VAHTS™ Total RNA-seq (H/M/R) Library Prep Kit for Illumina®

NR603

Version 8.1

Vazyme biotech co., ltd.

Introduction

The Vazyme VAHTS Total RNA-seq (H/M/R) Library Prep Kit for Illumina® is specially designed for the preparation of stranded transcriptome libraries for next generation sequencing (NGS) platforms of Illumina. The initial template is prepared with 0.1 μg-1 μg of total RNA of human, mouse, or rat. Compared with traditional transcriptome library preparation kits, this kit enables the depletion of ribosomal RNA (rRNA), including both cytoplasmic (28S, 18S, 5.8S, and5S) and mitochondrial (16S and 12S) rRNA, leaving mRNA and other non-coding RNA behind. This kit is suitable for subsequent non-coding RNA analysis such as lncRNA. Degraded RNA (i.e. FFPE RNA) could also be used to prepare the library with this kit. In addition, this kit enables the insertion of dUTP during the 2nd strand synthesis of cDNA. The double strand cDNA are digested by uracil-DNA glycosylase (UDG) to remove the second strand before sequencing. As a result, only information from the 1st strand cDNA is preserved. In addition to standard transcriptome information, strand-specific (i.e. from sense or anti-sense DNA) information can also be obtained from NGS data analysis.

Comtents of kits

	Component	NR603-01 (24 rxn)	NR603-02 (96 rxn)
NR 4	rRNA Probe (H/M/R)	24 μl	96 μl
	Probe Buffer	72 μl	288 μl
	RNase H Buffer	96 μl	384 μl
	RNase H	24 μl	96 μl
	DNase I Buffer	696 μl	4 × 696 μl
	DNase I	24 μl	96 μl
NR 5	Frag/Prime Buffer	468 μl	2×936 μl
	Actinomycin D (5 mg/ml)	24 μl	96 μl
	1st Strand Buffer	144 μl	576 μl
	1st Strand Enzyme Mix	48 μl	192 μl
	2nd Strand Marking Buffer	480 μl	2 × 960 μl
	2nd Strand/End Repair Enzyme Mix	120 μl	480 μl
NR 6	dA-Tailing Buffer Mix	240 μl	960 μl
	dA-Tailing Enzyme Mix	60 μl	240 μl
	Ligation Mix	60 μl	240 μl
	Stop Ligation Mix	120 μl	480 μl
	PCR Primer Mix	120 μl	480 μl
	Amplification Mix 1	600 μl	4 × 600 μl
	Heat-labile UDG	24 μl	96 μl

Storage

All components of this kit should be stored at -20℃.

Applications

Requirements for Starting Materials: 0.1 μg-1 μg total RNA of human, mouse. Degraded RNA (i.e. FFPE RNA) could also be used to prepare the library with this kit.

Information of Transcripts: : This kit is applicable to stranded mRNA or non-coding RNA (with the exception of rRNA) related analysis with RNA-seq, including gene expression analysis, single nucleotide variation calling, alternative splicing / fusion detection, target transcriptome analysis, and target genes prediction and functional analysis.

If conventional mRNA of animal, plant, or fungal is under consideration, please use the VAHTS mRNA-seq V2 Library Prep Kit for Illumina® (Vazyme, #NR601) for library construction. If stranded mRNA of animal, plant, or fungal is under consideration, please use the VAHTS Stranded RNA-seq Library Prep Kit for Illumina® (Vazyme, #NR602) for library construction.

Additional Materials Required

DNA Clean Beads: VAHTS DNA Clean Beads (Vazyme,#N411) or Agencourt® AMPure® XP Beads (Beckman Coulter, #A63880, #A63881, #A63882).

RNA Clean Beads: VAHTS RNA Clean Beads (Vazyme, #N412) or Agencourt® RNAClean® XP Beads(Beckman Coulter, #A63987).

