RNA Library Prep Kit

REF CAMC02-05 (48 Reactions)

PRODUCT NAME
RNA Library Prep Kit

INTENDED USE
RNA Library Prep Kit is an in vitro diagnostic test for the library construction of RNA in bronchoalveolar lavage (BAL), sputum, plasma, and cerebrospinal fluid (CSF) samples. It is used in conjunction with the adapted nucleic acid extraction kit and universal reaction kit for sequencing (Combinatorial Probe-Anchor Synthesis Sequencing Method) for qualitative detection of pathogens in the sample.

PRINCIPLE
The RNA Library Prep Kit f is based on combinatorial probe-anchor synthesis sequencing method (cPAS) to detect pathogenic metagenomics of RNA samples. The RNA extracted from clinical samples is subjected to fragment by heating, reverse transcription, cDNA double-strand synthesis and end repair, and then a specific adapter is ligated and amplified to prepare an RNA sequencing library. The sequencing library was used with the Universal Sequencing Kit to obtain DNA sequence information. The principle of library preparation is shown in Figure 1.

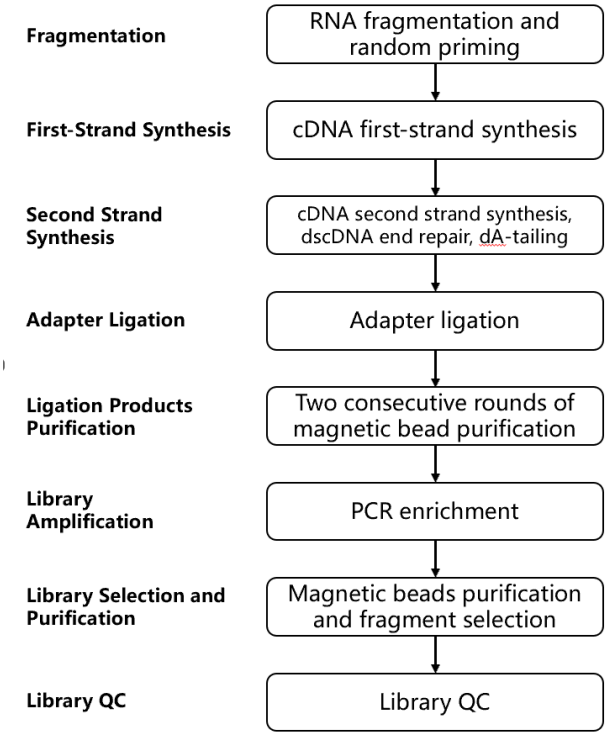

Figure 1: RNA sample Library Preparation Principle

STORAGE AND STABILITY
- Box 1 and 3 should be stored below -25℃~-15℃.
- Box 2 should be stored at 2~8℃.
- The shelf life is 12 months.
- Production date and expiration date are shown in the package label.

REAGENT AND MATERIALS PROVIDED

Kit	Component	Specification	Quantity
Box 1	Frag/Primer buffer	384 μL/ tube	1 tube
	Enhancer R	48 μL/ tube	1 tube
	1st Strand Buffer	288 μL/ tube	1 tube
	1st Strand Enzyme Mix	96 μL/ tube	1 tube
	2nd Strand Buffer	1200 μL/ tube	1 tube
	2nd Strand Enzyme Mix	720 μL/ tube	1 tube
	Ligation Buffer R	1200 μL/ tube	1 tube
	Ligase R	240 μL/ tube	1 tube
	PCR amplification Mix	1200 μL/ tube	1 tube
	MSI-PCR primer	240 μL/ tube	1 tube
Box 2	Purified beads	4.8 mL/ tube	2 tubes
	Elution Buffer	1.5 mL/ tube	1 tube
	Nuclease-free H2O	3 mL/ tube	2 tubes
Box 3	MSI-Adapter	10 μL/ tube	1 tube

Note: Kits with different batch numbers cannot be mixed for use.

MATERIALS REQUIRED BUT NOT PROVIDED
Equipment: Single-channel pipette (1000 μL, 200 μL, 100 μL, 10 μL, 2.5 μL), 8-channel pipette (300 μL or 200 μL, 50 μL, 10 μL), magnetic rack (adapted to 1.5 mL centrifuge tube), PCR instrument, shaker, short time centrifuge machine, etc.

