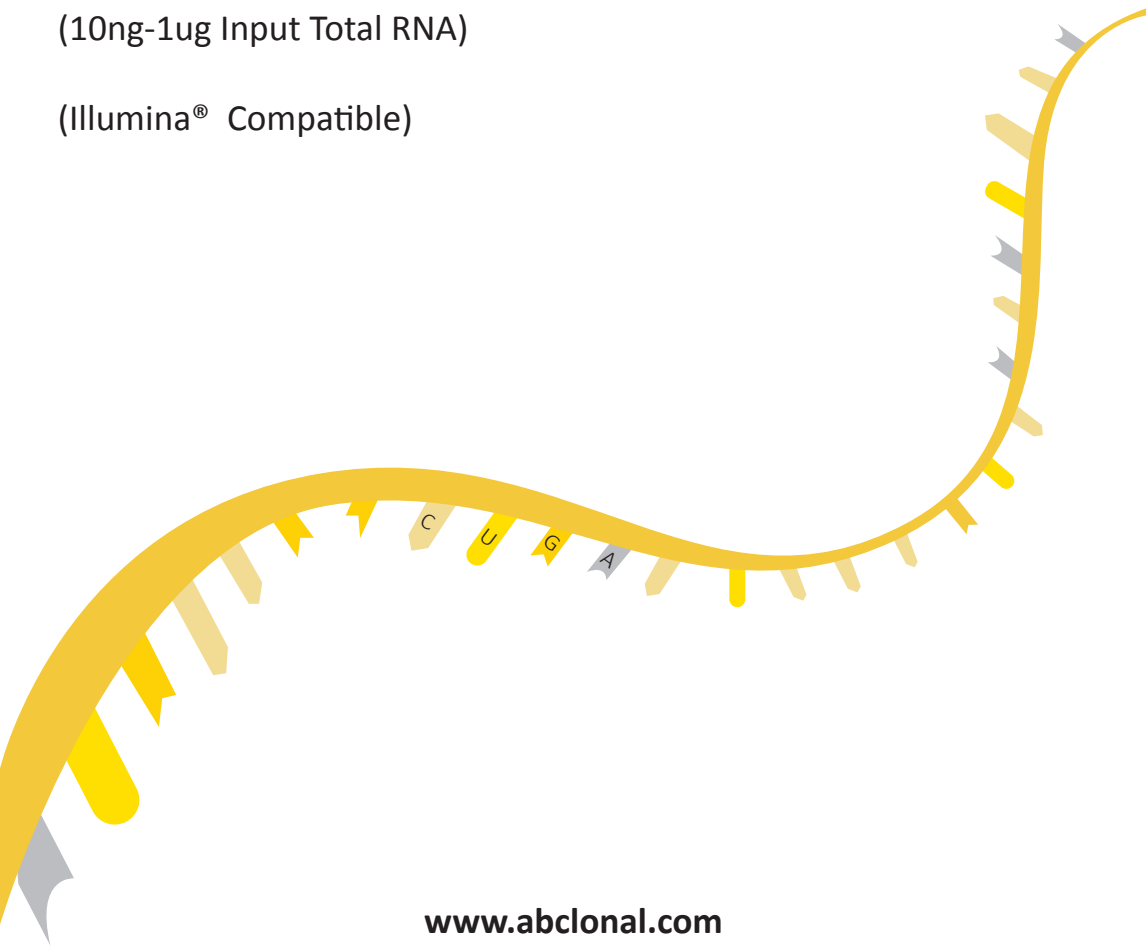
mRNA-seq Lib Prep Kit for Illumina®

RK20302

(10ng-1ug Input Total RNA)

(Illumina® Compatible)



www.abclonal.com

version: N12G13v1.0

Contents

1.Introduction

The ABclonal mRNA-seq Lib Prep Kit for Illumina® comprises four processing modules: Poly(A) mRNA Purification, First Strand Synthesis, Second cDNA Synthesis and DNA Library Preparation. The kit includes all of the enzymes and buffers for poly(A) mRNA enrichment and mRNA-seq library construction, from 10 - 1000 ng of total RNA isolated from a wide variety of eukaryotic species.

- The first Poly(A) mRNA Purification module effectively enriches poly(A) mRNA using poly(T)-oligo attached magnetic beads.
- The DNA Library Preparation module contains enzymes and buffers for end polishing, dA-addition, truncated adapter ligation, and PCR amplification. The truncated RNA adapters exhibit better ligation efficiency compared to full-length ones due to the absence of adapter dimers. The RNA Universal Primer and the RNA Index Primers are designed for the amplification of mRNA-seq library, flanked by the P5 sequence and the P7 / Index sequence.