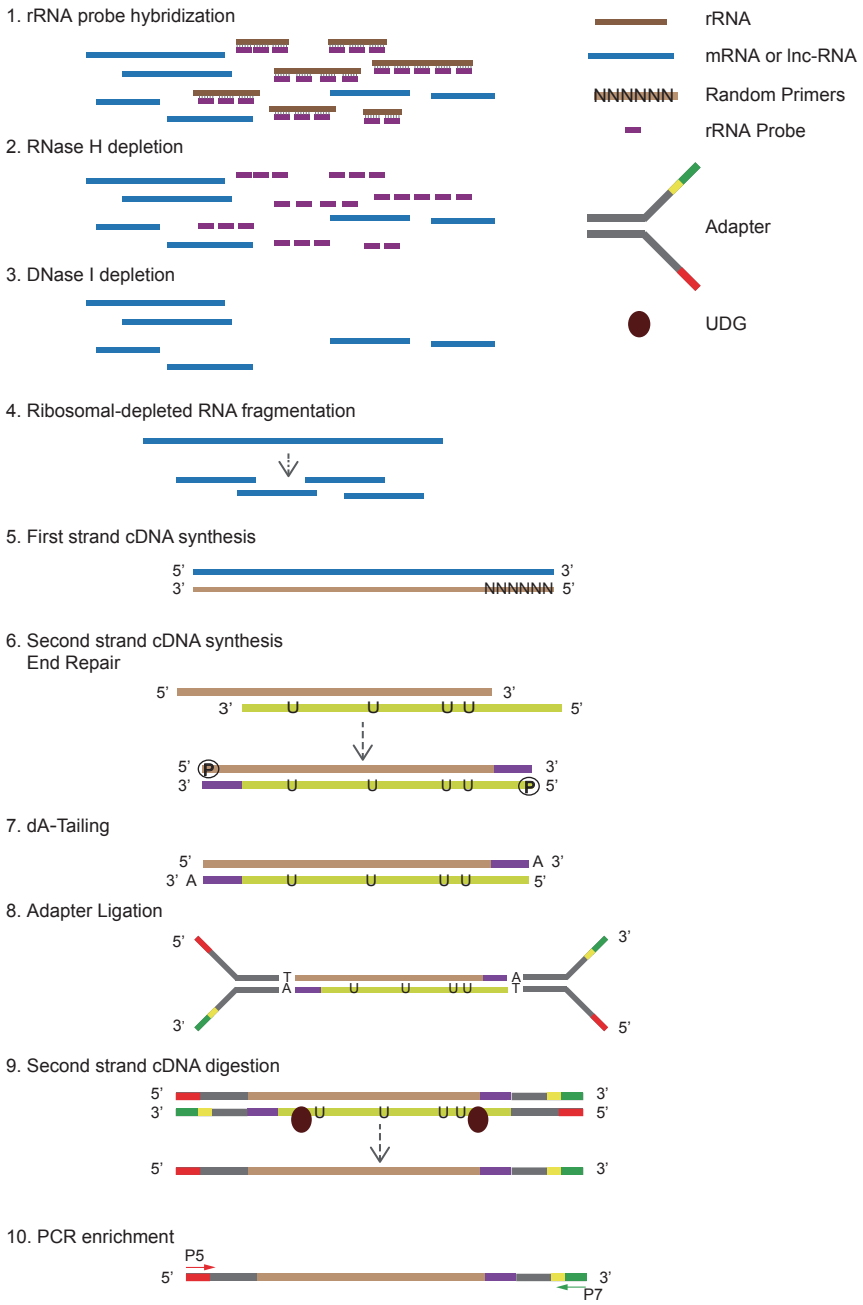
RNA Analysis： Agilent RNA 6000 Pico Kit (Agilent, #5067-1513).

