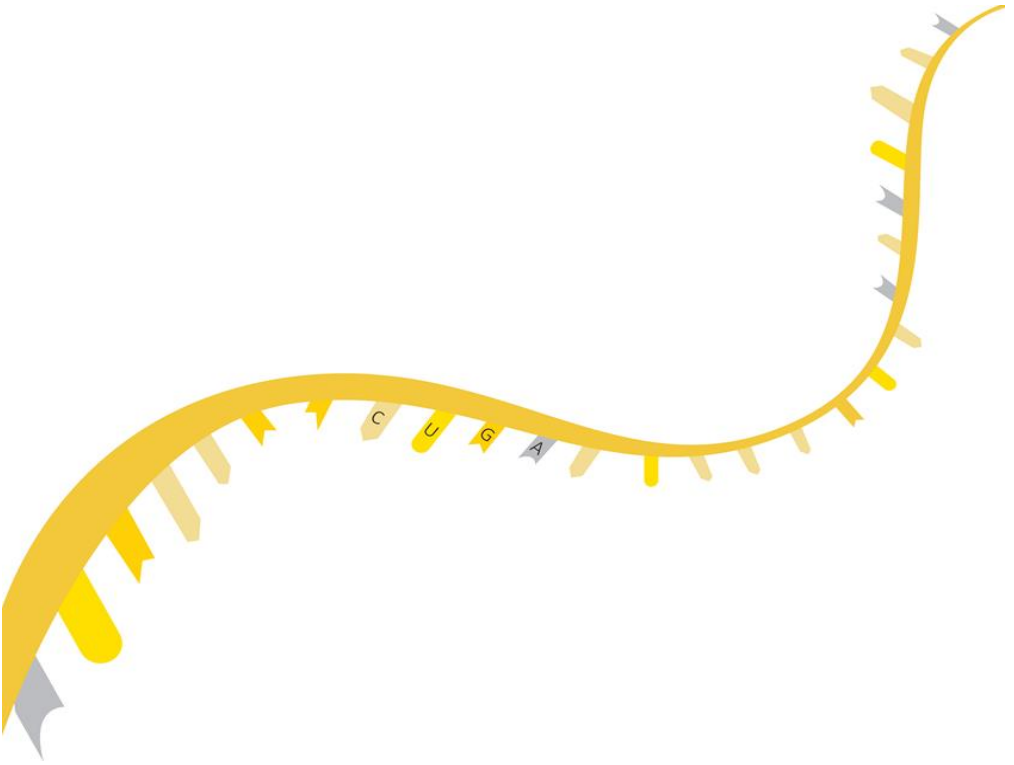
Stranded mRNA-seq Lib Prep Kit for Illumina®

RK20301



www.abclonal.com

Version: N16G01v3.5

Table of Contents

1. Product Overview

- The kit is compatible with Illumina sequencing platforms.
- The kit is applicable to animal, plant, fungi, and other eukaryote RNA samples.
- The initial total RNA input is 10 ng-1 μg, and the recommended RNA integrity number (RIN) is ≥ 7.
- The kit contains the RNA Truncated Adapter and needs to be combined with the PCR Index to add a unique index to each sample. The truncated adapter exhibits higher ligation efficiency and reduces adapter dimers compared with the full-length adapter.
- The Stranded mRNA-seq Lib Prep Kit for Illumina® contains the Poly(A) mRNA Purification Module, Stranded RNA-seq Lib Prep Kit for Illumina and ABclonal RNA Adapter Module. It contains all buffers and enzymes required for mRNA library preparation.
- The kit incorporates dUTP into the second strand cDNA during synthesis; the labeled strand is degraded with uracil-DNA glycosylase (UDG) before PCR-based library enrichment. This ensures that the final sequencing data comes exclusively from the first strand cDNA, maintaining mRNA strand specificity for stranded mRNA-seq.
- All reagents provided in the kit have undergone rigorous quality control. Each lot of the kits has been verified through library preparation and sequencing to ensure stable performance.

2. Kit Components

Kit Components	Tube Name and Color	24 rxns (RK20301M)	96 rxns (RK20301L)
Poly(A) mRNA Purification Module (RK20341)	Oligo d(T)$_{25}$ Capture Beads	480 µL	1,920 µL
	mRNA Binding Buffer	12 mL	48 mL
	Washing Buffer	19.2 mL	76.8 mL
	Tris Buffer	1.2 mL	4.8 mL
Stranded RNA-seq Lib Prep Kit for Illumina (RK20349)	2X Frag/Elute Buffer	144 µL	576 µL
	RT Strand Specificity Reagent	192 µL	768 µL
	First Strand Synthesis Enzyme Mix	48 µL	192 µL
	Second Strand Synthesis Reaction Buffer with dUTP	192 µL	768 µL
	Second Strand Synthesis Enzyme Mix	96 µL	384 µL
	Nuclease-free Water	1 mL × 2	8 mL
	End-prep Buffer	240 µL	960 µL
	End-prep Enzyme Mix	72 µL	288 µL
	Ligation Buffer	396 µL	1,584 µL
	Ligase Mix	72 µL	288 µL
	2X PCR Mix	600 µL	1,200 µL X 2
	UDG Enzyme	12 µL	48 µL
	Low EDTA TE	1 mL × 3	10 mL

denotes the color of the tube cap.

The kit contains the Truncated Adapter, so PCR is required to ensure an intact library structure and add a unique index to each sample. The kit can be combined with the following adapter modules for Illumina as needed:

Kit Name	Cat. No.
RNA Adapter Module 96 Index for Illumina MiniSet (8 Indices)	RK20355
RNA Adapter Module 96 Index for Illumina MidiSet (24 Indices)	RK20356
RNA Adapter Module 96 Index for Illumina Set_A (48 Indices)	RK20351
RNA Adapter Module 96 Index for Illumina Set_B (48 Indices)	RK20352
Dual DNA Adapter 96 Kit for Illumina	RK20287
Dual DNA Adapter 96 Kit Extended for Illumina	RK20296

3. Storage

Stranded mRNA-seq Lib Prep Kit for Illumina® contains three packages:

Kit Name	storage
Poly(A) mRNA Purification Module	2-8°C
Stranded mRNA-seq Lib Prep Module for Illumina	-20°C
RNA Adapter Module / Dual DNA Adapter Module	-20°C

4. Additional Materials Require

Magnetic beads for purification: Agencourt AMPure XP Beads (Beckman Coulter, Inc. #A63881).

RNA quality control: Agilent RNA 6000 Pico Chip.

