 µl of the supernatant to a new Nuclease-free PCR tube without disturbing the beads.
    ▲Do not disturb the beads when transferring the supernatant. Even trace amount of beads can affect the subsequent library yield.

**The purified product can be stored at -30 ~ -15°C for 24 h.**

### 08-5/Library Amplification

1. Prepare the PCR reaction system according to the selected adapters as follows:
   When VAHTS DNA Adapters Set 3 - Set 6 for Illumina (Vazyme #N805/N806/N807/N808), or VAHTS Maxi Unique Dual Index DNA Adapters Set 1 - Set 4 for Illumina (Vazyme #N34201/N34202/N34203/N34204) are used, please refer to Table 3.

Table 3. PCR Reaction System for Universal Primers

| Components | Volume | |
|---|---|---|
| Purified ligation products | 20 µl | |
| PCR Primer Mix 5 | 5 µl | ■ |
| VAHTS HiFi Amplification Mix 3 | 25 µl | ■ |
| Total | 50 µl | |

When VAHTS Multiplex Oligos Set 4 - Set 5 for Illumina (Vazyme #N321/N322) are used, please refer to Table 4.

Table 4. PCR Reaction System for Universal Primers

| Components | Volume | |
|---|---|---|
| Purified ligation products | 20 µl | |
| VAHTS i5 PCR Primer* | 2.5 µl | |
| VAHTS i7 PCR Primer* | 2.5 µl | |
| VAHTS HiFi Amplification Mix 3 | 25 µl | ■ |
| Total | 50 µl | |

▲For multiple samples, it is recommended to prepare a mixture of above components (except for purified ligation products) in a suitable size tube first, and then dispense into each PCR tube. It is recommended to prepare 1.1 × volumes of the actual volume to compensate for the loss.

*VAHTS i5 PCR Primer and VAHTS i7 PCR Primer are provides in VAHTS Multiplex Oligos Set 4 - Set 5 for Illumina (Vazyme #N321/N322).

2. Adjust the pipettor to a 30 µl range and mix thoroughly by gently pipetting up and down 10 times.

3. Put the sample into the PCR instrument and run the following PCR program:

| Program | Temperature | Time | Cycles |
|---|---|---|---|
| Heated lid | 105°C | On | |
| Initial denaturation | 98°C | 45 sec | 1 |
| Denaturation | 98°C | 15 sec | 9 - 18 |
| Annealing | 60°C | 30 sec | |
| Extension | 72°C | 30 sec | |
| Final extension | 72°C | 1 min | 1 |
| | 4°C | Hold | |

The mRNA ratio in the equal amount of total RNA extracted from different species and individuals is not necessarily the same. According to the actual situation of species, the number of PCR cycles should be adjusted appropriately, generally 9 - 18 cycles. Refer to the following table for the number of cycles for each amount of Input RNA.

| Input RNA | | Cycles | |
|---|---|---|---|
| Protocol A/B | Protocol C | Non-strand-specific RNA-seq | Strand-specific RNA-seq |
| >1 μg | | 9 | 9 |
| 1 μg | 100 ng | 10 | 11 |
| 500 ng | 10 ng | 12 | 13 |
| 100 ng | 1 ng | 14 | 15 |
| 10 ng | 0.5 ng | 17 | 18 |

4. Purification of the PCR products with VAHTS DNA Clean Beads:

   a. Equilibrate the VAHTS DNA Clean Beads to room temperature.

   b. Suspend the VAHTS DNA Clean Beads thoroughly by inverting or vortexing. Pipet 45 μl (0.9 ×) of beads into the above amplification products. Mix thoroughly by pipetting up and down 10 times.

   c. Incubate at room temperature for 10 min to make the DNA to bind to the beads.

   d. Place the tube on a magnetic rack. After the solution is clear (about 5 min), keep it on magnetic rack, and carefully discard the supernatant without disturbing the beads.

   e. Keep the tube on the magnetic rack, add 200 μl of freshly prepared 80% ethanol to rinse the beads. Incubate for 30 sec at room temperature and carefully discard the supernatant without disturbing the beads.

   f. Repeat Step e.

   g. Keep the tube on the magnetic rack, open the lid and air-dry the beads for 5 - 10 min.

   ▲Do not disturb the beads when adding 80% ethanol.