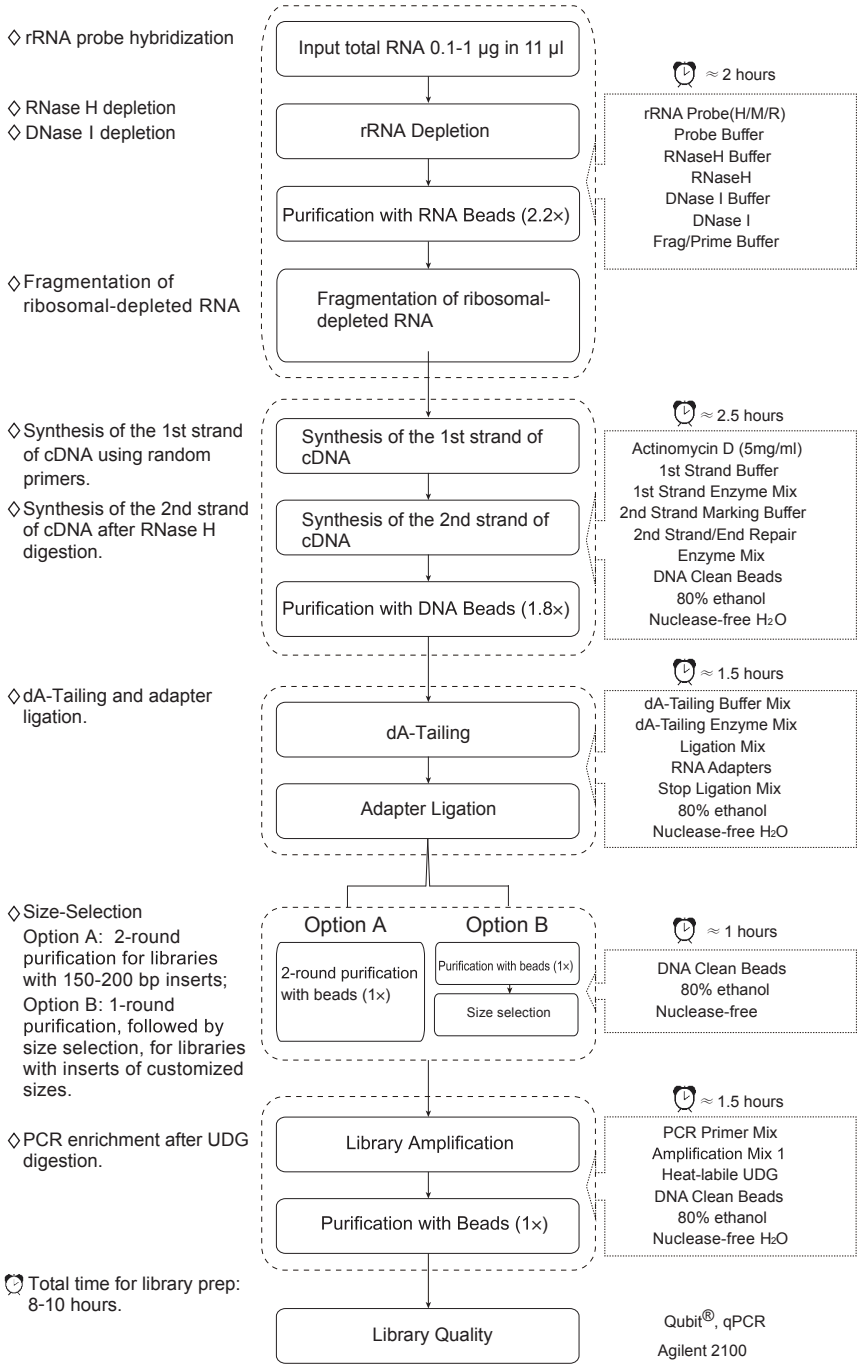
Library Analysis: Agilent DNA 1000 Kit (Agilent, #5067-1504).

Adapters: VAHTS RNA Adapters Set 1 - Set 2 for Illumina® (Vazyme, #N803, #N804), or VAHTS RNA Adapters Set 3 - Set 6 for Illumina® (Vazyme, #N809, #N810, #N811, #N812).

Other Materials: Fresh Ethanol (80%), Nuclease-free Water, Nuclease-free PCR tubes, Low absorption EP tubes, Agilent 2100 Bioanalyzer, Thermocyler (PCR instrument) ,Magnetic stand.

Mechanism & Workflow





Protocol

1. rRNA Depletion and Fragmentation

Preparation of total RNA sample

1.1. Dilute 0.1 - 1 μg of total RNA with 11 μl of Nuclease-free Water in a Nuclease-free PCR tube and keep on ice.

rRNA/Probe hybridization

1.2. Prepare the following reaction solution in a Nuclease-free PCR tube:

rRNA Probe (H/M/R)	1 μl	■
Probe Buffer	3 μl	■
Total RNA	11 μl	
Total	15 μl	

Mix by gently pipetting for 10 times.

