艾科瑞生物
Accurate Biology

Instruction for Use

AccuNext Stranded RNA-seq Library Kit for Illumina

AG12503 AG12504
Version.V1E1

Research Use Only
Not For Diagnosis Procedures

1. Description

This product is suitable for indexed cDNA library construction for Next-Generation Sequencing (NGS) on any Illumina platforms. This kit is compatible with input RNA of 100 pg ~ 100 ng. This kit consist of all required reagent components for RNA fragmentation, Reverse Transcription and Library Construction. With utilization of unique template switching capability from the Reverse Transcriptase and PCR amplification, this kit generates Illumina-compatible libraries without the need for enzymatic clean-up/End Repairing or adapter ligations. The final library preserves the strand orientation of the RNA, as it would be possible to retrieve strand-specific sequencing information from the final library.

It is recommended to use RNA template with ribosomal RNA depleted, to reduce sequencing cost and increase rate of efficient data.
This kit is recommended to be used along with the *AccuNext* Dual Index (CDI) Kit for Illumina (Cat. No AG 12505/12506/12507).
The final output of cDNA library could be directly used for Next-Generation Sequencing.

2. Kit Information

Kit Name	Cat. No	Specification
AccuNext Stranded RNA-seq Library Kit for Illumina	AG 12503	12 rxns
	AG 12504	48 rxns

3. Transportation and Storage

Storage	Package 2-1 Store at –80°C Package 2-2 Store at –20°C
Transportation	Transport at –20°C Dry Ice or Blue Ice Condition

4. Kit Components

Package 2-1 (-80°C Storage Component)

Kit Components	AG 12503 (12 rxns)	AG 12504 (48 rxns)
Control Total RNA* (1μg/μl)	20 μl	20 μl
5' Template Switching Oligo	54 μl	216 μl

*: Control Total RNA is 293T Cell Total RNA.

Package 2-2 (-20°C Storage Component)

Kit Components	AG 12503 (12 rxns)	AG 12504 (48 rxns)
RNase Inhibitor (40 U/μl)	6 μl	24 μl
3'- N6 Primer	12 μl	48 μl
5 X First-Strand Buffer	48 μl	192 μl
AccuNext Reverse Transcriptase(100U/μl)	24 μl	96 μl
2 X *AccuNext* PCR Buffer	300 μl	1.2 ml
AccuNext DNA Polymerase (1U/μl)	12 μl	48 μl
Nuclease Free Water	1 ml	1 ml x 4 pcs

Note: The Dual Index (CDI, Illumina) are required reagent but not included in this kit. AG12505, AG12506, AG12507 from Accurate Biotech are recommended, or equivalent kits from other suppliers.

Kit Components Information for AccuNext **Dual Index (CDI) Kit for Illumina**

Intended Use	Component Code	AG 12505 (12 rxns)	AG 12506 (48 rxns)	AG 12507 (96 rxns)	Cap Color
i5 Index Primer (AP501-AP508)	AP 501	12 µl	12 µl	12 µl	
	AP 502	-	12 µl	12 µl	
	AP 503	-	12 µl	12 µl	
	AP 504	-	12 µl	12 µl	
	AP 505	-	-	12 µl	
	AP 506	-	-	12 µl	
	AP 507	-	-	12 µl	
	AP 508	-	-	12 µl	
I7 Index Primer (AP701-AP712)	AP 701	5 µl	5 µl	8 µl	
	AP 702	5 µl	5 µl	8 µl	
	AP 703	5 µl	5 µl	8 µl	
	AP 704	5 µl	5 µl	8 µl	
	AP 705	5 µl	5 µl	8 µl	
	AP 706	5 µl	5 µl	8 µl	
	AP 707	5 µl	5 µl	8 µl	
	AP 708	5 µl	5 µl	8 µl	
	AP 709	5 µl	5 µl	8 µl	
	AP 710	5 µl	5 µl	8 µl	
	AP 711	5 µl	5 µl	8 µl	
	AP 712	5 µl	5 µl	8 µl	