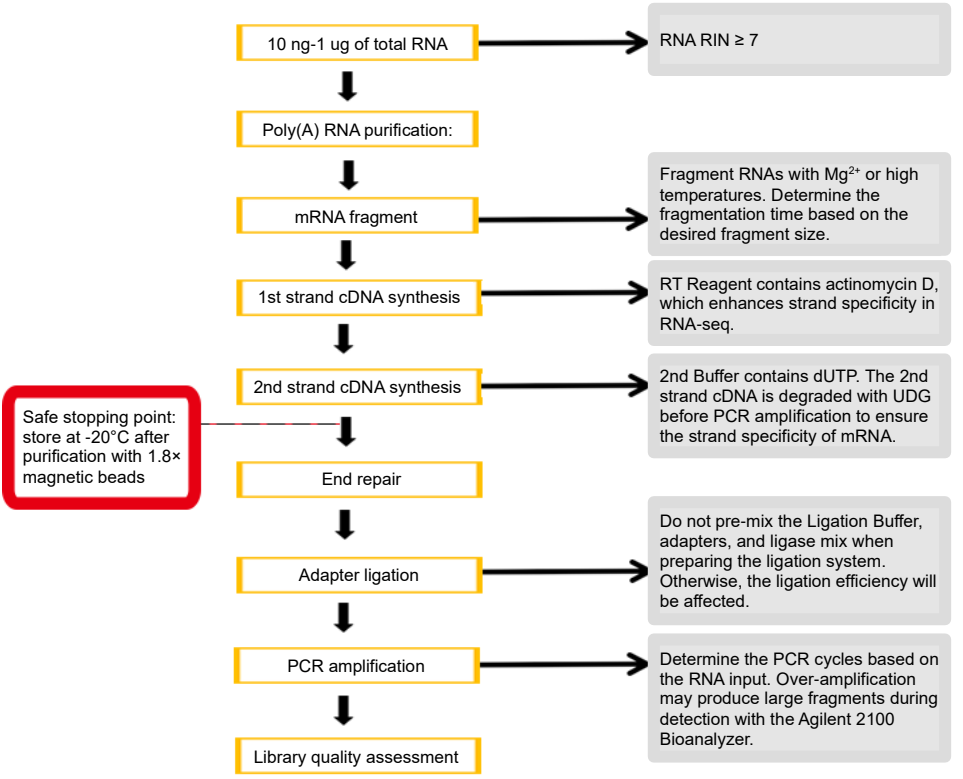
Library quality control: Agilent High Sensitivity DNA Chips and Agilent DNA 1000 Chip.

Other reagents: freshly prepared 80% ethanol.

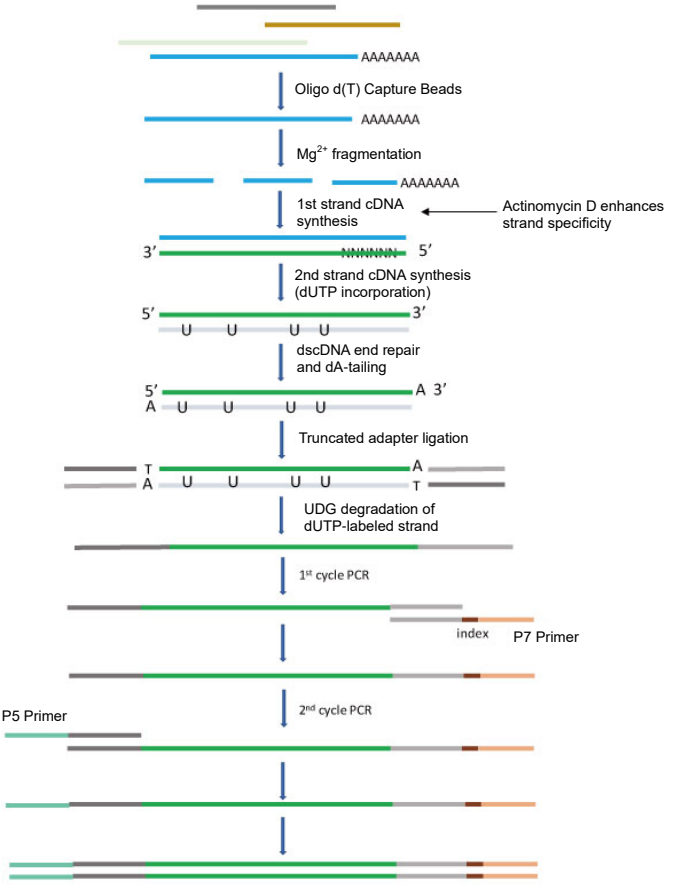
Other instruments: magnetic rack, PCR system, etc.

5. Workflow Diagram

Flow diagram:



Schematic diagram:



6. Precautions

◆ The total RNA input should be ≥ 10 ng. Less input may affect library preparation.

◆ The OD260/OD280 ratio of RNAs should be 1.8-2.1. A higher or lower ratio may indicate genome or protein contamination, and agarose gel electrophoresis should show three clear bands.

◆ Masks and gloves should be worn during mRNA library preparation. The mRNAs should be purified at room temperature. Diluted RNA samples should be placed on ice and used for the next step as soon as possible to avoid RNA degradation.

◆ The conditions for mRNA fragmentation and subsequent size selection should be determined according to the recommended parameter ranges in the Instructions for Use. Otherwise, the library size and yield will be compromised.

◆ The magnetic beads should be taken out half an hour in advance and brought to room temperature. During purification with magnetic beads, the Low EDTA TE must be added for elution after the ethanol has fully evaporated, namely when the bead color changes from bright brown to frosted brown. Residual ethanol or over-drying (leading to cracks) of beads may reduce the library yield.

◆ During purification with magnetic beads, the supernatant should be removed cautiously to avoid disturbing the magnetic beads. Otherwise, the library fragment size and library yield will be compromised.

◆ During size selection of the ligation products, the tubes should stand at room temperature for 5 min after each round of adding beads and then on the magnetic rack for another 5 min. The supernatant should not be discarded.

◆ The PCR Index should be used cautiously to avoid cross contamination between the reagent and the sample.

7. Protocol

1 mRNA Capture and Fragmentation

1.1 Take the Oligo d(T)$_{25}$ Capture Beads out of the 2-8°C environment, let it stand until it reaches room temperature, and mix it well by vortexing.