1.3. Collect the liquid to the bottom of the tube by a brief centrifugation. Put the sample into a PCR instrument and run the following program (Hot Lid Temperature: 105℃):

95℃	2 min
95-22℃	0.1℃/sec
22℃	5 min

Digestion with RNase H

1.4. Prepare the following reaction solution on ice:

RNase H Buffer	4 μl	■
RNase H	1 μl	■
Products of **Step1.3**	15 μl	
Total	20 μl	

Mix by gently pipetting.

1.5. Place the sample in a PCR instrument and incubate at 37℃ for 30 min (Hot Lid Temperature: 105℃).

Digestion with DNase I

1.6. Prepare the following reaction solution on ice:

DNase I Buffer	29 μl	■
DNase I	1 μl	■
RNase H Digested Products	20 μl	
Total	50 μl	

Mix by gently pipetting.

1.7. Place the sample in a PCR instrument and incubateat 37℃ for 30 min(Hot Lid Temperature: 105℃). Collect the liquid to the bottom of the tube by a brief centrifugation. Put the tube on ice and immediately proceed to the next procedure.

Purification of ribosomal-depleted RNA with VAHTS RNA Clean Beads

1.8. Suspend the **VAHTS RNA Clean Beads** thoroughly by inverting or vortexing, pipet 110 μl (2.2×)of beads into the RNA sample of **Step1.7.** Mix thoroughly by pipetting for 10 times.

1.9. Incubate the sample on ice for 15 min to make the RNA bind to the beads.

1.10. Put the sample onto a magnetic stand. Wait until the soultion clarifies (about 5 min). Then carefully discard the supernatant without disturbing the beads.

1.11. Keep the sample on the magnetic stand, add 200 μl of freshly prepared 80% ethanol to rinse the beads. DO NOT re-suspend the beads! Incubate at room temperature for 30 sec and carefully discard the supernatant without disturbing the beads.

Note: It is highly recommended to use a 10-μl pipettor to remove the residual supernatant in this step.

1.12. Repeat **Step 1.11**

1.13. Keep the sample on the magnetic stand, open the tube and air-dry the beads for 5-10 min.

1.14. Take the sample out of magnetic stand. Add 18.5 μl of Frag/Primer Buffer and mix thoroughly by pipetting for 6 times. Incubate at room temperature for 2 min. Put the tube back on the magnetic stand and wait until the soultion clarifies (about 5 min). Carefully transfer 16 μl of the supernatant to a new Nuclease-free PCR tube without disturbing the beads.

1.15. ncubate the sample in a PCR device and set programs according to the fragment size required:

- For **150-200 bp insert**: incubate at 94℃ for 8 min,then hold at 4℃.
- For **200-300 bp insert**: incubate at 94℃ for 5 min,then hold at 4℃.
- For **250-450 bp insert**: incubate at 85℃ for 6 min,then hold at 4℃.
- For **450-550 bp insert**: incubate at 85℃ for 5 min,then hold at 4℃.

Immediately proceed to **Step2. Synthesis of Double Strand cDNA.**

2. Synthesis of Double Strand cDNA

2.1. Tap the tube several times to mix thoroughly, collect the liquid at the bottom of the tube by a brief centrifugation. Dilute to 0.5 mg/ml by adding 1 μl Actinomycin D (5 mg/ml) to 9 μl Nuclease-free Water and use immediately.

Note: The Diluted Actinomycin D solution is highly sensitive to light and will attach to the surfaces of plastic and glass. Discard unused diluted solution.

2.2. Thaw the 1st Strand Buffer and mix it thoroughly by inverting the tube. Prepare the reaction solution to synthesize the first strand of cDNA as follows:

Fragmented mRNA	16 μl
Actinomycin D (0.5 mg/ml)	1 μl
1st Strand Buffer	6 μl
1st Strand Enzyme Mix	2 μl
Total	25 μl

Mix thoroughly by gently pipetting for 10 times.

2.3. Put the sample in a PCR instrument and run the following program (Hot Lid Temperature: 105℃) :

25 ℃	10 min
42 ℃	15 min
70 ℃	15 min
4 ℃	Hold

Immediately proceed to **Step2.4** for the 2nd Strand Synthesis of cDNA.