Sequence Information of the AccuNext **Dual Index (CDI) Kit for Illumina**

i5 Index Primer for Illumina

5' -AATGATACGGCGACCACCGAGATCTACAC ***[i5 Index]*** ACACTCTTTCCCTACACGACGCTCTTCCGATCT- 3'

i7 Index Primer for Illumina

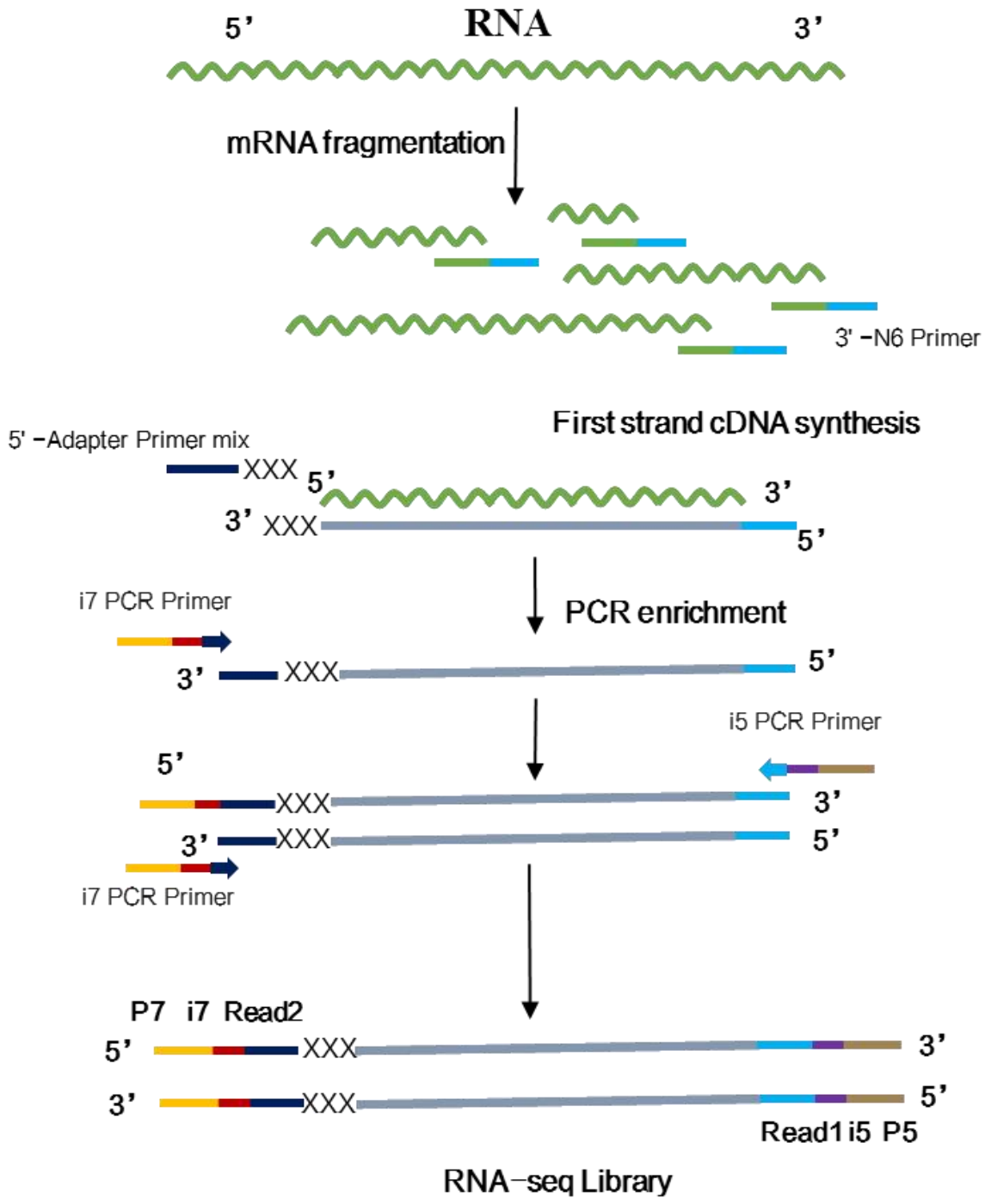
5' -CAAGCAGAAGACGGCATACGAGAT ***[i7 Index]*** GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT- 3'