1.2 Pre-process the magnetic beads: Transfer 20 μL of Oligo d(T)$_{25}$ Capture Beads into a 1.5 mL centrifuge tube, add 200 μL of mRNA Binding Buffer, mix well by vortexing, and microcentrifuge the tube. Let the centrifuge tube stand on the magnetic rack for about 5 min until the solution becomes clear, and remove and discard the supernatant. Add 200 μL of mRNA Binding Buffer, mix well by vortexing, and microcentrifuge the tube. Let the centrifuge tube stand on the magnetic rack for about 2 min until the solution becomes clear, and remove and discard the supernatant. Remove the centrifuge tube from the magnetic rack, add 50 μL of mRNA Binding Buffer, and mix the solution well by vortexing for later use.

1.3 Thaw RNAs on ice, dissolve 10 ng-1 μg of total RNA in 50 μL of nuclease-free water, and place the solution on ice for later use.

1.4 Mix the equilibrated Oligo d(T)$_{25}$ Capture Beads by vortexing, transfer 50 μL of the beads into the prepared RNA solution, and mix the solution by pipetting.

1.5 Incubate the mixture in the PCR system (heating lid temperature ≥ 75°C).

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

1.6 After incubation, let the centrifuge tube stand on the magnetic rack for about 2 min until the solution becomes clear, and remove and discard the supernatant.

1.7 Remove the centrifuge tube from the magnetic rack, add 200 μL of Washing Buffer, and mix the solution by pipetting. Let the centrifuge tube stand on the

magnetic rack until the solution becomes clear, and remove and discard the supernatant.

1.8 Remove the centrifuge tube from the magnetic rack, add 50 µL of Tris Buffer, mix the solution well by pipetting, and incubate the mixture in the PCR system (heating lid temperature 105°C).

Temperature	Time
80°C	2 min

1.9 After the solution reaches room temperature, add 50 µL of mRNA Binding Buffer, mix the solution well by pipetting, and let the mixture stand at room temperature for 5 min.

1.10 Let the centrifuge tube stand on the magnetic rack for about 2 min until the solution becomes clear, and remove and discard the supernatant.

1.11 Remove the centrifuge tube from the magnetic rack, add 200 µL of Washing Buffer, and mix the solution by pipetting. Let the centrifuge tube stand on the magnetic rack until the solution becomes clear, and remove and discard the supernatant.

1.12 Microcentrifuge the tube after capping, place it on the magnetic rack, and remove all the residual liquid with a 10 µL pipette and discard it.

1.13 Prepare the Frag/Elute Buffer according to the following table:

Reagent	Volume
2X Frag/Elute Buffer*	6 µL
Nuclease-free Water*	6 µL
Total Volume	**12 µL**

* : The pre-mix can be prepared in advance based on the number of samples. No additional amount is needed.

1.14 Add 11 μL of Frag/Elute Buffer, mix the solution well by pipetting, and elute and fragment RNAs as per the following table (heating lid temperature 105°C):

Target Fragment Size	Fragmentation Condition
200-300 nt	94°C 15 min, 4°C hold
300-450 nt	94°C 10 min, 4°C hold
400-700 nt	94°C 5 min, 4°C hold

1.15 After cooling to 4°C, take out the centrifuge tube, centrifuge it instantaneously, and let the tube stand on the magnetic rack until the solution becomes clear. Transfer 10 μL of the supernatant into a new PCR tube, and immediately use it for the first strand cDNA synthesis.

2 First Strand cDNA Synthesis

2.1 Thaw RT Strand Specificity Reagent at room temperature, and prepare the following system on ice:

Reagent	Volume
Fragmented mRNAs (Step 1.15)	10 μL
RT Reagent*	8 μL
First Strand Synthesis Enzyme Mix*	2 μL
Total Volume	**20 μL**

* : The pre-mix can be prepared in advance, and its volume should be 1.1 times the sample volume to make up for the natural loss.

2.2 Mix the prepared system well by pipetting, centrifuge it instantaneously, and incubate it in the PCR system (heating lid temperature 105°C).

Temperature	Time
25°C	10 min
42°C	15 min
70°C	15 min
4°C	Hold

3 Second Strand cDNA Synthesis

3.1 Take Second Strand Synthesis Reaction Buffer with dUTP out of the refrigerator, thaw it on ice, and successively add the reagents listed in the table below.

Reagent	Volume
First strand cDNA (Step 2.2)	20 μL
Second Strand Synthesis Reaction Buffer with dUTP *	8 μL
Second Strand Synthesis Enzyme Mix*	4 μL
Nuclease-free Water*	48 μL
Total Volume	**80 μL**

* : The pre-mix can be prepared in advance, and its volume should be 1.1 times the sample volume to make up for the natural loss.

3.2 Mix the prepared system well by pipetting, centrifuge it instantaneously, and incubate it in the PCR system (with the heating lid disabled).

Temperature	Time
16°C	1 h

3.3 Take Agencourt AMPure XP Beads out of the 2-8°C environment in advance, let it stand until it reaches room temperature, and mix well by vortexing or oscillation prior to use.

3.4 Add 144 μL of Agencourt AMPure XP Beads (1.8X) into each incubated sample, and mix them well by pipetting.

3.5 Let the mixture stand at room temperature for 5 min and then on the magnetic rack for another 5 min until the solution becomes clear, and cautiously remove and discard the supernatant.

3.6 Hold the centrifuge tube on the magnetic rack, add 200 μL of 80% ethanol, let it stand for 30s, and remove and discard all the supernatant.

3.7 Repeat Step 3.6, wash the magnetic beads once with 80% ethanol, and remove all of the residual liquid with a 10 μL pipette.

3.8 Dry the magnetic beads for 2-3 min, add 40 μL of Low EDTA TE after the ethanol has fully evaporated (when the bead color changes from bright brown to frosted brown), and mix them well by pipetting.

3.9 Let the mixture stand at room temperature for 2 min and then on the magnetic rack for another 1 min until the solution becomes clear, and cautiously transfer 37 μL of the supernatant into a new centrifuge tube.

♦ The double-stranded cDNA elute can be stored at -20°C for no more than 24 hours.

4 End Repair

4.1 Take the End-prep Buffer out of the refrigerator, thaw it on ice, and prepare the systems listed in the table below.

Reagent	Volume
Double-stranded cDNA (Step 3.9)	37 μL
End-prep Buffer*	10 μL
End-prep Enzyme Mix*	3 μL
Total Volume	**50 μL**

* : The pre-mix can be prepared in advance, and its volume should be 1.1 times the sample volume to make up for the natural loss.