2.4. Thaw the 2nd Strand Buffer and mix it thoroughly by inverting the tube. Prepare the reaction solution to synthesize the 2nd strand of cDNA as follower:

1st Strand cDNA	25 μl
2nd Strand Buffer	20 μl
2nd Strand Enzyme Mix	5 μl
Total	50 μl

Mix thoroughly by gently pipetting up and down for 10 times.

2.5. Put the sample in a PCR instrument and run the following program (Hot Lid Temperature: 30℃) :

16 ℃	60 min
4 ℃	Hold

Note: The double strand cDNA products can be stored at 4℃ for less than 60 min.

2.6. Purification of the double strand cDNA

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

b. Suspend the VAHTS DNA Clean Beads thoroughly by inverting or vortexing. Pipet 90 ul (1.8x) of beads into the above sample. Mix thoroughly by pipetting up and down for 10 times.

c. Incubate at room temperature for 10 min.

d. Place the sample on a magnetic stand. Wait until the solution clarifies (about 5 min). Keep it on magnetic stand and carefully discard the supernatant without disturbing the beads.

e. Keeping the sample on the magnetic stand, add 200 ul of freshly prepared 80% ethanol to rinse the beads. **DO NOT re-suspend the beads!** Incubate at room temperature for 30 sec and carefully discard the supernatant without disturbing the beads.

f. Repeat the **Step e.**

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

h. Take the sample out of the magnetic stand. Add 20 ul of Nuclease-free Water to elute the cDNA. Mix thoroughly by vortexing or pipetting, and incubate for 2 min at room temperature. Put the tube on the magnetic stand and wait until the solution clarifies (about 5 min). Carefully transfer 17.5 ul of supernatant to a new Nuclease-free PCR tube without disturbing the beads.

Note: The dilution can be stored at -20℃ for 24 hours.

3. dA-Tailing

3.1. Thaw the dA-tailing Buffer Mix and mix it thoroughly by inverting the tube. Prepare the reaction solution as follows:

Purified End-Repair Products	17.5 μl	
dA-tailing Buffer Mix	10 μl	■
dA-tailing Enzyme Mix	2.5 μl	■
Total	30 μl	

Mix thoroughly by gently pipetting for 10 times.

3.2. Put the sample in a PCR instrument and run the following program for dA-Tailing (Hot Lid Temperature 105℃):

37℃	30 min
70℃	5 min
4℃	Hold

Immediately proceed to **Step 4. Adapter Ligation** .

Note: The dA-tailing products can be stored at 4℃ for less than 60 min.

4. Adapter Ligation

4.1. Thaw the RNA Adapter and mix it thoroughly by inverting the tube. Prepare the reaction solution as follows:

Purified dA-ailing Products	30 μl	
Ligation Mix	2.5 μl	■
RNA Adapter*	2.5 μl	□
Total	35 μl	

*VAHTS RNA Adapters Set 1 for Illumina® (Vazyme, #N803) contains 12 adapters (Adapter 1 to 12).

VAHTS RNA Adapters Set 2 for Illumina® (Vazyme, #N804) contains 12 adapters (Adapter 13 to 27).

VAHTS RNA Adapters Set 3 for Illumina® (Vazyme, #N809) contains 24 adapters (Adapter 96-01 to 96-24).

VAHTS RNA Adapters Set 4 for Illumina® (Vazyme, #N810) contains 24 adapters (Adapter 96-25 to 96-48).

VAHTS RNA Adapters Set 5 for Illumina® (Vazyme, #N811) contains 24 adapters (Adapter 96-49 to 96-72).

VAHTS RNA Adapters Set 6 for Illumina® (Vazyme, #N812) contains 24 adapters (Adapter 96-73 to 96-96).

Mix thoroughly by gently pipetting for 10 times.

4.2. Put the sample in a PCR instrument and run the following program for adapter ligation (Hot Lid Temperature: 105℃):

30℃	10 min
4℃	Hold

Immediately proceed to the next step.

4.3. Add 5 μl of the Stop Ligation Mix to 35 μl of ligation products, mix thoroughly by gently pipetting up and down for 10 times to terminate the ligation reaction.

Note: The adapter-ligation products can be stored at 4℃ for less than 60 min.

5.Purification and Size Selection of Adapter-ligated DNA

Option A for libraries with 150-200 bp inserts (for mRNA fragmented by incubation at 94℃ for 8 min)

5A.1. Equilibrate the VAHTS DNA Clean Beads to room temperature.