2.Components

Box	Module Name	Tube Color & Name		24 Reactions (RK20302M)	96 Reactions (RK20302L)
Box-1	Poly(A) mRNA Purification Module (RK20341)	●	Oligo $d(T)_{25}$ Capture Beads	480 µl	1920 µl
		●	mRNA Binding Buffer	12 ml	48 ml
		●	Washing Buffer	19.2 ml	76.8 ml
		●	Tris Buffer	1.2 ml	4.8 ml
Box-2	First Strand Synthesis Module (RK20353)	●	2X Frag/Elute Buffer	144 µl	576 µl
		●	RT Reagent	192 µl	768 µl
		●	First Strand Synthesis Enzyme Mix	48 µl	192 µl
	Second Strand Synthesis Module (RK20346)	●	Second Strand Synthesis Reaction Buffer	192 µl	768 µl
		●	Second Strand Synthesis Enzyme Mix	96 µl	384 µl
		●	Nuclease-free Water	2 ml	8 ml
	DNA Lib Prep Module (RK20347)	●	End Prep Buffer	240 µl	960 µl
		●	End Prep Enzyme Mix	72 µl	288 µl
		●	Ligation Buffer	396 µl	1584 µl
		●	Ligase Mix	72 µl	288 µl
		●	2X PCR Mix	600 µl	2×1200 µl
		●	Low-EDTA TE	2.5 ml	10 ml

Box	Module Name	Tube Color & Name		24 Reactions (RK20302M)	96 Reactions (RK20302L)
Box-3	RNA Adapter Module (24 Indices) (RK20345)	●	RNA Truncated Adapter	60 µl	240 µl
		●	RNA Universal Primer	60 µl	240 µl
		●	RNA Index Primer	2.5 µl*	10 µl*

*RNA index primers contain 24 Illumina® sequencing indices.

Storage Conditions

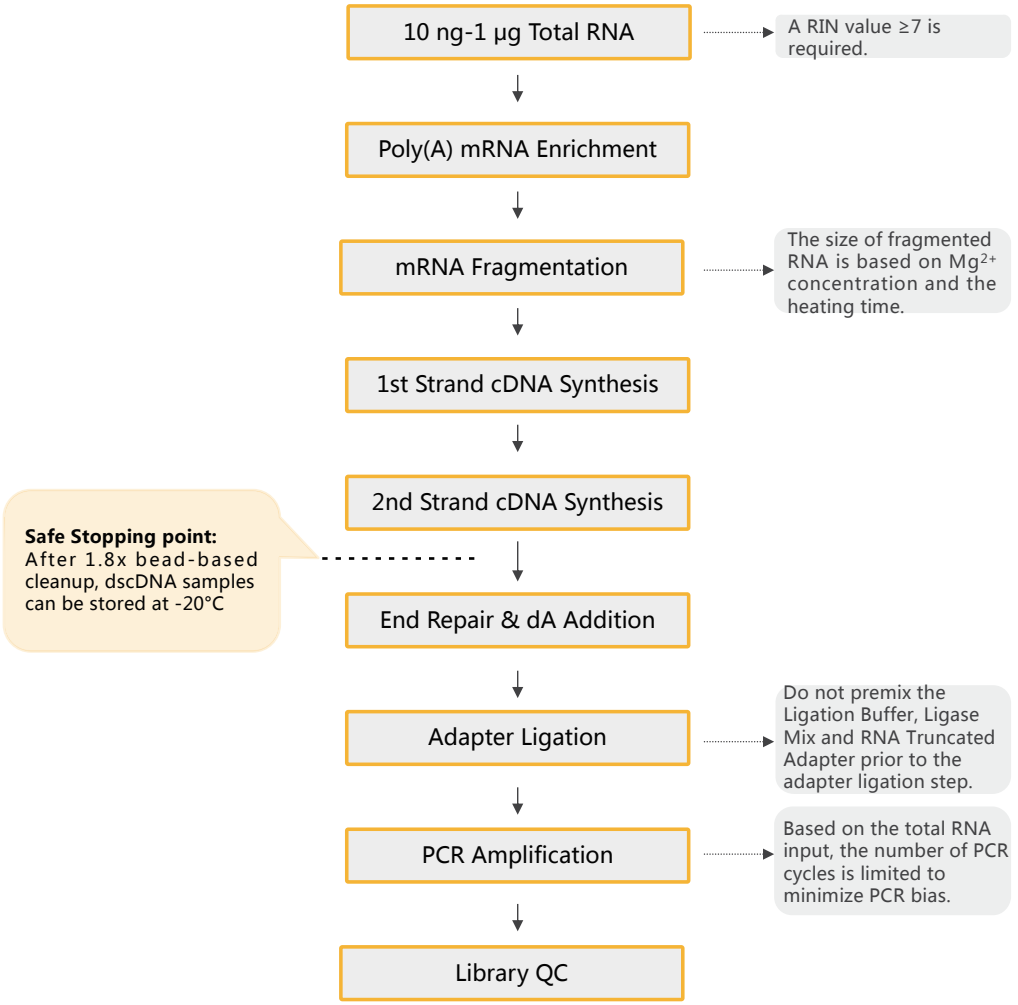
- Box-1: 2-8°C;
  Do NOT freeze the Oligo (dT)25 Capture Beads.
- Box-2: -20°C;
- Box-3: -20°C.