4.2 Mix the prepared system by pipetting, centrifuge it instantaneously, and incubate it in the PCR system according to the table below (heating lid temperature 75°C).

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

5 Adapter Ligation

5.1 Thaw the Ligation Buffer and RNA Truncated Adapter on ice and prepare the adapter ligation system on ice.

Reagent	Volume
End-prep DNA (Step 4.2)	50 μL
Ligation Buffer*	16.5 μL
RNA Truncated Adapter**	2.5 μL
Ligase Mix	3 μL
Total Volume	**About 70 μL**

* : Ligation Buffer is viscous as it contains PEG, so it needs to be pipetted slowly to avoid volume errors that may affect the subsequent size selection.

** : The adapter is a truncated one and is therefore not applicable to PCR-free library preparation, so the ligation products must be amplified.

Notes: Do not pre-mix the Ligase Mix and RNA Truncated Adapter when preparing the ligation system. Otherwise, adapter dimers will be produced, thus affecting the ligation efficiency.

5.2 Mix the ligation system by pipetting, centrifuge it instantaneously, and incubate it in the PCR system (with the heating lid disabled).

Temperature	Time
22°C	15 min

6 Size Selection

After the end of ligation, the ligation products can be purified with two options: direct purification or size selection:

When the total RNA input < 100 ng, direct purification is preferred.

When the total RNA input ≥ 100 ng, size selection is preferred according to the following table (Option 1).

Fragmentation Condition	94°C 15 min	94°C 10 min	94°C 5 min
RNA fragment size	200-300 nt	300-450 nt	400-600 nt
Library fragment size	320-420 bp	420-570 bp	520-720 bp
Proportion of Beads (1st Round)	0.35X (35 μL)	0.3X (30 μL)	0.25X (25 μL)
Proportion of Beads (2nd Round)	0.2X (20 μL)	0.2X (20 μL)	0.15X (15 μL)

Refer to the Appendix for more size selection options (Options 1, 2, and 3).

Both Options 1 and 2 are cautiously performed in Ligation Buffer. The fragments obtained with Option 2 are slightly larger and have a narrower size distribution range compared with those in Option 1. Option 3 is implemented in the aqueous phase. In this option, the ligation products are purified first (Step 5.2) before size selection (refer to the Appendix for specific procedures).

Direct Purification of Ligation Products

6.1 Take Agencourt AMPure XP Beads out of the 2-8°C environment in advance, let it stand until it reaches room temperature, and mix well by vortexing or oscillation prior to use.

6.2 After ligation, add 56 μL of Agencourt AMPure XP Beads (0.8X) into the ligation products, and mix them well by pipetting.

6.3 Let the mixture stand at room temperature for 5 min and then on the magnetic rack for another 5 min until the solution becomes clear, and cautiously remove and discard the supernatant.

6.4 Hold the centrifuge tube on the magnetic rack, add 200 μL of 80% ethanol, let it stand for 30s, and remove and discard all the supernatant.

6.5 Repeat Step 6.4, wash the magnetic beads once with 80% ethanol, and remove all the residual liquid with a 10 μL pipette.

6.6 Dry the magnetic beads for 2-3 min, add 22 μL of Low EDTA TE after the ethanol has fully evaporated (when the bead color changes from bright brown to frosted brown), and mix them well by pipetting.

6.7 Let the mixture stand at room temperature for 2 min and then on the magnetic rack for another 1 min until the solution becomes clear, and cautiously transfer 19.5 μL of the supernatant into a new PCR tube for later use.

Size Selection of Ligation Products (fragmentation conditions: 94°C 10 min)

6.1 Take Agencourt AMPure XP Beads out of the 2-8°C environment in advance, let it stand until it reaches room temperature, and mix well by vortexing or oscillation prior to use.

6.2 **Add 30 μL of nuclease-free water into the ligation system to get a 100 μL volume.**

6.3 Add 30 μL of Agencourt AMPure XP Beads (0.30X), and mix the solution well by pipetting.

6.4 Let the mixture stand at room temperature for 5 min and then on the magnetic rack for another 5 min until the solution becomes clear (**Do not discard the supernatant)**.

6.5 Transfer the supernatant into a new centrifuge tube, add 20 μL of Agencourt AMPure XP Beads (0.2X), and mix them well by pipetting.

6.6 Let the mixture stand at room temperature for 5 min and then on the magnetic rack for another 5 min until the solution becomes clear, and cautiously remove and discard the supernatant.

6.7 Hold the centrifuge tube on the magnetic rack, add 200 μL of 80% ethanol, let it stand for 30s, and remove and discard all the supernatant.

6.8 Repeat Step 6.7, wash the magnetic beads once with 80% ethanol, and remove all the residual liquid with a 10 μL pipette.

6.9 Dry the magnetic beads for 2-3 min, add 22 μL of Low EDTA TE after the ethanol has fully evaporated (***when the bead color changes from bright brown to frosted brown***), and mix them well by pipetting.

6.10 Let the mixture stand at room temperature for 2 min and then on the magnetic rack for another 1 min until the solution becomes clear, and cautiously transfer 19.5 μL of the supernatant into a new PCR tube for later use.

7 PCR Amplification

7.1 After purification of ligation products, enrich the library with the Adapter Kit.

- When the RNA Adapter Module 96 Index for Illumina is used, prepare the PCR reaction system as follows.

Reagent	Volume
Purification products	19.5 μL
2X PCR Mix	25 μL
UDG Enzyme	0.5 μL
Universal PCR Primer	2.5 μL
PCR Index *	2.5 μL
Total Volume	**50 μL**

* PCR Index Primer is for index labeling at the P7 end. It needs to be pipetted very cautiously. Each pipette tip is for single use to avoid cross contamination between samples and reagents.

- When the Dual DNA Adapter 96 Kit for Illumina is used, prepare the PCR reaction system as follows.

Reagent	Volume
Purification products	19.5 μL
2X PCR Mix	25 μL
UDG Enzyme	0.5 μL
i5xx Primer*	2.5 μL
i7xx Primer*	2.5 μL
Total Volume	**50 μL**

*: i5xx is the primer for index labeling at the P5 end, and i7xx is the primer for index labeling at the P7 end. Both need to be pipetted very cautiously. Each pipette tip is for single use to avoid cross contamination between samples and reagents.

7.2 Mix the reaction system by pipetting, microcentrifuge the tube, and incubate it in the PCR system according to the table below (heating lid temperature 105°C).