[i5 Index] represents the i5 Index sequence of 8 bp length; [i7 Index] represents the i7 Index Sequence of 8 bp length

i5 Index Primer	i5 Index Sequence	I7 Index Primer	i7 Index Sequence
AP 501	TATAGCCT	AP 701	ATTACTCG
AP 502	ATAGAGGC	AP 702	TCCGGAGA
AP 503	CCTATCCT	AP 703	CGCTCATT
AP 504	GGCTCTGA	AP 704	GAGATTCC
AP 505	AGGCGAAG	AP 705	ATTCAGAA
AP 506	TAATCTTA	AP 706	GAATTCGT
AP 507	CAGGACGT	AP 707	CTGAAGCT
AP 508	GTACTGAC	AP 708	TAATGCGC
-	-	AP 709	CGGCTATG
-	-	AP 710	TCCGCGAA
-	-	AP 711	TCTCGCGC
-	-	AP 712	AGCGATAG

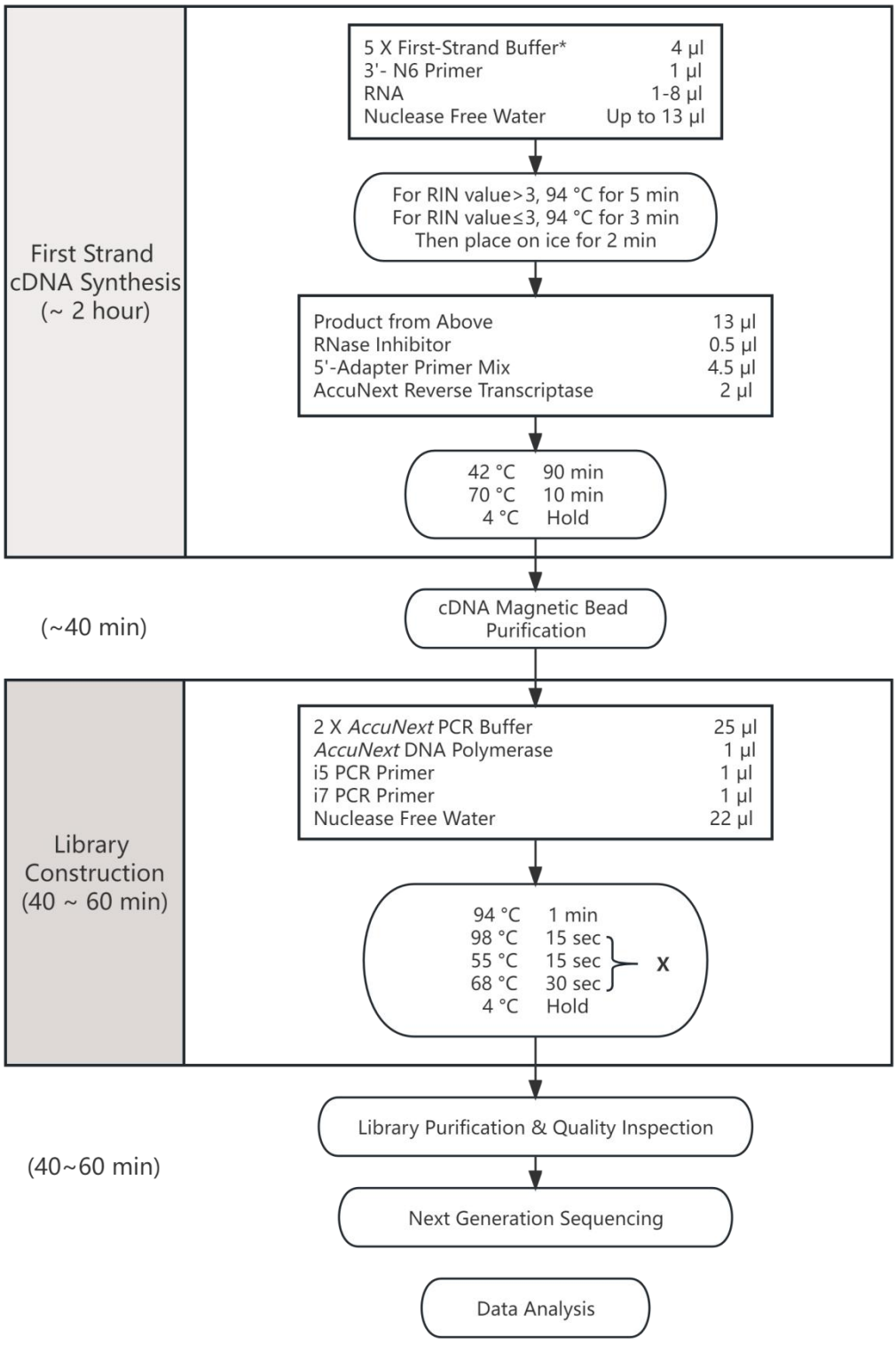
5. Experimental Principle and Workflow

Figure (1) Library Construction/Preparation Principle of AG12503/AG12504



Note: Recommend to use AG12505,AG12506,or AG12507 as for the i5 PCR Primer and i7 PCR Primer.

Figure (2) Experimental Workflow



6. Required Material not Included

Intended Use	Required Materials
mRNA Enrichment	*SteadyPure Mag* mRNA Purification Kit (Cat.No AG 21204) or other equivalent product
Magnetic Bead Purification	AMPure XP Reagent (Beackman Coulter Life Science, A63881) or other equivalent product, Magnetic Rack/Stand
DNA Quality Inspection	High Sensitivity DNA Kit (Agilent， Code. 5067-4626), Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific, Cat. No. Q32854) or other equivalent product
RNA Quality Inspection	Agilent RNA 6000 Pico Kit (Agilent, Code. No 5067-1513) or other equivalent product
Others	80% Ethanol (freshly prepared), Nuclease-free Water, RNase free PCR strip tubes (0.2 ml), 1.5 ml centrifuge tubes, Thermal Cycler, Vortex Mixer, Microcentrifuger, Agilent® Bioanalyzer® or similar fragment analyzer and associated consumables, Qubit 4 Fluorometer

7. Recommendation and Precaution

7.1 Anti-Contamination Procedures