3.Additional Materials Required

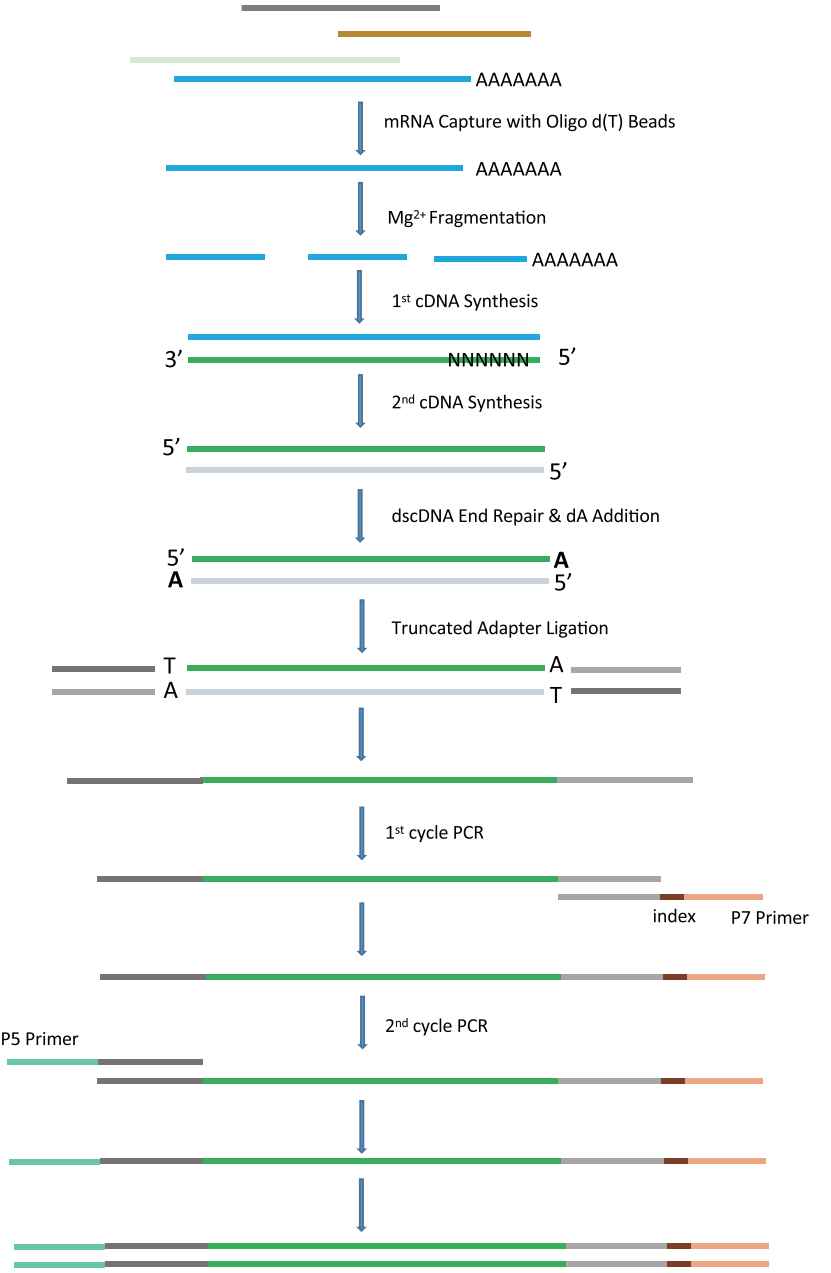
- 100% ethanol (ACS grade)
- Nuclease-free water
- Nuclease-free PCR tubes or plates
- Magnetic stand
- Thermocycler
- Agencourt™ AMPure XP bead (Beckman Coulter Inc., cat. no. A63880)
- Pipettes and multichannel pipettes
- Aerosol resistant pipette tips
- Microcentrifuge
- Vortex mixer
- Agilent Bioanalyzer or comparable method to assess the quality of stranded mRNA-seq library