Temperature	Time	Cycles
37°C	10 min	1
98°C	1 min	1
98°C	10s	8-16*
60°C	15s	
72°C	30s	
72°C	1 min	1
4°C	Hold	

*** : Recommended PCR cycles:**

Total RNA Input	Direct Purification PCR Cycles	Size Selection PCR Cycles
10 ng	15-16	-
100 ng	12-13	14-15
1 μg	8-9	10-11

7.3 Take Agencourt AMPure XP Beads out of the 2-8°C environment in advance, let it stand until it reaches room temperature, and mix well by vortexing or oscillation prior to use.

7.4 After the end of reaction, add 40 μL of Agencourt AMPure XP Beads (0.8X) into each reaction tube, and mix them well by pipetting.

7.5 Let the mixture stand at room temperature for 5 min and then on the magnetic rack for another 5 min until the solution becomes clear, and carefully remove and discard the supernatant.

7.6 Hold the centrifuge tube on the magnetic rack, add 200 μL of 80% ethanol, let it stand for 30s, and remove and discard all the supernatant.

7.7 Repeat Step 7.6, wash the magnetic beads once with 80% ethanol, and remove all the residual liquid with a 10 μL pipette.

7.8 Dry the magnetic beads for 2-3 min, add 31 μL of Low EDTA TE after the ethanol has fully evaporated (when the bead color changes from bright brown to frosted brown), and mix them well by pipetting.

7.9 Let the mixture stand at room temperature for 2 min and then on the magnetic rack for another 1 min until the solution becomes clear, and cautiously transfer 30 μL of the library into a new centrifuge tube for later use.

8. Appendix

1. Size Distribution of RNA Fragments

Fragment mRNAs purified from 1 μg of mouse tissue total RNA using 1X Frag/Elute Buffer at 94°C for 5, 10, and 15 min separately, place them on the magnetic rack, take the supernatant, and use Agencourt RNAClean XP Beads (2.2X volume) for purification. Use the Agilent RNA 6000 Pico Chip to analyze the fragment size distribution.

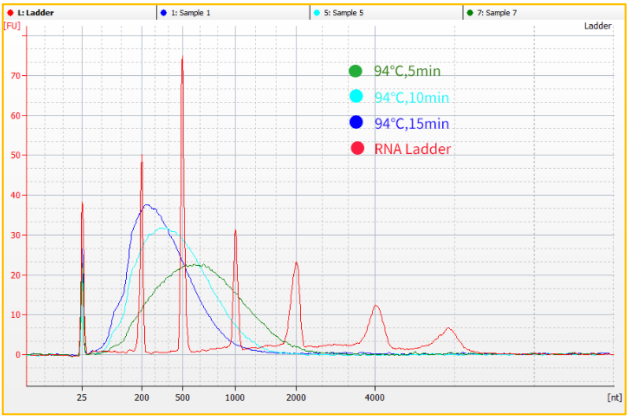


Figure 1. Size Distribution of mRNA Fragments (Agilent 2100 Bioanalyzer)

2. Library Size of Different RNA Fragmentation Time and Size Selection Programs

Different size selection options can help obtain fragments with varying main-peak patterns and size distribution ranges. ABclonal provides multiple size selection options to meet different needs. Options 1 and 2 are carried out in Ligation Buffer. The fragments obtained with Option 2 have smaller size distribution ranges and narrower library peak patterns compared with Option 1.

Option 3 is implemented in the aqueous phase, and library fragments selected with this option show stable size distribution ranges. Refer to Appendix 2.3 for specific procedures.

2.1 Size Selection of Adapter Ligation Products (Option 1)

Option 1. Bead Proportions for Size Selection and Library Size Distribution

mRNA Fragmentation Condition	Proportion of Beads (1st Round)	Proportion of Beads (2nd Round)	Library Fragment Size (bp)
94°C 5 min	0.25X (25 μL)	0.15X (15 μL)	520-720
94°C 10 min	0.30X (30 μL)	0.2X (20 μL)	420-570
94°C 15 min	0.35X (35 μL)	0.2X (20 μL)	320-420

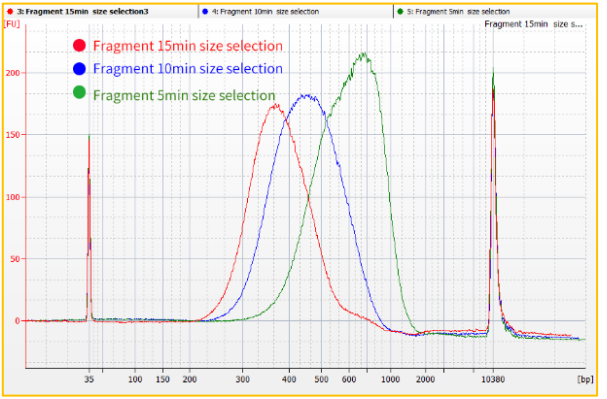


Figure 2. Library Fragment Size Distribution of Option 1 (Agilent 2100 Bioanalyzer)

Use 1 μg of mouse cell total RNA to obtain ligation products, select target fragments using different size selection conditions, and amplify the selected fragments by 10 PCR cycles to obtain libraries. Dilute the libraries to 2 ng/μL and analyze them using Agilent High Sensitivity DNA Chips and the Agilent 2100 Bioanalyzer.

2.2 Size Selection of Adapter Ligation Products (Option 2)

Option 2. Bead Proportions for Size Selection and Library Size Distribution

mRNA Fragmentation Condition	Proportion of Beads (1st Round)	Proportion of Beads (2nd Round)	Library Fragment Size (bp)
94°C 5 min	0.25X (25 μL)	0.1X (10 μL)	500-700
94°C 10 min	0.3X (30 μL)	0.1X (10 μL)	450-550
94°C 15 min	0.35X (35 μL)	0.1X (10 μL)	350-450

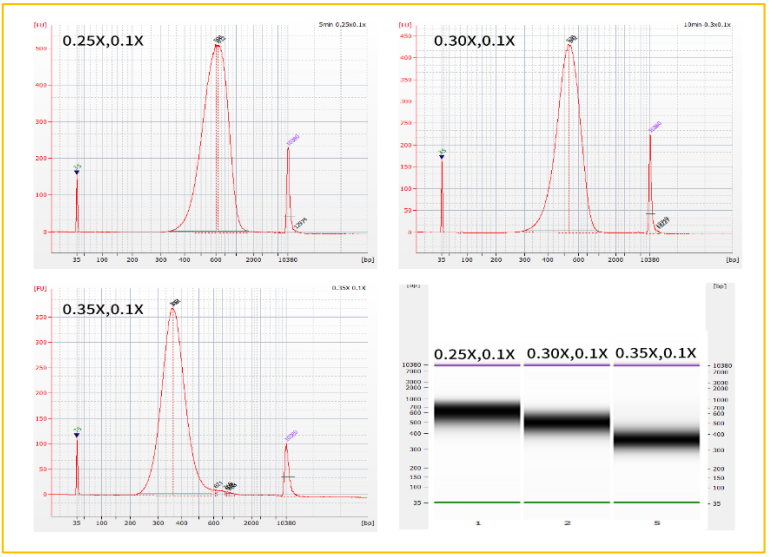


Figure 3. Library Fragment Size Distribution of Option 2 (Agilent 2100 Bioanalyzer)