   ▲Use a 10 μl pipette to remove all the residual liquid.

   ▲Over-drying of beads will result in the reduction of recovery efficiency. Please avoid over-drying.

h. Take the tube out of the magnetic rack. Add 25 µl of Nuclease-free dd$H_2O$ to elute the DNA. Mix thoroughly by vortexing or pipetting and Incubate 2 min at room temperature. Place the tube on the magnetic rack, after the solution is clear (about 5 min), and carefully transfer 23 µl of supernatant to a new Nuclease-free PCR tube without disturbing the beads.

▲ Do not disturb the beads when transferring the supernatant. Even trace amount of beads can affect the subsequent library yield.

### 08-6/Library Quality Control

Analyze 1 µl of the purified PCR product using Agilent DNA 1000 chip (Agilent #5067-1504). For example, as shown in **Fig 1-A/B and Fig 2.** A library with high quality should exhibit a narrow peak at the expected size. A narrow peak at 128 bp indicates the contamination of adapter dimers. To eliminate this contamination, dilute the library to 50 µl with Nuclease-free dd$H_2O$ and repeat 08-5/Library Amplification/Step 4 for another purification.

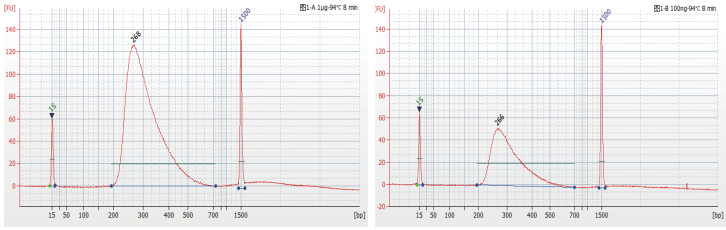


Fig 1-A/B. 1 µg/100 ng RNA of 293T cells was fragmented at 94°C for 8 min and purified with 0.6 × VAHTS DNA Clean Beads, respectively.

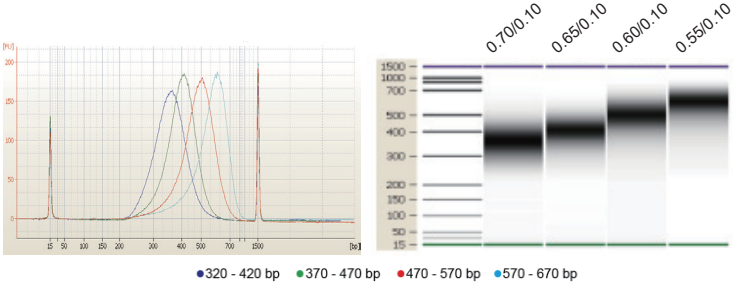



Fig 2. 200 ng RNA of 293T cells was fragmented under different conditions, purified once using 0.6 × VAHTS DNA Clean Beads, and size-selected using VAHTS DNA Clean Beads at different ratios.

## 09/FAQ & Troubleshooting

◇ Methods to solve the problem of low concentration of library.

It is recommended to use high-quality RNA samples as templates for libraries construction to make library concentration meet the requirements for sequencing. If you cannot provide qualified RNA samples, try to use the following methods to make up:

① Amount of Input RNA: Increase the amount of Input RNA.

② Prepare several duplicate samples, merge them after the fragmentation step, or before PCR step.

③ Construct libraries without size selection: Though RNA fragmented at 94°C for 8 min is short, its distribution is concentrated and the homogeneity is also well.

◇ High rRNA residue

① The compatible input amount varies for different mRNA enrichment methods. Select the total RNA input within the specification range.

② Check whether rRNA depletion kit is compatible with the species from which the Input RNA is isolated.

◇ Questions for library quantification

There are two methods for library quantification: Qubit and qPCR are used for determining library concentration and library molarity, respectively. qPCR can truly reflects the number of DNA fragments used for sequencing owing to the theory of clustered primers performing amplification quantification. Therefore, the library quantitative results measured by qPCR are more reliable. The single-stranded portion cannot be detected by the Qubit, but can be effectively measured by qPCR, thus the concentration measured by Qubit is lower than that measured by qPCR at about 10% - 50%. These two methods can be used at the same time to quantify libraries and correct each other.