4.Workflow Chart

Workflow Chart



The Scheme of Technologies



5.Precautions

- High-quality RNA is essential for sequencing library construction. The integrity and size distribution of total RNA can be accessed using an Agilent Bioanalyzer to address the RNA integrity number (RIN) score. An RNA sample with a RIN score lower than 7 is NOT recommended in this protocol.
- To avoid contamination, keep all the reagents and samples in closed tubes on ice and use RNasezap® to clean the workspace.
- To avoid cross contamination, always carefully add the RNA index primer to the PCR reaction.
- Prepare fresh 80% Ethanol.

6.Protocol

1. mRNA Isolation and Fragmentation

1.1 Equilibrate mRNA capture beads before starting.

1.1.1 Resuspend the oligo d(T) capture beads thoroughly by pipetting up and down several times.

1.1.2 Add 200 µl of mRNA Binding Buffer to 20 µl of the Oligo dT beads, and mix thoroughly by pipetting up and down several times.

1.1.3 Pellet the beads on a magnetic stand at room temperature (RT) for 2 minutes and carefully remove and discard the supernatant.

1.1.4 Wash the beads with 200 µl of mRNA Binding Buffer and mix thoroughly by pipetting.

1.1.5 Pellet the beads on a magnetic stand at RT for 2 minutes and discard the supernatant.

1.1.6 Add 50 µl of mRNA Binding Buffer to the beads and mix thoroughly by pipetting.

1.2 Dilute 10-1000 ng of total RNA with nuclease-free water to a final volume of 50 µl.

1.3 Add the diluted RNA to the beads mixture (step 1.1.6) and mix thoroughly by

pipetting.

1.4 Incubate the reaction tubes in a thermocycler at 65°C for 5 minutes with the heated lid set to ≥ 75°C and then cool to 4°C.

1.5 Mix thoroughly by pipetting and place at RT for 5 minutes, enhancing the mRNA binding to the beads.

1.6 Pellet the beads on a magnetic stand at RT for 2 minutes and carefully remove the supernatant.

1.7 Wash the beads with 200 µl of mRNA Washing Buffer and mix thoroughly by pipetting. Pellet the beads on a magnetic stand at RT for 2 minutes and carefully remove the supernatant.

1.8 Repeat step 1.7 for a total of two washes.

1.9 Resuspend the beads with 50 µl of Tris Buffer and mix thoroughly by pipetting.

1.10 Incubate at 80°C for 2 minutes with a heated lid set to ≥ 90°C, then hold at 25°C.

1.11 Add 50 µl of mRNA Binding Buffer to the mixture of capture beads and mix thoroughly by pipetting.

1.12 Incubate at RT for 5 minutes, allowing the mRNA binding to the beads.

1.13 Pellet the beads on a magnetic stand at RT for 2 minutes and carefully remove the supernatant.

1.14 Wash the beads with 200 µl of mRNA Washing Buffer and mix thoroughly by pipetting. Pellet the beads on a magnetic stand at RT for 2 minutes and carefully remove the supernatant.

1.15 Repeat Step 1.14 for a total of two washes.

1.16 Prepare the 1X Frag/Elute Buffer as the Table 1. below

Table 1. 1X Frag/Elute Buffer Preparation

Component	Volume
2X Frag/Elute Buffer	6 µl
Nuclease-free Water	6 µl
Total Volume	12 µl

1.17 Resuspend the beads with 11 µl of 1X Frag/Elute Buffer and mix thoroughly by pipetting

1.18 Incubate the samples in a thermocycler, carry out the fragmentation and priming program according to the Table 2. Below.

Table 2. RNA Fragmentation and Priming Condition

Average RNA Library Size	Fragmentation and Priming Conditions
200-300 nt	94°C 15 min
300-450 nt	94°C 10 min
400-700 nt	94°C 5 min

1.19 When the tubes are cool enough to handle (~65°C), immediately pellet the beads on a magnetic stand for 2 minutes to avoid the re-hybridization of mRNA to the beads.