Use 1 μg of mouse cell total RNA to obtain ligation products, select target fragments using different size selection conditions in Option 2, and amplify the selected fragments by 10 PCR cycles to obtain libraries. Dilute the libraries to 2 ng/μL and analyze their peak patterns using the Agilent 2100 Bioanalyzer.

2.3 Size Selection of Adapter Ligation Products (Option 3)

During adapter ligation, PEG contained in Ligation Buffer enables very sensitive size selection. Thus, a bead volume error tends to cause fragment size deviations. If high-quality fragments are required, it is recommended to conduct size selection in the aqueous phase. Specifically, purify the ligation products with 1.0X magnetic beads after ligation, elute them with 103 μL of water, and take 100 μL of the elution product for size selection as per the following table.

Option 3. Bead Proportions for Size Selection and Library Size Distribution

Fragmentation Condition	Purification of Ligation Products	Proportion of Beads (1st Round)	Proportion of Beads (2nd Round)	Library fragment size (bp)
94°C 5 min	Purify with 1.0X magnetic beads	0.55X (55 μL)	0.1X (10 μL)	600-720
94°C 10 min		0.6X (60 μL)	0.1X (10 μL)	500-600
		0.65X (65 μL)	0.1X (10 μL)	420-500
94°C 15 min		0.75X (75 μL)	0.1X (10 μL)	360-420
		0.8X (80 μL)	0.1X (10 μL)	320-360

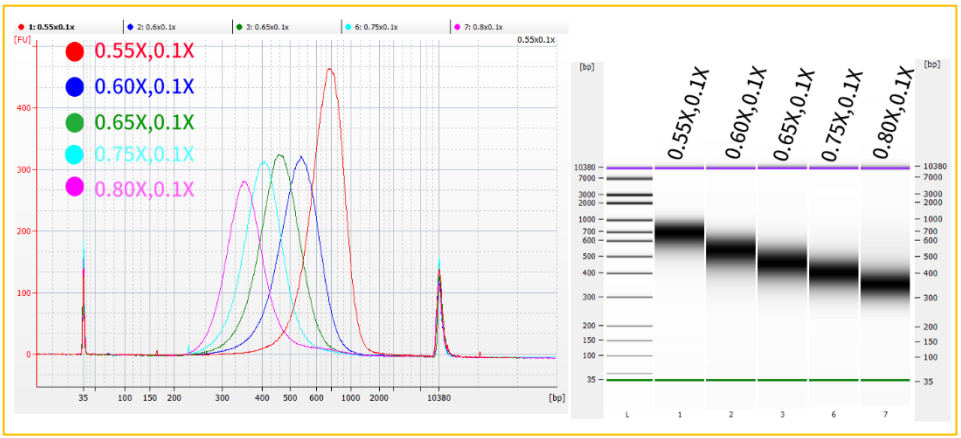


Figure 4. Library Fragment Size Distribution of Option 3 (Agilent 2100 Bioanalyzer)

Option 3: Operating Procedures for Size Selection in Aqueous Phase

2.3.1 Take Agencourt AMPure XP Beads out of the 2-8°C environment in advance, let it stand until it reaches room temperature, and mix well by vortexing or oscillation prior to use.

2.3.2 After ligation, add 70 μL of Agencourt AMPure XP Beads (1.0X) into the ligation products, and mix them well by pipetting.

2.3.3 Let the mixture stand at room temperature for 5 min and then on the magnetic rack for another 5 min until the solution becomes clear, and carefully remove and discard the supernatant.

2.3.4 Hold the centrifuge tube on the magnetic rack, add 200 μL of 80% ethanol, let it stand for 30s, and remove and discard all the supernatant.

2.3.5 Repeat Step 2.3.4, wash the magnetic beads once with 80% ethanol, and remove all the residual liquid with a 10 μL pipette.

2.3.6 Dry the magnetic beads for 2-3 min, add 102.5 μL of Low EDTA TE after the ethanol has fully evaporated (when the bead color changes from bright brown to frosted brown), and mix them well by pipetting.

2.3.7 Let the mixture stand at room temperature for 2 min and then on the magnetic rack for another 1 min until the solution becomes clear, and cautiously transfer 100 μL of the supernatant into a new PCR tube for size selection.

2.3.8 Add 65 μL of Agencourt AMPure XP Beads (0.65X 100 μL) into the purified ligation products, and mix them well by pipetting.

2.3.9 Let the mixture stand at room temperature for 5 min and then on the magnetic rack for another 5 min until the solution becomes clear (Do not discard the supernatant).

2.3.10 Transfer 160 μL of the supernatant into a new centrifuge tube, add 10 μL of Agencourt AMPure XP Beads (0.1X 100 μL), and mix them well by pipetting.

2.3.11 Let the mixture stand at room temperature for 5 min and then on the magnetic rack for another 5 min until the solution becomes clear, and cautiously remove and discard the supernatant.

2.3.12 Hold the centrifuge tube on the magnetic rack, add 200 μL of 80% ethanol, let it stand for 30s, and remove and discard all the supernatant.

2.3.13 Repeat Step 2.3.12, wash the magnetic beads once with 80% ethanol, and remove all the residual liquid with a 10 μL pipette.

2.3.14 Dry the magnetic beads for 2-3 min, add 22 μL of Low EDTA TE after the ethanol has fully evaporated (when the bead color changes from bright brown to frosted brown), and mix them well by pipetting.

2.3.15 Let the mixture stand at room temperature for 2 min and then on the magnetic rack for another 1 min until the solution becomes clear, and cautiously transfer 20 μL of the supernatant into a new PCR tube for later use.

2.4 The above results are internal test data. The ligation system is sensitive and closely associated with the size selection system, so the operating habits of operators or pipette errors may cause fragment size deviations.