5A.2. Suspend the VAHTS DNA Clean Beads thoroughly by inverting or vortexing. Pipet 40 μl (1×) of beads into the above sample. Mix thoroughly by pipetting up and down for 10 times.

5A.3. Incubate at room temperature for 10 min.

5A.4. Place the sample on a magnetic stand. Wait until the soultion clarifies (about 5 min). Keep it on magnetic stand, and carefully discard the supernatant without disturbing the beads.

5A.5. Keeping the sample on the magnetic stand, add 200 µl of freshly prepared 80% ethanol to rinse the beads. **DO NOT re-suspend the beads!** Incubate for 30 sec at room temperature and carefully discard the supernatant without disturbing the beads.

5A.6. Repeat the **Step 5A.5.**

5A.7. Keep the sample on the magnetic stand, open the tube and air-dry the beads for 5-10 min.

5A.8. Take the sample out of the magnetic stand. Add 52.5 µl of Nuclease-free Water to elute DNA. Mix thoroughly by vortexing or pipetting and incubate for 2 min at room temperature. Place the tube back on the magnetic stand and wait until the soultion clarifies (about 5 min).Carefully transfer 50 µl of the supernatant to a new Nuclease-free PCR tube without disturbing the beads.

5A.9. Suspend the VAHT DNA Clean Beads thoroughly by inverting or vortexing. Pipet 50 µl (1×) of the suspended beads to the product above. Mix thoroughly by pipetting up and down for 10 times.

5A.10. Incubate at room temperature for 10 min.

5A.11. Place the sample on the magnetic stand. Wait until the solution clarifies (about 5 min). Keep it on the magnetic stand, and carefully discard the supernatant without disturbing the beads.

5A.12. Keep the sample on the magnetic stand, and add 200 µl of freshly prepared 80% ethanol to rinse the beads. **DO NOT re-suspend the beads!** Incubate for 30 sec at room temperature and carefully discard the supernatant without disturbing the beads.

5A.13. Repeat the Step 5A.12.

5A.14. Keep the sample on the magnetic stand, open the tube and air-dry the beads for 5-10 min.

5A.15. Take the sample out of the magnetic stand. Add 21.5 µl of nuclease-free water to elute the DNA. Mix thoroughly by vortexing or pipetting and place for 2 min at room temperature. Place the tube on the magnetic stand and wait until the solution clarifies (about 5 min). Carefully transfer 19 µl of supernatant to a new Nuclease-free PCR tube without disturbing the beads.

Note: Immediately proceed to **Step 6. Library Amplification.**

Note: The dilution can be stored at -20˚C for 24 hours.

Note: DO NOT disturb the beads while drawing samples from the supernatant. Even trace amount of beads will affect the quality of the final library.

Option B for libraries with > 200 bp inserts (for mRNA fragmented by incubation at 94˚C for 5 min, 85˚C for 6 min, or 85˚C for 5 min)

B-1.Purfication of ligation proucts using 1 × VAHTS DNA Clean Beads

5B.1. Equilibrate the VAHTS DNA Clean Beads to room temperature.

5B.2. Suspend the VAHTS DNA Clean Beads thoroughly by inverting or vortexing. Pipet 40 µl (1×) of beads into the sample above. Mix thoroughly by pipetting up and down for 10 times.

5B.3. Incubate at room temperature for 10 min.

5B.4. Place the sample on a magnetic stand. Wait until the solution clarifies (about 5 min). Keep it on the magnetic stand and carefully discard the supernatant without disturbing the beads.

5B.5. Keeping the sample on the magnetic stand, add 200 µl of freshly prepared 80% ethanol rinse the beads. **DO NOT re-suspend the beads!** Incubate for 30 sec at room temperature and carefully discard the supernatant without disturbing the beads.

5B.6. Repeat the **Step 5B.5.**

5B.7. Keep the sample on the magnetic stand, open the tube and air-dry the beads for 5-10 min.

5B.8. Take the sample out of the magnetic stand. Add 102.5 µl of Nuclease-free Water to elute DNA. Mix thoroughly by vortexing or pipetting and incubate for 2 min at room temperature. Place the tube back on the magnetic stand. Wait until the solution clarifies (about 5 min).Carefully transfer 100 µl of the supernatant to a new Nuclease-free PCR tube without disturbing the beads.