1.20 Carefully transfer 10 µl of the supernatant to a new PCR tube.

1.21 Place the tube on ice and proceed to the first strand cDNA synthesis.

2. First Strand cDNA Synthesis

2.1 Set up the first strand cDNA synthesis reaction on ice according to the Table 3. below.

Table 3. First Strand cDNA Synthesis Reaction Setup

Component	Fragmentation and Priming Conditions
Fragmented and Primed mRNA (Step 1.21)	10 µl
RT Reagent	8 µl
First Strand Synthesis Enzyme Mix	2 µl
Total Volume	20 µl

2.2 Mix thoroughly by pipetting up and down several times and incubate the reaction tube in a thermocycler using the conditions listed in the Table 4. (A heated lid is set to 105°C).

Table 4. Reverse Transcription Program

Temperature	Fragmentation and Priming Conditions
25°C	10 min
42°C	15 min
70°C	15 min
4°C	Hold

2.3 Proceed to the second strand cDNA synthesis immediately.

3. Second Strand cDNA Synthesis

3.1 Set up the Second Strand cDNA Synthesis reaction on ice according to the Table 5. Below

Table 5. Second Strand cDNA Synthesis Reaction Setup

Components	Volume
First Strand cDNA Product (Step 2.2)	20 µl
Second Strand Synthesis Reaction Buffer	8 µl
Second Strand Synthesis Enzyme Mix	4 µl
Nuclease-free Water	48 µl
Total Volume	80 µl

3.2 Keep the tube on ice, mix thoroughly by pipetting the reaction up and down several times.

3.3 Incubate in a thermocycler at 16°C for 60 minutes without a heated lid.

3.4 Clean up the second strand synthesis products.

3.4.1 Resuspend the Agencourt™ AMPure XP beads by vortexing and keep at RT for at least 15 minutes.

3.4.2 Add 144 µl (1.8X) of resuspended beads to the second strand synthesis reaction (~80 µl). Mix thoroughly by pipetting.

3.4.3 Incubate at RT for 5 minutes.

3.4.4 Pellet the beads on a magnetic stand at RT for 5 minutes.

3.4.5 Carefully remove and discard the supernatant.

3.4.6 Wash the beads with 200 µl of fresh 80% ethanol. Pellet the beads on a magnetic stand and carefully remove the ethanol.

3.4.7 Repeat the step 3.4.6 for a total of two washes.

3.4.8 Air dry the beads on a magnetic stand for 5 minutes.

3.4.9 Resuspend the beads in 39 µl of Low-EDTA TE buffer and mix thoroughly by pipetting.

3.4.10 Incubate at RT for 2 minutes.

3.4.11 Pellet the beads on a magnetic stand and carefully transfer 37 µl of supernatant to a new PCR tube.

The purified dscDNA samples can be stored at −20°C for 24 hours.

4.End Preparation of cDNA Library

4.1 Set up the end prep reaction on ice according to Table 6. below.

Table 6. End Preparation Reaction Setup

Components	Volume
Second Strand Synthesis Product (Step 3.4.11)	37 µl
End-prep Buffer	10 µl
End-prep Enzymes Mix	3 µl
Total Volume	50 µl

4.2 Mix thoroughly by pipetting up and down several times.

4.3 Incubate the samples in a thermocycler using the program listed in the Table 7. (A heated lid is set to 75°C).

Table 7. End Preparation Reaction Program

Temperature	Time
20°C	30 min
65°C	30 min
4℃	Hold

5. Adapter Ligation

5.1 Set up the adapter ligation reaction on ice according to the Table 8. below.

Table 8. Adapter Ligation Reaction Setup

Components	Volume
End-prep DNA Product (Step 4.3)	50 µl
Ligation Buffer	16.5 µl
RNA Truncated Adapter*	2.5 µl
Ligase Mix	3 µl
Total Volume	71 µl

*** The truncated adapters can NOT be used for PCR-free DNA library preparation.**

Note: Do NOT premix the Ligation Buffer, Ligase Mix and the RNA Truncated Adapter prior to the Adapter Ligation step.

5.2 Mix thoroughly by pipetting up and down several times.

5.3 Incubate in a themocycler for 15 minutes at 22°C, without a heated lid. For larger size fragments (> 200nt), size selection is recommended and proceed to step 5.5.

Note: Size selection for < 100 ng input total RNA is not recommended.