- When larger library fragments are obtained, it is recommended to increase the volume of beads in the first round.
- When smaller library fragments are obtained, it is recommended to reduce the volume of beads in the first round.
- The bead proportion can be adjusted by 0.01X-0.05X based on the size deviations.

3. FAQs

◆ What if Box 1 is mistakenly stored at -20°C?

The Oligo $d(T)_{25}$ Capture Beads may be damaged at -20°C, so Box 1 cannot be used any longer. It is recommended to purchase the Poly(A) mRNA Purification Module (RK20341).

◆ Can RNA degraded to RIN < 7 be used for RNA-seq library preparation?

For severely degraded human/mouse/rat RNA samples (e.g., FFPE samples), it is recommended to prepare libraries using the ABclonal Whole RNA-seq Lib Prep Kit for Illumina (RK20303).

For plant or other eukaryote cell RNA samples, if RNAs are degraded but both 28S and 18S bands are observed in agarose gel electrophoresis, it is recommended to increase the total RNA input and increase PCR cycles to obtain desired libraries.

◆ How should the quality of prepared libraries be determined?

Before sequencing, the prepared library is assayed using the Agilent 2100 Bioanalyzer. The library quality is acceptable if there is no abnormal jagged peak, no detectable peak at 130 bp (adapter dimer), or no fragment peak with a large peak area at the right side of the library peak.

The library concentration is determined with the Qubit fluorometer. The library molarity determined with the Agilent Bioanalyzer can be calibrated by the qPCR, while the library molarity obtained with the qPCR can be calibrated by the Agilent Bioanalyzer.

◆ What if library preparation with qualified total RNA (RIN > 7) fails?

The RIN of total RNA is a metric for total RNA quality assessment and cannot fully reflect the abundance and integrity of poly(A) RNAs. For some samples, the total RNA is intact, but many mRNAs are degraded. The degradation causes significant loss during poly(A) RNA purification. Thus, library preparation fails.

In this case, the abundance and integrity of purified poly(A) RNAs can be assessed as follows: After mRNA capture, add 6 μL of Tris Buffer, heat the solution at 80°C for 2 min, let the tube stand on the magnetic rack until the solution becomes clear, and take 1 μL of the supernatant (intact poly(A) RNAs) for analysis with the Agilent 2100 Bioanalyzer and Agilent RNA 6000 Pico Chip.

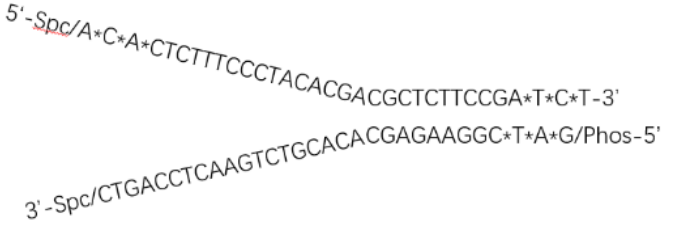
Determine solutions based on analysis results:

- If the mRNA abundance is low, increase the total RNA input.
- If the mRNA integrity is poor, adjust the mRNA fragmentation time.

For more information, please consult our technical support team.

4. Sequence Information of Adapter and PCR Index Primer

Truncated Adapter:



Universal PCR Primer:

5′-

Spc/A*A*T*GATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGA*T*C*T-3′

PCR Index:

5′-

Spc/C*A*A*GCAGAAGACGGCATACGAGAT**XXXXXXXX**GTGACTGGAGTTCAGACGTGTGCTCTTC

CGA*T*C*T-3′

Notes: XXXXXXXX is the index sequence.

i501 Primer:

5′-

Spc/A*A*T*GATACGGCGACCACCGAGATCTACAC**TATAGCCT**ACACTCTTTCCCTACACGACGCTC

TTCCGGA*T*C*T-3′

i701 Primer:

5′-

Spc/C*A*A*GCAGAAGACGGCATACGAGAT**CGAGTAAT**GTGACTGGAGTTCAGACGTGTGCTCTTC

CGA*T*C*T-3′

- Components of RNA Adapter Module 96 Index for Illumina (ABclonal, Cat. No. RK20351/RK20352):

Module	Tube Name and Color
RNA Adapter Module 96 Index for Illumina	RNA Truncated Adapter
	Universal PCR Primer
	PCR Index

- Components of Dual DNA Adapter 96 Kit for Illumina (ABclonal, Cat. No. RK20287):

Module	Tube Name and Color
Dual DNA Adapter 96 Kit for Illumina	Truncated Adapter
	i5xx Primer
	i7xx Primer

◆ Components of Dual DNA Adapter 96 Kit Extended for Illumina (ABclonal, Cat. No. RK20296):

Module	Tube Name and Color
Dual DNA Adapter 96 Kit for Illumina	Truncated Adapter
	i5xx Primer
	i7xx Primer

5. Index Sequence Information

Index Table 1. RNA Adapter Module 96 Index for Illumina MiniSet (8 Indices) (RK20355)

PCR Index Name	Index
PCR Index 1	TTACCGAC
PCR Index 2	AGTGACCT
PCR Index 3	TCGGATTC
PCR Index 4	CAAGGTAC
PCR Index 5	TCCTCATG
PCR Index 6	GTCAGTCA
PCR Index 7	CGAATACG
PCR Index 8	TCTAGGAG

Index Table 2. RNA Adapter Module 96 Index for Illumina MidiSet (24 Indices)

(RK20356)

PCR Index Name	Index	PCR Index Name	Index
PCR Index 1	TTACCGAC	PCR Index 13	TCGTCTGA
PCR Index 2	AGTGACCT	PCR Index 14	AGCCTATC
PCR Index 3	TCGGATTC	PCR Index 15	CTGTACCA
PCR Index 4	CAAGGTAC	PCR Index 16	AGACCTTG
PCR Index 5	TCCTCATG	PCR Index 17	AGGATAGC
PCR Index 6	GTCAGTCA	PCR Index 18	CCTTCCAT
PCR Index 7	CGAATACG	PCR Index 19	GTCCTTGA
PCR Index 8	TCTAGGAG	PCR Index 20	TGCGTAAC
PCR Index 9	CGCAACTA	PCR Index 21	CACAGACT
PCR Index 10	CGTATCTC	PCR Index 22	TTACGTGC
PCR Index 11	GTACACCT	PCR Index 23	CCAAGGTT
PCR Index 12	CGGCATTA	PCR Index 24	CACGCAAT

Index Table 3. RNA Adapter Module 96 Index for Illumina Set_A (48 Indices)