◇ Is this kit suitable for small RNA library preparation?

No. Small RNA is only about 22 nt long, but the beads in this kit capture RNA at least 100 bp in size and cannot effectively enrich small RNA.

◇ Is this kit suitable for FFPE samples?

FFPE samples typically yield degraded, low-integrity RNA. We recommend using Ribo-off rRNA Depletion Kit (Human/Mouse/Rat) (Vazyme #N406) during library preparation.

◇ Using the instruction manual for size selection, why is the actual insert size different?

There are various reasons that cause the amount of magnetic beads added less than the specified value, resulting in the larger sorting insertions: the magnetic beads are not equilibrated to room temperature or not mixed thoroughly; the pipette is inaccurate, and the tip of the pipette is severely attached.

◇ How many cycle numbers at most can be used for library amplification?

The number of cycles can be adjusted according to the amount of Input RNA; it is recommended to take 1 μl for Qubit test and then make additional 1 - 2 cycles, but the maximum cycle numbers should be no more than 19.

◇ Why are there double peaks in the graph when the library was tested on the Agilent 2100 Bioanalyzer?

There are residual impurities and degrades of RNA during library construction; the amount of effective template is low when PCR, causing non-specific amplification. It is recommended to heat RNA sample at 65°C for 15 min for degradation test. If RNA is unqualified, please re-extract the RNA.

The species are special. The RNA fragments are not continuous and uniform after fragmentation, and two ranges of fragments might be obtained.

High-sensitivity chips are used for high concentration detection. It is recommended to use the Agilent DNA 1000 kit for detection or to dilute the library to the appropriate concentration and test with the Agilent DNA High Sensitivity kit.

◇ The explanations for over-amplified high-yield libraries after being tested on the Agilent 2100 Bioanalyzer, Qubit and qPCR.

High-yield libraries usually exhibit varying degrees of over-amplification. Because at the later period of library amplification, primers are usually exhausted. Therefore, a large number of library fragments can't be combined with primers, and the fragments are incorrectly annealed through incomplete matching. Thus, a hybrid strand mixed with partial double strand and partial single strand is formed in larger size. According to the corresponding principles of different detection methods, excessive amplified products show slight tailing after the upper marker in the analysis graph of Agilent 2100 Bioanalyzer. The above phenomenon is normal and would not affect the library sequencing and data analysis.

## Appendix I: Direct Size Selection of Adapter Ligation Products

For libraries with >200 bp inserts, applicable for mRNA fragmented at 94°C for 5 min, 85°C for 6 min, or 85°C for 5 min (The following is an illustration with a fragmentation condition of 85°C for 6 min, and an insert size of 350 - 450 bp. For other insert sizes, choose the volume of beads according to the tables below).

When VAHTS DNA Adapters Set 3 - Set 6 for Illumina (Vazyme #N805/N806/N807/N808), or VAHTS Maxi Unique Dual Index DNA Adapters Set 1 - Set 4 for Illumina (Vazyme #N34201/N34202/N34203/N34204) are used, please refer to Table 5 for size selection.

Table 5. Size Selection Conditions for Full Length Adapter Ligation Products

| Insert size (bp) | 200 - 300 | 250 - 350 | 350 - 450 | 450 - 550 |
|---|---|---|---|---|
| Library size (bp) | 320 - 420 | 370 - 470 | 470 - 570 | 570 - 670 |
| Fragmentation condition | 94°C 5 min | 85°C 6 min | 85°C 6 min | 85°C 5 min |
| Volume of beads for 1st round (μl) | 22 | 20 | 17 | 15 |
| Volume of beads for 2nd round (μl) | 8 | 8 | 8 | 8 |

When VAHTS Multiplex Oligos Set 4 - Set 5 for Illumina (Vazyme #N321/N322) are used, please refer to Table 6 for size selection.