5.4 Cleanup the ligation reaction (without size selection).

5.4.1 Resuspend the Agencourt™ AMPure XP beads by vortexing and keep at RT for at least 15 minutes.

5.4.2 Add 56 µl (0.8X) of the resuspended beads to the adapter-ligated DNA product (~70 µl) from Step 5.3. Mix thoroughly by pipetting up and down several times.

5.4.3 Incubate at RT for 5 minutes.

5.4.4 Pellet the beads on a magnetic stand at RT for 5 minutes.

5.4.5 Carefully remove and discard the supernatant.

5.4.6 Wash the beads with 200 µl of fresh 80% ethanol. Pellet the beads on a magnetic stand and carefully remove the ethanol.

5.4.7 Repeat step 5.4.6 for a total of two washes.

5.4.8 Air dry the beads on a magnetic stand for 5 minutes.

5.4.9 Resuspend the beads in 21 µl of Low-EDTA TE buffer and mix thoroughly by pipetting.

5.4.10 Incubate at RT for 2 minutes.

5.4.11 Pellet the beads on a magnetic stand and carefully transfer 20 µl of supernatant to a new PCR tube for PCR amplification.

5.5 Size selection of adapter-ligated DNA.

Table 9. Amount of Agencourt™ AMPure XP Beads for DNA Size Selection

Fragmentation	94°C 15 min	94°C 10 min	94°C 5 min
RNA Insert Size	200-300 nt	300-450 nt	400-600 nt
Final Library Size	320-420 bp	420-570 bp	520-720 bp
1st Binding Beads	35 µl	30 µl	25 µl
2nd Binding Beads	20 µl	20 µl	15 µl

Take the samples with 10 min of fragmentation at 94°C as an example

5.5.1 Resuspend the Agencourt™ AMPure XP beads by vortexing and keep at RT for at least 15 minutes.

5.5.2 Add 30 µl of nuclease-free water to the ligation reaction at step 5.3 to a final volume of 100 µl.

5.5.3 Add 30 µl of Agencourt™ AMPure XP beads (0.30X) and mix thoroughly by pipetting up and down several times.

5.5.4 Incubate at RT for 5 minutes.

5.5.5 Pellet the beads on a magnetic stand at RT for 5 minutes (Do NOT discard the supernatant).

5.5.6 Carefully **TRANSFER** the supernatant to a new PCR tube (Do NOT disturb the beads).

5.5.7 Add 20 µl of Agencourt™ AMPure XP beads (0.20×) to the collected supernatant and mix thoroughly by pipetting up and down several times.

5.5.8 Incubate at RT for 5 minutes.

5.5.9 Pellet the beads on a magnetic stand at RT for 5 minutes.

5.5.10 Carefully remove and discard the supernatant.

5.5.11 Wash the beads with 200 µl of fresh 80% ethanol. Pellet the beads on a magnetic stand and carefully remove the ethanol.

5.5.12 Repeat step 5.5.11 for a total of two washes.

5.5.13 Air dry the beads on a magnetic stand for 5 minutes.

5.5.14 Resuspend the beads in 21 µl of Low-EDTA TE buffer and mix thoroughly by pipetting.

5.5.15 Incubate at RT for 2 minutes.

5.5.16 Pellet the beads on a magnetic stand and carefully transfer 20 µl of supernatant to a new PCR tube for PCR amplification.

6. PCR Amplification

6.1 Set up the PCR amplification reaction on ice according to the Table 10. below.

Table 10. PCR Amplification Reaction Setup

Components	Volume
Purified Adapter Ligated DNA (Steps 5.4.11 or 5.5.16)	20 µl
2X PCR Mix	25 µl
RNA Universal Primer	2.5 µl
RNA Index Primer (X)*	2.5 µl
Total Volume	50 µl

*RNA index primers contain 24 Illumina® sequencing index.

6.2 Mix thoroughly by pipetting up and down several times.

6.3 Incubate the samples in a thermocycler using the conditions listed in the Table 11. and Table 12.. (A heated lid is set to 105°C).