(1) Recommend to adopt adequate anti-contamination lab procedures and wear protective gears. Operate in clean lab space and Use sterile consumables to avoid RNase contamination.

(2) Due to high sensitivity of the kit, avoid cross-contamination with other experiment and samples. Separate sample processing and reagent processing in two isolated lab spaces.

(3) All components of the kit shall be stored in sterile environment.

(4) Recommend to use pipette tips with sterile filters.

(5) Run on two different thermal cyclers for first-strand cDNA sysnthesis and PCR amplification.

7.2 Sample Requirement

(1) The RNA samples should be free of salts (e.g., Mg2+), divalent cation chelating agents (e.g., EDTA), or organics (e.g., ethanol). Use RNA Template with high purify.

(2) Inspect quality of the input RNA by running it on an Agilent Bioanalyzer to determine the RNA integrity number (RIN).

(3) Recommend to deplete ribosomal RNA before using RNA template for library construciton, to reduce sequencing cost and increase efficient rate of sequencing data.

(4) For degraded RNA or RNA template with no Poly A tailing, recommend to use the NEBNext rRNA Depletion Kit (Human/Mouse/Rat) to deplete rRNA before library construction to enhance efficiency.

7.3 Template Fragmentation

(1) If encounter severe degradation of RNA template, adjust running time of fragmentation accordingly; If range of fragment distribution is overly large or with unexpected peak of large length fragment, then prolong the running time of fragmentation; if range of fragment distribution is unsatisfactorily small or with unexpected peak of short length fragment, reduce the running time accordingly.

7.4 Recommendation for Bead Purification (cDNA and Library)

(1) Thaw bead to room temperature before use, otherwise will affect the DNA recovery rate.

(2) Vortex and mix bead well, each time before use.