Reagents:

Function	Name	Manufacture
Sequencing	DNA Circularization Kit	Fuzhou Agenmic Biotechnology Co., Ltd
	MGIEasy Circularization Kit	MGI Tech Co., Ltd.
	Universal Sequencing Kit (cPAS)	MGI Tech Co., Ltd.
Others	Nucleic Acid Remover	Fuzhou Agenmic Biotechnology Co., Ltd
	Qubit™ dsDNA HS Assay Kit	Thermo Fisher Scientific
	Nuclease-free H2O	Thermo Fisher Scientific
	Absolute Ethanol	——
	UltraPure Sterile Water	——
	TE buffer	——

APPLICABLE TEST SYSTEM
Suitable for Sequencer DNBSEQ-G50, DNBSEQ-G400 FAST, DNBSEQ-G99 from MGI Tech Co., Ltd.

SPECIMEN COLLECTION AND REQUIREMENTS
1. Applicable sample types: bronchoalveolar lavage (BAL), sputum, plasma, and cerebrospinal fluid (CSF).
2. Sample collection process: collect sample according to the clinical test sample collection guidelines, perform aseptic operation, and place the collected samples in sterile tubes.
3. Precautions for sample collection: avoid contamination during

sample collection, storage and transfer.
4. Sample storage and transportation: after sample collection, it is recommended to test within 12 hours. If it cannot be tested in time, store it at -18°C or below, and complete the test within one month. Store it at -70°C or below for long-term storage, valid within 1 year. Samples should be shipped on dry ice. Freezing and thawing times should not exceed 3 times. Frozen samples should be thawed at room temperature before testing, and mixed thoroughly before use.
5. Sample safety: all samples are considered to be potentially infectious, and operations should be performed in accordance with relevant national standards.
6. Storage of nucleic acid: The extracted nucleic acid should be prepared for library immediately. If it cannot be detected in time, it should be stored at 2°C~8°C and processed within 12 hours.
Perform library preparation, or store at -18°C or below. Sequence should be performed immediately after library preparation, if sequencing cannot be done in time, store at -18°C or below, and complete the detection within 30 days.

TEST PROCEDURE

Refer to manual before the test. Confirm that the test kit is not open or broken before use, and the test should be performed at a temperature between 2-30°C.

1. Reagent preparation:

1.1 Take the reagents from box 1. The enzyme reagents (1st Strand Enzyme Mix, 2nd Strand Enzyme Mix, Ligase R, PCR amplification Mix) need to be briefly centrifuged and placed on ice for later use. Other reagents should be melted on ice, vortexed to mix, and briefly centrifuged for later use.
1.2 The magnetic beads should be balanced at room temperature for 30 min before use, and mixed well before use.
1.3 Prepare 80% ethanol with absolute ethanol and molecular grade water, and prepare and use according to the dosage.

2. Nucleic acid extraction:

It is recommended to use the "RNA Extraction Kit" produced by Fuzhou Agenmic Biotechnology Co., Ltd and strictly follow the instructions to extract the sample nucleic acid.
When extracting each batch of clinical samples, use a Nuclease-free H2O as a negative quality control sample for extraction.

3. Nucleic acid sample quantification and preparation:

It is recommended to use a Qubit Fluorometers or an instrument with equivalent functions to measure the concentration of the extracted nucleic acid (DNA & RNA), and operate in strict accordance with the instructions. The concentration of nucleic acid should be greater than or equal to 0.1ng/μL, otherwise the extraction is considered unqualified and needs to be re-extracted.

4. Library preparation:

4.1 Fragmentation:
1) Take out the Frag/Prime Buffer, thaw and mix well, and briefly centrifuge to collect to the bottom of the tube. All the following steps should be performed on ice.
2) Take the PCR tube and mark it, and prepare the following reactions according to the table below:

Components	Volume
Frag/Prime Buffer	8 μL
Input RNA	8 μL

3) Vortex the PCR tube and place it in a PCR machine to run the following program (the total volume of the system is 16 μL):

Temperature	Time
Heated lid 105°C	On
94°C	8 minutes
4 °C	Hold

4.2 First-strand synthesis:
1) Prepare Enhancer R: Take a 1.5 mL centrifuge tube, add 48.8 μL Nuclease-free H2O and 1.2 mL Enhancer R, vortex to mix, and centrifuge briefly.
2) According to the number of samples N, prepare first-strand synthesis Mix according to the following table, vortex and mix, then centrifuge briefly, and place on ice for later use:

Components	Volume
Enhancer R (after dilution)	1 μL * (N+1)
1st Strand Synthesis Buffer	6 μL * (N+1)
First-strand synthetase	2 μL * (N+1)

3) Briefly centrifuge the fragmented product, and add 9 μL of first-strand synthesis Mix to each tube, vortex to mix, briefly centrifuge and put on the PCR machine, the program is as follows (total system volume 25 μL):