Table 11. PCR Amplification Program

Temperature	Time	Cycles
98°C	1 min	1
98°C	10s	
60°C	15s	8-16*
72°C	30s	
72°C	1 min	1
4°C	Hold	

*Recommended PCR cycles based on the total RNA input amount

Table 12. Recommended Number of PCR Cycles for Various Sample Inputs

Input Total RNA	PCR Cycles (No Size Selection)	PCR Cycles (Size Selection)
10 ng	15-16	N/A
100 ng	12-13	14-15
1 µg	8-9	10-11

6.4 Cleanup PCR products.

6.4.1 Resuspend the Agencourt™ AMPure XP beads by vortexing and keep at RT for at least 15 minutes.

6.4.2 Add 50 µl (1.0X) of resuspended beads to the PCR amplification reaction (~50 µl). Mix thoroughly by pipetting.

6.4.3 Incubate at RT for 5 minutes.

6.4.4 Pellet the beads on a magnetic stand at RT for 5 minutes.

6.4.5 Carefully remove and discard the supernatant.

6.4.6 Wash the beads with 200 µl of fresh 80% ethanol. Pellet the beads on a magnetic stand and carefully remove the ethanol.

6.4.7 Repeat step 6.4.6.

6.4.8 Air dry the beads on a magnetic stand for 5 minutes.

6.4.9 Resuspend the beads in 31 µl of Low-EDTA TE buffer and mix thoroughly by pipetting.

6.4.10 Incubate at RT for 2 minutes.

6.4.11 Pellet the beads on a magnetic stand and carefully transfer 30 µl of supernatant to a new PCR tube.

7.1.Introduction

• RNA Fragmentation

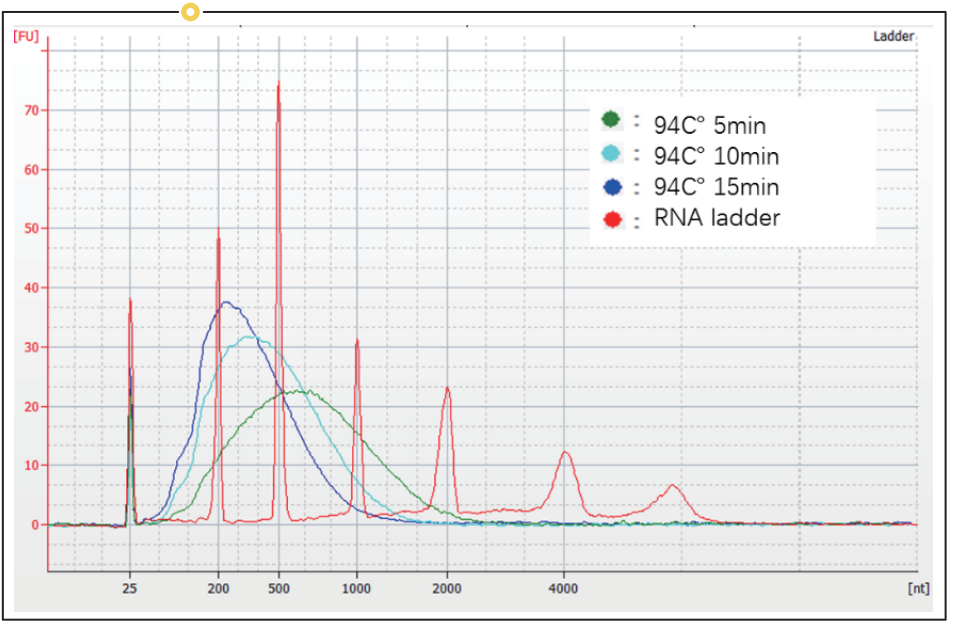


Figure 1: Electropherogram results of fragmented mRNAs from Agilent 2100 Bioanalyzer with an RNA 6000 Pico Chip. The samples were treated according to the protocol of mRNA Capture Module, and then fragmented at 94°C for 5, 10 and 15 minutes.