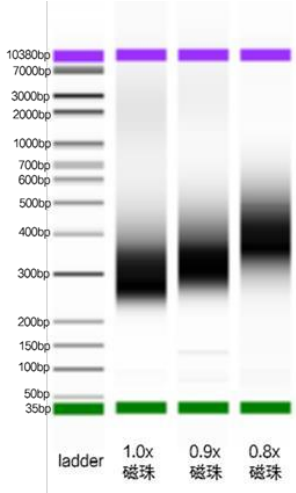
(3) Use and make 80% ethanol freshly, before each experiment.

(4) Use the product after ethanol fully volatilized, otherwise will affect the product recovery rate.

(5) Do not touch bead precipitation when removing the supernatant.

(6) Recommend to repeat the cDNA purification steps twice, to enhance reduction of leftovers.

(7) Select different bead:input ratio for different size of DNA fragment. Recommend to use 1:1 ratio for cDNA and library construction. For short fragment, recommend to use ratio of 0.9X or 0.8X for purification. Example of difference using different ratio is as shown below.



7.5 Library Construction and PCR Amplification

(1) Select the adequate number of running cycles per experimental conditions. Recommend to keep the result within the exponential stage of amplification. While securing enough quantity of library product, use the lesser number of cycles.

(2) The quality of constructed library would be negatively affected by too high number of cycles/long amplification time.

(3) If the quantity of library product is not satisfactory following recommended number of cycles, retry with adding 2 ~ 3 cycles.

(4) Please follow the instruction of this protocol or instructions of the *AccuNext* Dual Index (CDI) Kit for Illumina (AG12505/AG12506/AG12507) to select the adequate Index Primers.

7.6 Recommendation for Sequencing Data Analysis

(1) For Illumina sequencing platforms, most common requirement for final concentration of library is ≥2 nM or ≥4 nM. Recommend to construct and prepare the library with ≥7.5 nM firstly for long term storage.

(2) Read 2 is the reading of forward direction of initial RNA, Read 1 is the reading of reverse direction of initial RNA.

(2) For Read 2 reading, the first 3 bases shall be excluded for dual Index analysis. The first 3 bases are introduced external sequences by 5'-Adapater Primer Mix.

8. Protocol

8.1 RNA Fragmentation

(1) Thaw reagents on ice, centrifuge briefly and mix well. Place on ice for further use.

Note: Do not vortex for the 5' -Adapter Primer Mix, *AccuNext* Reverse Transcriptase and RNase Inhibitor.

Then Prepare reaction mix as below table. Mix well and centrifuge.

Components	Volume
5 X First-Strand Buffer	4 μL
3' - N6 Primer	1 μL
RNA*	1 ~ 8 μL
Nuclease Free Water	Up to 13 μL

*: Recommended RNA input is 100pg ~ 100ng. Higher RNA template input would grant better result within recommended range.

Note: Component Control Total RNA included in this kit is 1 μg/μL of Mouse Liver Total RNA, dilute to 2ng/μL, then add 5 μL for each use. For experimental use, dilute to 20ng/μL, 2 ng/μL, 200 pg/μL, 20 pg/μL, and then add 5 μL for each control reaction.

(2) Run Fragmentation step following program setup as below. Place on ice for 2 min after done.

RIN Value	Time	Temperature
>3	5 min	94°C
≤3	3 min	94°C

***Hint** for enhancing experimental results

While running reaction for RNA fragmentation, prepare reaction mix for Step(2) Reverse Transcription. If encounter severe degradation of RNA template, adjust running time of fragmentation accordingly; If range of fragment distribution is overly large or with unexpected peak of large length fragment, then prolong the running time of fragmentation; if range of fragment distribution is unsatisfactorily small or with unexpected peak of short length fragment, reduce the running time accordingly.

(3) Start **Step 8.2 Reverse Transcription** right after finishing fragmentation.

8.2 Reverse Transcription

(1) Prepare Reverse Transcription reaction mix as below.