Temperature	Time
Heated lid 105°C	On
25°C	10 minutes
42°C	15 minutes
70°C	15 minutes
4 °C	Hold

4.3 Second-strand synthesis:
1) According to the number of samples N, prepare second-strand synthesis Mix according to the following table, vortex and mix, then centrifuge briefly, and place on ice for later use:

Components	Volume
2nd Strand Synthesis Buffer	25 μL * (N+1)
2nd Strand Enzyme Mix	15 μL * (N+1)

2) Briefly centrifuge the first-strand synthesis product, and add 40 μL of second-strand synthesis Mix to each tube, vortex to mix, briefly centrifuge and put on the PCR machine, the program is as follows (total system volume 65 μL):

Temperature	Time
Heated lid 105°C	On
16°C	30 minutes
65°C	15 minutes
4 °C	Hold

4.4 Adapter Ligation:
1) According to the number of samples N, prepare the adapter ligation Mix according to the following table, vortex and mix, then centrifuge briefly, and place on ice for later use:

Components	Volume
Ligation Buffer R	25 μL * (N+1)
Ligase R	5 μL * (N+1)

2) Briefly centrifuge the second-strand synthesis product, and add 30 μL of adapter ligation Mix to each tube, and add 5 μL of MSI-adapter, vortex to mix, briefly centrifuge and then put on the PCR machine, the program is as follows (total system volume 100 μL):

Temperature	Time
Heated lid	Off
20°C	15 minutes
4 °C	Hold

4.5 Ligation products purification 1:
1) After the purified magnetic beads are balanced to room temperature, vortex to mix, and pipette 45 μL of purified magnetic beads to a new 1.5 mL centrifuge tube.
2) Add 100 μL adapter ligation product, vortex to mix, and incubate at room temperature for 5 min.
3) Centrifuge the centrifuge tube briefly and place it on a magnetic rack, let it stand for 3 min until the solution is clear, then carefully remove the supernatant.
4) Keep the centrifuge tube on the magnetic rack all the time, add 200 μL of freshly prepared 80% ethanol to rinse the magnetic beads, incubate at room temperature for 30 sec, and carefully remove the supernatant.
5) Repeat step 4 for a total of two rinses.
6) Keep the centrifuge tube on the magnetic rack all the time, open the caps and air-dry until the surface of the magnetic beads has no water reflection and is matte.

7) Remove the centrifuge tubes from the magnetic rack, add 52 μL of Nuclease-free H2O, vortex to mix, and incubate at room temperature for 5 min.
8) Centrifuge the centrifuge tube briefly and place it on the magnetic rack for 2 min. After the magnetic beads are completely absorbed, pipette 50 μL of the supernatant into a new 1.5 mL centrifuge tube.

4.6 Ligation products purification 2:
1) After the purified magnetic beads are balanced to room temperature, vortexed to mix, and add 50 μL purified magnetic beads to the purified product in the last step.
2) Vortex to mix and incubate at room temperature for 5 min.
3) Centrifuge the centrifuge tube briefly and place it on a magnetic rack, let it stand for 3 min until the solution is clear, then carefully remove the supernatant.
4) Keep the centrifuge tube on the magnetic rack all the time, add 200 μL of freshly prepared 80% ethanol to rinse the magnetic beads, incubate at room temperature for 30 sec, and carefully remove the supernatant.
5) Repeat step 4 for a total of two rinses.
6) Keep the centrifuge tube on the magnetic rack all the time, open the caps and air-dry until the surface of the magnetic beads has no water reflection and is matte.
7) Remove the centrifuge tube from the magnetic rack, add 22 μL of Nuclease-free H2O, vortex to mix, and incubate at room temperature for 5 min.
8) Centrifuge the centrifuge tube briefly and place it on the magnetic rack for 2 min. After the magnetic beads are completely adsorbed, pipette 20 μL of the supernatant into a new centrifuge tube and proceed to the next step of library amplification.