• Size Selection of DNA Libraries

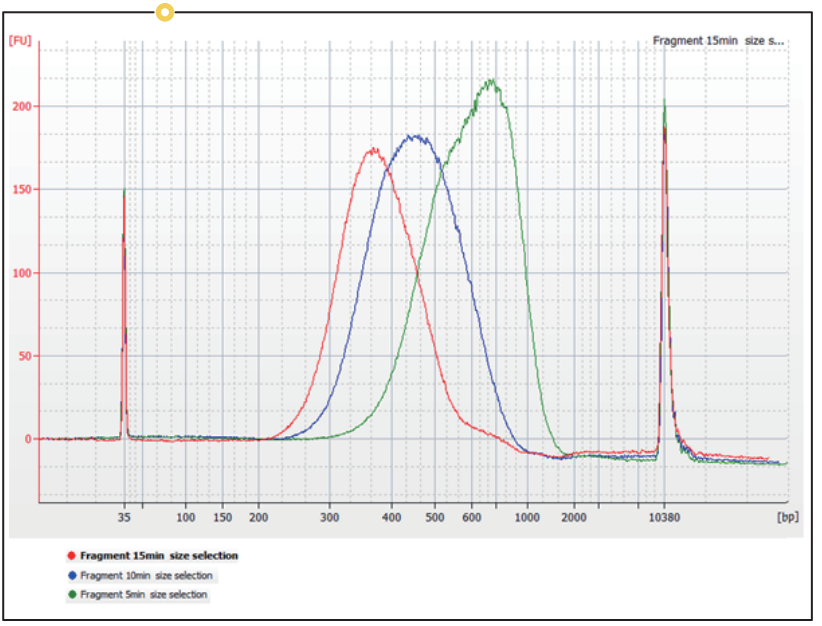


Figure 2: Eletropherogram results of size-selected DNA libraries from Agilent 2100 Bioanalyzer with a dsDNA HS Chip. 1 µg mouse total RNA input was treated according to the protocol for the ABclonal Stranded mRNA-seq Lib Prep Kit. Different strategies for size selection were employed as described in Table 9.

• The Sequences of Adapter and Index Primers Used in the Kit

Truncated Adaptor:

Universal PCR Primer:

5'-Spc/A*A*T*GATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTC CGA*T*C*T-3'

RNA Index 1 Primer (ATCACG):

5'-Spc/C*A*A*GCAGAAGACGGCATACGAGATCGTGATGTGACTGGAGTTCAGACGTG TGCTCTTCCGA*T*C*T-3'

Index Table: RNA Adapter Module 24 Indices (RK20345)

RNA Index Primer name	Index	RNA Index Primer name	Index
RNA_Index_1_Primer	ATCACG	RNA_Index_13_Primer	AGTCAA
RNA_Index_2_Primer	CGATGT	RNA_Index_14_Primer	AGTTCC
RNA_Index_3_Primer	TTAGGC	RNA_Index_15_Primer	ATGTCA
RNA_Index_4_Primer	TGACCA	RNA_Index_16_Primer	CCGTCC
RNA_Index_5_Primer	ACAGTG	RNA_Index_18_Primer	GTCCGC
RNA_Index_6_Primer	GCCAAT	RNA_Index_19_Primer	GTGAAA
RNA_Index_7_Primer	CAGATC	RNA_Index_20_Primer	GTGGCC
RNA_Index_8_Primer	ACTTGA	RNA_Index_21_Primer	GTTTCG
RNA_Index_9_Primer	GATCAG	RNA_Index_22_Primer	CGTACG
RNA_Index_10_Primer	TAGCTT	RNA_Index_23_Primer	GAGTGG
RNA_Index_11_Primer	GGCTAC	RNA_Index_25_Primer	ACTGAT
RNA_Index_12_Primer	CTTGTA	RNA_Index_27_Primer	ATTCCT

- Frequently Asked Questions

Q : I accidentally stored Box-1 (Poly(A) mRNA Purification Module) at -20°C, can I still use it?

A : -20°C storage will affect the performance of the Oligo-dT beads for mRNA-binding.

Q : How do I construct the RNA-seq library if the RNA is degraded (RIN < 7)?

A : In the case of FFPE-derived RNA samples, which typically have low RIN score, the ABclonal's Whole RNA-seq Lib Prep Kit for Illumina® (RK20303) is recommended alternatively.

In the cases of the degraded RNA samples from other eukaryotic cells, while both 28S and 18S bands are presented in the agarose gel, appropriate increase of total RNA input and the number of PCR cycles are recommended.

Q : How do I determine the quality of the RNA-seq library?

A : The size distribution of prepared RNA-seq library can be verified by performing analysis on Agilent 2100 Bioanalyzer. Check for the correct size distribution of library fragments and the absence of adapter dimers or any abnormal peaks more than 1000bp, which represented large assemblies of improperly annealed, partially double-stranded, and heteroduplex DNA. Qubit® or qPCR quantification is highly recommended before proceeding to sequencing.

Q : Can I use the kit for more than 24 RNA samples?

A : Yes, additional index primers are provided in RNA Adapter Module 96 Index for Illumina® Set_A (48 indices) (Index Primer 1-48, cat.no. RK20351) or RNA Adapter Module 96 Index for Illumina® Set_B (48 indices) (Index Primer 49-96, cat. no. RK20352).

United States
www.abclonal.com
Address: 86 Cummings Park Dr ,Woburn,MA 01801， United States
Phone: 888.754.5670, +1 857.259.4898 (Int'l)
Email: service@abclonal.com