Components	Volume
Product solution from **Step 8.1 RNA Fragmentation**	13 μL
RNase Inhibitor	0.5 μL
5'-Adapter Primer Mix	4.5 μL
AccuNext Reverse Transcriptase	2 μL
Total	20 μL

***Hint**

Prepare a premix solution of RNase Inhibitor, 5'-Adapater Primer Mix, *Accunext* Reverse Transcriptase firstly, mix gently with pipette. Then add 7 μL of the premix solution to prepare the full reaction system. Gently mix and centrifuge for further use.

(2) Run following program setup as below. Keep at 4°C, or store at -20°C for further use. (To preserve cDNA from degradation)

Temperature	Time
42°C	90 min
70°C	10 min
4°C	Hold

8.3 cDNA Purification

Purify cDNA product using magnetic beads. Recommend to use the AMPure XP Reagent (Beckman Coulter Life Sciences, A63881)

Note: Recommend to premix the beads into 1.5 ml centrifuge tubes, store at 4 °C, before experimental use;

Note: Prepare 80% ethanol before experimental use, 400 µL of 80% ethanol is needed per sample.

(1) Vortex and mix the beads well, then place at room temperature for 30 min, before use.

(2) Add 20 µL of beads into 20 µL of cDNA products from upstream steps (ratio of 1:1), vortex 5 sec and mix well, then centrifuge briefly.

(3) Incubate the mix at room temperature for 8 mins.

(4) Place the mix on the magnetic stand for at least 5 min to precipitate. Carefully remove supernatant.

(5) (Keep the mix tube on the magnetic stand) add 200 µL of 80% ethanol. Incubate at room temperature for 30 sec, then remove supernatant again carefully.

(6) Repeat step (5) once.

(7) Centrifuge briefly, settle for 30 secs then remove supernatant again carefully. (Keep the mix tube on the magnetic stand) Keep caps open for 3 ~ 5 min until ethanol all volatilized.

(8) Remove tubes from the magnetic stand. Add 20 µL of Nuclease Free Water, covering the beads. Use pipettes to gently mix beads, then incubate at room temperature for 2 min.

(9) Centrifuge briefly. Place on the magnetic stand again to precipitate until supernatant is clear (Approximately 5 min).

(10) Retrieve the supernatant carefully to new tubes, store at -20°C. Discard the leftover and beads.

(11) If needed, Repeat step (1) ~ (7) to reduce contamination from primer leftovers.

(12) Process to **Step 8.4 Library Construction**.

8.4 Library Construction

(1) Prepare PCR reaction mix as below.

Components	Volume
AccuNext DNA Polymerase	1 µL
2 X *AccuNext* PCR Buffer	25 µL
i5 PCR Primer*	1 µL
i7 PCR Primer*	1 µL
Nuclease Free Water	Up to 50 µL

***Hint**

Prepare a premix solution of all components except the *AccuNext* DNA Polymerase firstly, mix gently. Then add the *AccuNext* DNA Polymerase to prepare the full reaction system. Gently mix and centrifuge for further use.

*: Recommend to use the *AccuNext* Dual Index (CDI) Kit for Illumina (Product Code. AG 12505/AG12506/AG12507).

(2)Add 50 µL of prepared PCR reaction mix to the cDNA purification product (from Step 8.3), vortex and gently mix, centrifuge.

(3) Run PCR program as below.

Step	Temperature	Time	Number of Cycles
Initial-Denaturation	94°C	1 min	1
Denaturation	98°C	15 sec	
Annealing	55°C	15 sec	X*
Extension	68°C	30 sec	
End	4 °C	Hold	-

*:Please select adequate number of running cycles per following recommendation table. Following recommendation is based upon Mouse Liver mRNA and Mouse Liver Total RNA. Actual experimental requirement could vary and shall be adjusted accordingly.

Initial Total RNA Input	Recommended Number of Cycles
100 pg	18 ~ 21
1 ng	15 ~ 17
10 ng	12 ~ 14
100 ng	6 ~ 9

(4)Place the product on ice for further purification. Or store at -20°C/-80°C.

8.5 Library Purification

Purify library using magnetic beads. Recommend to use the AMPure XP Reagent (Beckman Coulter Life Sciences, A63881)

Note: Recommend to premix the beads into 1.5 ml centrifuge tubes, store at 4 °C, before experimental use;

Note: Prepare 80% ethanol before experimental use, 400 µL of 80% ethanol is needed per sample.