Table 6. Size Selection Conditions for Stubby Adapter Ligation Products

| | | | | |
|---|---|---|---|---|
| Insert size (bp) | 200 - 300 | 250 - 350 | 350 - 450 | 450 - 550 |
| Library size (bp) | 320 - 420 | 370 - 470 | 470 - 570 | 570 - 670 |
| Fragmentation condition | 94°C 5 min | 85°C 6 min | 85°C 6 min | 85°C 5 min |
| Volume of beads for 1st round (μl) | 28 | 22 | 17 | 14 |
| Volume of beads for 2nd round (μl) | 8 | 8 | 8 | 8 |

1. Suspend the VAHTS DNA Clean Beads thoroughly by inverting or vortexing. Transfer 17 μl (0.17 ×) of the beads into the samples above. Mix thoroughly by pipetting up and down 10 times.
2. Incubate at room temperature for 10 min to make the DNA to bind to the beads.
3. Place the tube on a magnetic rack. After the solution is clear (about 5 min), keep the tube on magnetic rack, and carefully transfer 100 μl of the supernatant (**DO NOT discard**) to a new Nuclease-free PCR tube.
   ▲ If the beads are drawn out with the supernatant, the large DNA fragment residuals on the beads will result in unexpected large fragments in the final library.
4. Add 8 μl (0.08 ×) of VAHTS DNA Clean Beads and mix thoroughly by gently pipetting up and down 10 times.
5. Incubate at room temperature for 10 min to make the DNA to bind to the beads.
6. Place the tube on a magnetic rack. After the solution is clear (about 5 min), keep the tube on magnetic rack, and carefully discard the supernatant without disturbing the beads.
7. Keep the tube on the magnetic rack, add 200 μl of freshly prepared 80% ethanol to rinse the beads. Incubate for 30 sec at room temperature and carefully discard the supernatant without disturbing the beads.
8. Repeat Step 7.
9. Keep the tube on the magnetic rack, open the lid and air-dry the beads for 5 - 10 min.
   ▲ Do not disturb the beads when adding 80% ethanol.
   ▲ Use a 10 μl pipette to remove all the residual liquid.
   ▲ Over-drying of beads will result in the reduction of recovery efficiency. Please avoid over-drying.
10. Take the tube out of the magnetic rack. Add 22 μl of Nuclease-free dd$H_2O$ to elute DNA. Mix thoroughly by vortexing or pipetting and incubate for 2 min at room temperature. Place the tube back on the magnetic rack, after the solution is clear (about 5 min), carefully transfer 20 μl of the supernatant to a new Nuclease-free PCR tube without disturbing the beads.
    ▲ Do not disturb the beads while drawing samples from the supernatant. Even trace amout of beads will affect the quality of the final library.

## Appendix II: FFPE Library Preparation Protocol

FFPE RNA is derived from formalin-fixed and paraffin-embedded (FFPE) tissues. FFPE RNA is difficult to isolate (tightly cross-linked with histones) and has low quality (severe degradation). FFPE samples can be obtained from a wide range of sources and are easy to preserve, offering great value in medical applications.

Notes:

1. mRNA in FFPE samples has poor integrity due to varying degrees of degradation. We recommend increasing the template input amount (≥100 ng) and optimizing library preparation conditions to ensure a qualified library for sequencing.
2. When mapping raw data obtained from FFPE libraries, the mapping rate may decrease with increasing extent of degradation.

Library Preparation Workflow:

1. FFPE RNA quality analysis

   RNA from FFPE samples is severely fragmented due to formalin fixation, and standard RIN values may not be fully applicable to these samples. RNA quality can be evaluated with $DV_{200}$ instead.

   $DV_{200}$ measures the percentage of RNA fragments >200 nt in total RNA. $DV_{200}$>70% indicates high RNA quality; 50% - 70% indicates medium RNA quality; <50% indicates severe RNA degradation. There is a direct correlation between $DV_{200}$ and successful RNA library preparation.

2. rRNA depletion

   FFPE samples yield poor RNA integrity. We recommend using Ribo-off rRNA Depletion Kit (Human/Mouse/Rat) (Vazyme #N406) for RNA enrichment.

3. RNA fragmentation

| DV200 | Insert Size (bp) | Fragmentation Condition |
|---|---|---|
| >70% | 150 - 250 | 95°C 4min, 4°C hold |
| | 250 - 350 | 85°C 6 min, 4°C hold |
| 50% - 70% | 150 - 300 | 65°C 2 - 5 min, 4°C hold |
| <50% | / | Fragmentation not recommended |

4. Size selection conditions

   We recommend performing purification directly after ligation. Refer to the purification protocol in 08-4/Purification/Size-Selection Protocol for one round of purification.

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