(RK20351)

PCR Index Name	Index	PCR Index Name	Index
PCR Index 1	TTACCGAC	PCR Index 25	TTCCAGGT
PCR Index 2	AGTGACCT	PCR Index 26	TCATCTCC
PCR Index 3	TCGGATTC	PCR Index 27	GAGAGTAC
PCR Index 4	CAAGGTAC	PCR Index 28	GTCGTTAC
PCR Index 5	TCCTCATG	PCR Index 29	GGAGGAAT
PCR Index 6	GTCAGTCA	PCR Index 30	AGGAACAC
PCR Index 7	CGAATACG	PCR Index 31	CAGTGCTT
PCR Index 8	TCTAGGAG	PCR Index 32	CTTGCTAG
PCR Index 9	CGCAACTA	PCR Index 33	TGGAAGCA
PCR Index 10	CGTATCTC	PCR Index 34	AGCTAAGC
PCR Index 11	GTACACCT	PCR Index 35	GAACGGTT
PCR Index 12	CGGCATTA	PCR Index 36	GGAATGTC
PCR Index 13	TCGTCTGA	PCR Index 37	TACGGTCT
PCR Index 14	AGCCTATC	PCR Index 38	CCAGTATC
PCR Index 15	CTGTACCA	PCR Index 39	TCTACGCA
PCR Index 16	AGACCTTG	PCR Index 40	GTAACCGA
PCR Index 17	AGGATAGC	PCR Index 41	GACGTCAT
PCR Index 18	CCTTCCAT	PCR Index 42	CTTACAGC
PCR Index 19	GTCCTTGA	PCR Index 43	TCCATTGC
PCR Index 20	TGCGTAAC	PCR Index 44	AGCGAGAT
PCR Index 21	CACAGACT	PCR Index 45	CAATAGCC
PCR Index 22	TTACGTGC	PCR Index 46	AAGACACC
PCR Index 23	CCAAGGTT	PCR Index 47	CCAGTTGA
PCR Index 24	CACGCAAT	PCR Index 48	TGGTGAAG

Index Table 4. RNA Adapter Module 96 Index for Illumina Set_B (48 Indices)

(RK20352)

PCR Index Name	Index	PCR Index Name	Index
PCR Index 49	AAGACCGT	PCR Index 73	TAGGAGCT
PCR Index 50	TTGCGAGA	PCR Index 74	CGAATTGC
PCR Index 51	GCAATTCC	PCR Index 75	GTCCTAAG
PCR Index 52	GAATCCGT	PCR Index 76	CTTAGGAC
PCR Index 53	CCGCTTAA	PCR Index 77	TCCACGTT
PCR Index 54	TACCTGCA	PCR Index 78	CAACACAG
PCR Index 55	GTCGATTG	PCR Index 79	GCCTTAAC
PCR Index 56	TATGGCAC	PCR Index 80	GTAAGGTG
PCR Index 57	CTCGAACA	PCR Index 81	AGCTACCA
PCR Index 58	CAACTCCA	PCR Index 82	CTTCACTG
PCR Index 59	GTCATCGT	PCR Index 83	GGTTGAAC
PCR Index 60	GGACATCA	PCR Index 84	GATAGCCA
PCR Index 61	CAGGTTCA	PCR Index 85	TACTCCAG
PCR Index 62	GAACGAAG	PCR Index 86	GGAAGAGA
PCR Index 63	CTCAGAAG	PCR Index 87	GCGTTAGA
PCR Index 64	CATGAGCA	PCR Index 88	ATCTGACC
PCR Index 65	GACGAACT	PCR Index 89	AACCAGAG
PCR Index 66	AGACGCTA	PCR Index 90	GTACCACA
PCR Index 67	ATAACGCC	PCR Index 91	GGTATAGG
PCR Index 68	GAATCACC	PCR Index 92	CGAGAGAA
PCR Index 69	GGCAAGTT	PCR Index 93	CAGCATAC
PCR Index 70	GATCTTGC	PCR Index 94	CTCGACTT
PCR Index 71	CAATGCGA	PCR Index 95	CTTCGGTT
PCR Index 72	GGTGTACA	PCR Index 96	CCACAACA

Index Table 5. Dual DNA Adapter 96 Kit for Illumina (RK20287)

Name	Index	Name	Index
i501 Primer	TATAGCCT	i701 Primer	ATTACTCG
i502 Primer	ATAGAGGC	i702 Primer	TCCGGAGA
i503 Primer	CCTATCCT	i703 Primer	CGCTCATT
i504 Primer	GGCTCTGA	i704 Primer	GAGATTCC
i505 Primer	AGGCGAAG	i705 Primer	ATTCAGAA
i506 Primer	TAATCTTA	i706 Primer	GAATTCGT
i507 Primer	CAGGACGT	i707 Primer	CTGAAGCT
i508 Primer	GTACTGAC	i708 Primer	TAATGCGC
		i709 Primer	CGGCTATG
		i710 Primer	TCCGCGAA
		i711 Primer	TCTCGCGC
		i712 Primer	AGCGATAG

Index Table 6: Dual DNA Adapter 96 Kit Extended for Illumina (RK20296)

Name	Index	Name	Index
i509 Primer	GACCTGTA	i713 Primer	CGTGTAGG
i510 Primer	ATGTAACT	i714 Primer	GACACTAC
i511 Primer	GTTTCAGA	i715 Primer	TGCATACA
i512 Primer	CACAGGAT	i716 Primer	CAGTCTGG
i513 Primer	TAGCTGCC	i717 Primer	TGGCACCT
i514 Primer	AGCGAATG	i718 Primer	CAAGGTGA
i515 Primer	TATGCTGC	i719 Primer	AAAGATAC
i516 Primer	AGAAGACT	i720 Primer	TGGAGCTG
		i721 Primer	TAAGGCGA
		i722 Primer	CGTACTAG
		i723 Primer	GCTACGCT
		i724 Primer	CGAGGCTC

China

www.abclonal.com.cn

Headquarters: Building 5, Precision Medicine Industry Base Project I, Gaokeyuan 3rd Road, Donghu New Technology Development Zone, Jiangxia District, Wuhan, Hubei, China

Shanghai R&D Center: F4, Building 2, Zizhu High-Tech Industrial Development Park, No. 58 Yuanmei Road, Minhang District, Shanghai, China

US R&D Center: 86 Cummings Park Dr, Woburn, MA 01801, United States

Tel: 400-999-6126

Email: cn.market@abclonal.com