(1) Vortex and mix the beads well, then place at room temperature for 30 min, before use.

(2) Add 50 µL of beads into 50 µL of library products from upstream steps (ratio of 1:1), vortex 5 sec and mix well, then centrifuge briefly.

(3) Incubate the mix at room temperature for 8 mins.

(4) Place the mix on the magnetic stand for at least 5 min to precipitate until the supernatant is clear. Carefully remove supernatant.

(5) (Keep the mix tube on the magnetic stand) add 200 µL of 80% ethanol. Incubate at room temperature for 30 sec, then remove supernatant again carefully.

(6) Repeat step (5) once.

(7) Centrifuge briefly, settle for 30 secs then remove supernatant again carefully. (Keep the mix tube on the magnetic stand) Keep caps open for 3 ~ 5 min until ethanol all volatilized.

(8) Remove tubes from the magnetic stand. Add 17 µL of Nuclease Free Water, covering the beads. Use pipettes to gently mix beads, then incubate at room temperature for 2 min.

(9) Centrifuge briefly. Place on the magnetic stand again to precipitate until supernatant is clear (Approximately 5 min).

(10) Retrieve 15 µL of the clear supernatant carefully to new tubes, store at -20°C. Discard the leftover and beads.

8.6 Quality Inspection

Note: If precipitation occurs in the 5 X First-Strand Synthesis Buffer, vortex and mix well until precipitation dissolved before use.

(1) Take 2 µL of retrieved library product from above, to be tested for concentration on the Qubit 4 Fluorometer, using the Qubit 1X dsDNA HS Assay Kit (Thermo Fisher, Code.Q33231). Please refer to the corresponding instrument and kit instructions.

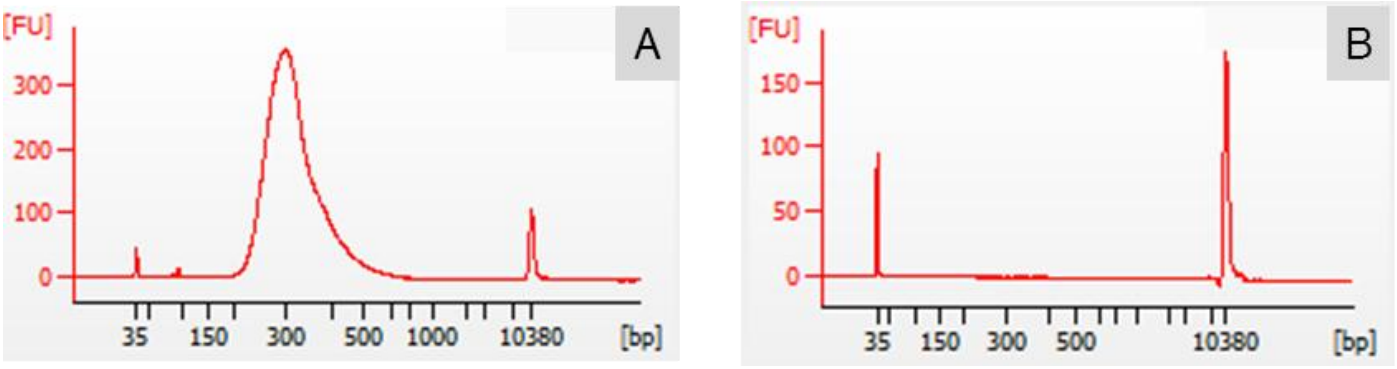
(2) Take 1 µL of retrieved library product from above, to be tested for purity on the Agilent Technologies 2100 Bioanalyzer, using the Agilent High Sensitivity DNA Kit (Agilent, Code.5067-4626). Please refer to the corresponding instrument and kit instructions.

Note: Typical molar concentration of retrieved product is more than 7.5nM. Length of fragment is expected to be 200 ~ 700 bp, with a peak on approximately 300 bp. Not amplification product should be observed for negative controls.