4.7 Library amplification:
1) According to the number of samples N, prepare the library amplification Mix according to the following table, vortex and mix well, then centrifuge briefly, and place on ice for later use:

Components	Volume
PCR amplification Mix	25 μL * (N+1)
MSI-PCR primer	5 μL * (N+1)

2) Briefly centrifuge the purified product in the last step, and add 30 μL of library amplification Mix to each tube, vortex to mix, briefly centrifuge, and then put on a PCR machine. The program is as follows (total system volume 50 μL):

Temperature	Time	Number of cycles
Heated lid 105°C	On	——
98 °C	30 sec	1
98 °C	10 sec	17
60°C	30 sec	
72°C	30 sec	
72°C	5 min	1
4 °C	Hold	——

4.8 Library fragment selection and purification:
1) After the purified magnetic beads are balanced to room temperature, vortex to mix, and pipette 70 μL of purified magnetic beads to a new 1.5 ml centrifuge tube.
2) Add 50 μL Nuclease-free H2O and 50 μL amplification product, vortex to mix, and incubate at room temperature for 5 min.
3) Briefly centrifuge the centrifuge tube and place it on a magnetic rack, let it stand for 3 min until the solution is clear, carefully pipette the supernatant to a new 1.5 mL centrifuge tube, and discard the magnetic beads.
4) Add 30 μL of purified magnetic beads to the supernatant, vortex to mix, and incubate at room temperature for 5 min.
5) Centrifuge the centrifuge tube briefly and place it on a magnetic rack, let it stand for 3 min until the solution is clear, then carefully remove the supernatant.
6) Keep the centrifuge tube in the magnetic rack all the time, add 200 μL of freshly prepared 80% ethanol to rinse the magnetic beads, incubate at room temperature for 30 sec, and carefully remove the supernatant.
7) Repeat step 6 for a total of two rinses.
8) Keep the centrifuge tubes on the magnetic rack all the time, open the cap and air-dry until the surface of the magnetic beads has no water reflection and is matte.
9) Remove the centrifuge tube from the magnetic stand, add 22 μL of Elution Buffer, vortex and mix well, and incubate at room temperature for 5 min.
10) Centrifuge the centrifuge tube briefly and place it on the magnetic rack for 2 min. After the magnetic beads are completely absorbed, pipette 20 μL of the supernatant into a new 1.5 mL centrifuge tube and store at -20°C.
(Note: It is recommended that the PCR laboratory use Nucleic Acid Remover to clean the biological safety cabinet, PCR machine, pipette, etc, in order to eliminate the contamination of DNA products during library amplification on the test results.)

4.9 Library QC:
Use Qubit™ dsDNA HS Assay Kit to test the concentration of the library. When the concentration of the library is lower than 0.5ng/μL, it is recommended to rebuild the library.

5. Sequencing:
5.1 Circularization
It is recommended to use the "MGIEasy Circularization Kit (REF: 100005259)" produced by MGI Tech Co., Ltd. or the "DNA Circularization Kit" produced by Fuzhou Agenmic Biotechnology Co., Ltd. The kit needs to evaluate the suitability if using the other brands. For the "MGIEasy Circularization Kit" produced by MGI Tech Co., Ltd., it should perform the library circularization as follows:
5.1.1 Circularization reagent preparation
Take out the circularization buffer and ligase, put them on ice. Vortex and mix for 5 sec after they melt, then centrifuge briefly and put them on ice for later use.
Note: Do not thaw the ligase at room temperature, and do not touch the tube wall with your hands for a long time.
5.1.2 According to the library quantification results, in a new centrifuge tube, mix the tested library in equal amounts based on the index adapter number, take 200 ng of the mixed library, and supplement the volume to 48 μL with TE buffer (if the volume of the mixed library is greater than 48 μL, please re-prepare the library). Mix thoroughly and centrifuge briefly for 5 sec, place on a PCR machine and incubate at 95°C for 5 min, immediately take out the centrifuge tube and place it on ice for 5 min after incubation.
5.1.3 Add 11.6 μL circularization buffer and 0.5 μL ligase to the above reaction system. Mix thoroughly, centrifuge briefly, and incubate at 37°C for 30 min. The reaction product can be used in the next reaction or frozen at -20°C or below.

5.2 Sequencing
It is recommended to use the DNBSEQ-G50, DNBSEQ-G400 FAST, DNBSEQ-G99 from MGI Tech Co., Ltd., and the adapted sequencing universal kit for library DNB preparation and sequencing.
It is also possible to use the DNBSEQ-G50, DNBSEQ-G400 FAST, DNBSEQ-G99 from other manufactures and the adapted sequencing universal kit for library DNB preparation and sequencing. But it is necessary to perform the adaptability test by itself.
If you use the DNBSEQ-G99 sequencer, it is recommended to use the sequencing universal kit corresponding to the DNBSEQ-G 99 platform produced by MGI Tech Co., Ltd. for library DNB preparation and sequencing.

6. Bioinformatics analysis:
The raw data obtained by the sequencing were sent to the local server, and the data will be analyzed and QC by the "Pathogen Management and Analysis System" from Fuzhou Agenmic Biotechnology Co., Ltd.