B-2.Size selection with VAHTS DNA Clean Beads

The following protocol is for a library with 350-450 bp inserts (as an example). Please refer to **Table 1** for the appropriate volume of beads for libraries with inserts of other sizes.

Table 1. Recommended conditions for bead-based size selection

Insertion Length (bp)	200 - 300	250 - 350	350 - 450	450 - 550
Library Length (bp)*	320 - 420	370 - 470	470 - 570	570 - 670
Fragmentation Condition	94℃, 5 min	85℃, 6 min	85℃, 6 min	85℃, 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 ×)

*Full library length means the peak size range determined by Agilent 2100 Bioanalyzer. Library length is equal to insertion length plus adapter length (120 bp). Please refer to **Step 7.4** for further information.

5B.9. Suspend the VAHTS DNA Clean Beads thoroughly by inverting or vortexing. Transfer 60 μl (0.6 ×) of beads into the sample above. Mix thoroughly by pipetting up and down for 10 times.

5B.10. Incubate at room temperature for 10 min.

5B.11. Place the sample on a magnetic stand. Wait until the solution clarifies (about 5 min). Keep it on magnetic stand and carefully transfer 155 μl of the supernatant into a new Nuclease-free PCR tube.

5B.12. Add 10 μl (0.1 ×) of VAHTS DNA Clean Beads, mix thoroughly by pipetting up and down for 10 times.

5B.13. Incubate at room temperature for 10 min.

5B.14. Place the sample on the stand. Wait until the solution clarifies (about 5 min). Keep it on the magnetic stand and carefully discard the supernatant without disturbing the beads.

5B.15. Keeping the sample on the magnetic stand, add 200 μl of freshly prepared 80% ethanol to rinse the beads. **DO NOT re-suspend the beads!** Incubate for 30 sec at room temperature and carefully discard the supernatant without disturbing the beads.

5B.16. Repeat the **Step 5B.15.**

5B.17. Keep the sample on the magnetic stand, open the tube and air-dry the beads for 10 min.

5B.18. Take the sample out of the magnetic stand. Add 21.5 μl of nuclease-free water to elute DNA. Mix thoroughly by vortexing or pipetting and incubate for 2 min at room temperature. Place the tube back on the magnetic stand and wait until the solution clarifies (about 5 min). Carefully transfer 19 μl of the supernatant to a new Nuclease-free PCR tube without disturbing the beads.

Note: Immediately proceed to **Step 6. Library Amplification.**

Note: The dilution can be stored at -20℃ for 24 hours.

Note: DO NOT disturb the beads while drawing samples from the supernatant. Even trace amount of beads will affect the quality of the final library.

6. Library Amplification

6.1. Thaw the PCR Primer Mix and Amplification Mix 1 thoroughly by inverting the tube. Prepare the reaction solution as follows:

Purified Ligation Product	19 μl	
PCR Primer Mix	5 μl	
Amplification Mix 1	25 μl	
Heat-labile UDG	1 μl	
Total	50 μl	

Mix thoroughly by gently pipetting up and down for 10 times.

6.2. Put the sample in a PCR instrument and run the following PCR program (Hot Lid Temperature: 105℃)

Procedure	Temperature	Time	Cycles
UDG Digestion	37℃	10 min	1
Pre-denaturation	98℃	30 sec	1
Denaturation	98℃	10 sec	15
Annealing	60℃	30 sec	
Extension	72℃	30 sec	
Complete Extension	72℃	5 min	1
Hold	4℃		

Note: The recommended PCR cycle number is 15, which can be adjust between 12 and 15 according to user's needs.

Note: The amplified library can be stored at 4℃ for 60 min.

6.3. Purification of the PCR product 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. Transfer 50 μl (1×) of the beads into the PCR product. Mix thoroughly by pipetting up and down for 10 times.

c. Incubate at room temperature for 10 min.

d. Place the sample on a magnetic stand. Wait until the solution clarifies (about 5 min). Keep it on the magnetic stand and carefully discard the supernatant without disturbing the beads.

e. Keeping the sample on the magnetic stand, add 200 μl of freshly prepared 80% ethanol to rinse the beads. **DO NOT re-suspend the beads!** Incubate for 30 sec at room temperature and carefully discard the supernatant without disturbing the beads.

f. Repeat the **Step e**.