POSITIVE JUGEMENT VALUE

By detecting 250 clinical samples with negative and positive results of known target pathogens, divided them to different groups according to different types of pathogens, and use SPSS software to analyze the receiver operating characteristic curve (ROC) to determine the positive judgment of different types of pathogens value. Use the Pathogen Management and Analysis System to calculate the number of detected reads and comparison rate of each pathogen when the data is normalized to 15M to judge the results.

QC OF TEST RESULT

The test result of the negative quality control should be negative. If the pathogenic microorganisms significantly higher than the background level are detected, it indicates that there may be contamination in the environment.

The results of this experiment are not reliable and need to be retested.

There should be 3 internal standard sequences detected in each sample, otherwise the results of this experiment are unreliable and need to be tested again.

The sequencing reads should be ≥15M.

LIMITATIONS

1. False negative results may occur when the concentration of pathogen nucleic acid contained in the sample to be tested is below the limit of detection(LoD).
2. The test results are for clinical reference only, and the clinical diagnosis and treatment of patients should be considered in combination with their symptoms/signs, medical history, other laboratory tests, and treatment responses.
3. This kit can only detect the types of pathogens covered in the manual. Therefore, when the test result of this kit is negative, it cannot be ruled out that the tested sample carries other pathogens outside the detection range of this kit.
4. Incorrect sample collection, transfer, storage, and nucleic acid extraction processes may lead to inaccurate test results.
5. Only limited to the sample types and applicable systems specified in the manual.
6. The test results of this kit should be comprehensively analyzed and interpreted in combination with the patient's symptoms/signs, medical history, and other laboratory diagnosis results, and should not be used as the sole basis for clinical diagnosis, treatment or management of patients.
7. Possibility leading to false negative results:

7.1 Incorrect sample collection, processing, transportation and storage conditions.

7.2 Variation of gene sequence or sequence change caused by other reasons.

7.3 Antibiotic treatment taken by patients before sampling will reduce the concentration of pathogens, and below the limit of detection.

7.4 The titer of the target substance in the sample is too low, lower than the limit of detection.

7.5 Other unvalidated interferences, such as substances introduced into the sample either endogenously or exogenously.

8. Possibility leading to false positive result:

8.1 Cross-contamination between samples.

8.2 Other cross-reactive substances not validated.

PERFORMANCE CHARACTERISTICS

1. Positive coincidence rate: Tested the positive reference materials, the test results were positive for corresponding pathogens, and the positive coincidence rate is 100%.
2. Negative coincidence rate: Tested the negative reference materials, the test results were negative for corresponding pathogens, and the negative coincidence rate is 100%.
3. Limit of detection (Lod): Tested the positive reference materials, the test results were positive for corresponding pathogens. The Lod of this test kit is as follows:

Bacteria: 100 CFU /mL
Fungus: 100 CFU /mL
Viruses: 1000 copies /mL
Parasites: 10 parasites / mL
Others (mycoplasma, chlamydia, etc.): 1000 copies / mL

4. Repeatability: Test the repeatable reference materials, and repeat 10 times for each reference, the test results were all positive.

WARNINGS AND PRECAUTIONS

The warnings and precautions are included, but not limited to the following:

1. Thaw all components of the kit on ice before use. After thawing, invert up and down several times to mix thoroughly, centrifuge briefly and place on ice for later use.
2. It is recommended to use pipette tips with filters and replace the tips when drawing different samples.
3. It is recommended to use Nucleic Acid Remover to remove nucleic acid from the laboratory after each experiment.
4. The consumables used before the second-strand synthesis must be RNase-free, and the consumables used after the second-strand synthesis must be DNase-free.
5. Be sure to use fresh Nuclease-free H2O during the experiment. It is recommended to divide into small tubes and take them one by one, and discard them after use.
6. Be sure to wear gloves for operation. After touching equipment outside the RNase-free space or other working areas, change gloves.
7. All reagents must be capped immediately after use to avoid contamination.
8. It is recommended to perform the reactions of each step in a PCR with a heated lid, and the PCR should be preheated to near the reaction temperature before use.
9. The preparation table in the operation steps is the standard dosage, and the loss must be considered during the preparation.
10. Contact manufacture for any questions.

INDEX OF SYMBOLS

IVD	In vitro diagnostic medical device	LOT	Batch code
	Date of manufacture		Use-by date
	Do not re-use		Consult instructions for use
EC REP	Authorized representative in the European Community / European Union		Manufacturer
	Temperature limits	REF	Catalogue number
	Caution	CE	CE marking

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