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

h. Take the sample out of the magnetic stand. Add 25 μl of Nuclease-free Water to elute DNA. Mix thoroughly by vortexing or pipetting and incubate for 2 min at room temperature. Place the tube back on the magnetic stand and wait until the solution clarifies (about 5 min).Carefully transfer 22.5 μl of the supernatant to a new Nuclease-free PCR tube without disturbing the beads.

Note:The dilution can be stored at -20℃.

Note: **DO NOT** disturb the beads while drawing samples from the supernatant. Even trace amount of beads will affect the quality of the final library.

6.4. Library Quality Determination Using an Agilent Technologies 2100 Bioanalyzer.

Analyze 1 μl of purified PCR product using a Agilent DNA 1000 chip (Agilent, #5067-1504). As shown in **Fig.1**, a library with high quality should exbit a narrow peak at the expected size. A narrow peak at 128 bp suggests the contamination of adapter-dimer. To eliminate this contamination, dilute the library to 50 μl with Nuclease-free Water and repeat **Step 6.3** for further purification.

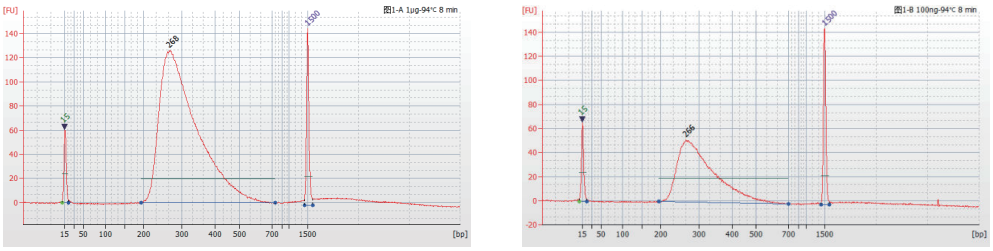


Fig1.100 ng universal human reference RNA, fragmented at 94℃ for 8 min and purified twice with VAHTS DNA Clean Beads (1×).

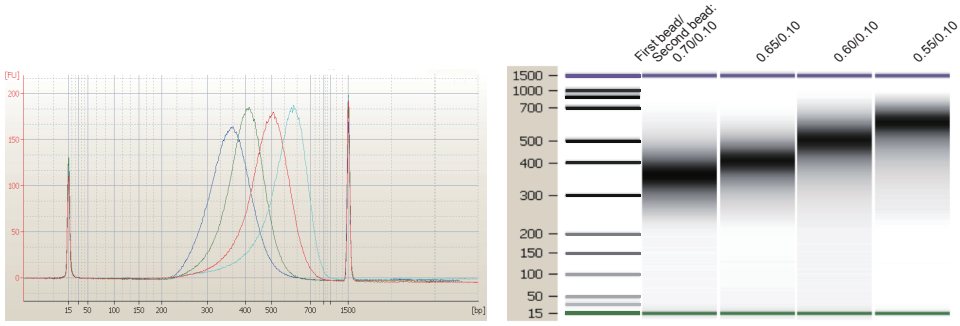


Fig1.200 ng universal human reference RNA, fragmented under different conditions, and purified once with VAHTS DNA Clean Beads (1×), followed by size selection steps according to **Table 1.**

Tips

1. VAHTS DNA Clean Beads Tips

Equilibrate the beads to room temperature before use.
Mix the beads thoroughly every time before pipetting.
Thoroughly mix the beads with DNA samples.
All the DNA size selection and procedures using beads should be performed at room temperature.
Do not pipet any when transferring the supernatant.
Prepare fresh 80% ethanol and discard after use.
Try to remove all the 80% ethanol after washing.
Thoroughly air-dry the beads before DNA elution.

2. Avoid cross contamination between samples.

Change tips between samples.
Use filtered pipette tips.

3. Aliquot reagents after the first use to avoid repeated freeze-thaw cycles.

4. Prevent contamination of PCR products.

Isolate the experimental area and carefully clean all equipments and instruments (e.g. clean with 0.5% sodium hypochlorite or 10% bleach) to avoid possible contamination in PCR system.

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