Appendix Experimental Sample Result

Appendix 1 Example of constructed library size distribution(A) and clean negative control result (B) on a Bioanalyzer

100 pg of T293T Cell mRNA used and amplified using 19 cycles. 1:1 ratio of bead purified



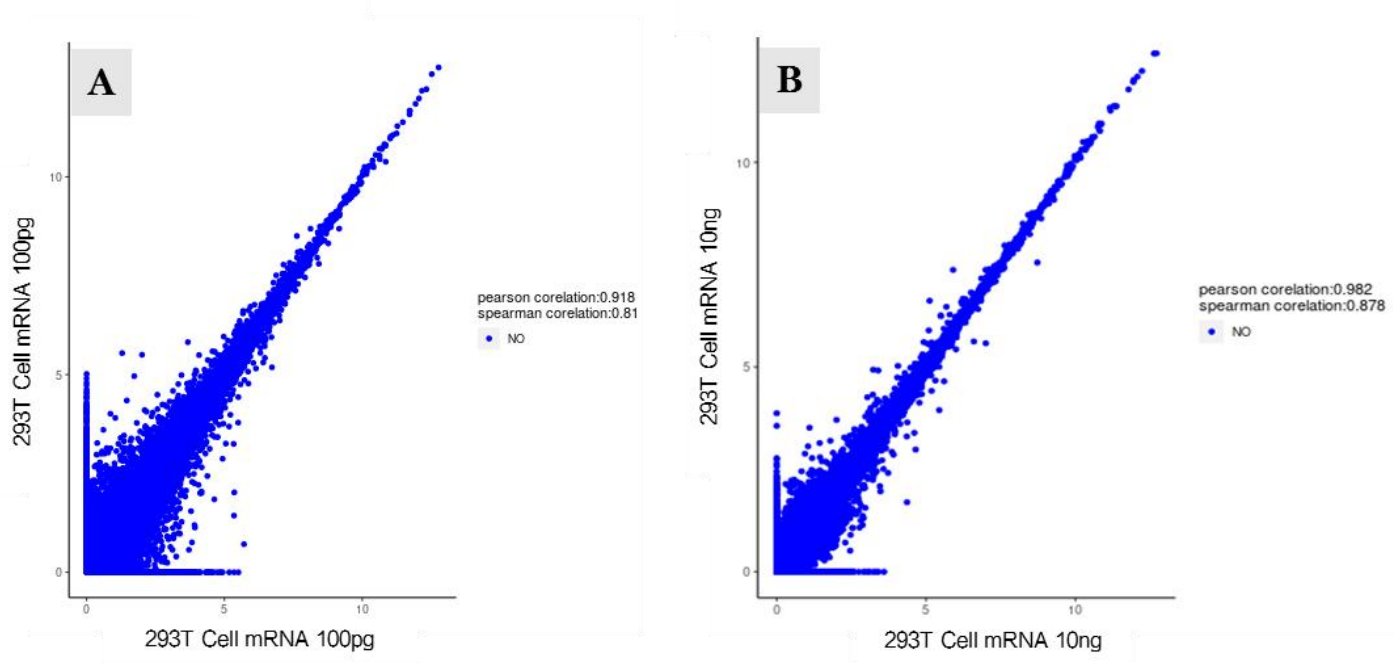
Appendix 2 Example Sequencing Data Analysis

Using 293T Cell mRNA on the Illumina NovaSeq 6000

Sequencing metrics comparing input mRNA amounts				
mRNA source	293T cell mRNA			
Input mRNA	100pg		10ng	
Replicate	1	2	1	2
Q30 Bases rate（%）	93.2	92.62	91.91	92.5
Total gene number(FPKM>0.1)	17455	17348	21522	21399
Total gene number(FPKM>1)	12968	12986	13118	13090
Percentage of reads(%)				
rRNA residue rate	2.49	2.25	2.63	2.66
Mapped to genome	90.51	90.16	88.06	88.69
Mapped unique to genome	85.19	85.02	82.88	83.38
exon	89.97	90.08	90.54	90.5
Intron	6.4	6.39	5.91	5.93
Intergenic	3.63	3.53	3.55	3.57
Pearson	0.918		0.982	
Spearman	0.81		0.878	

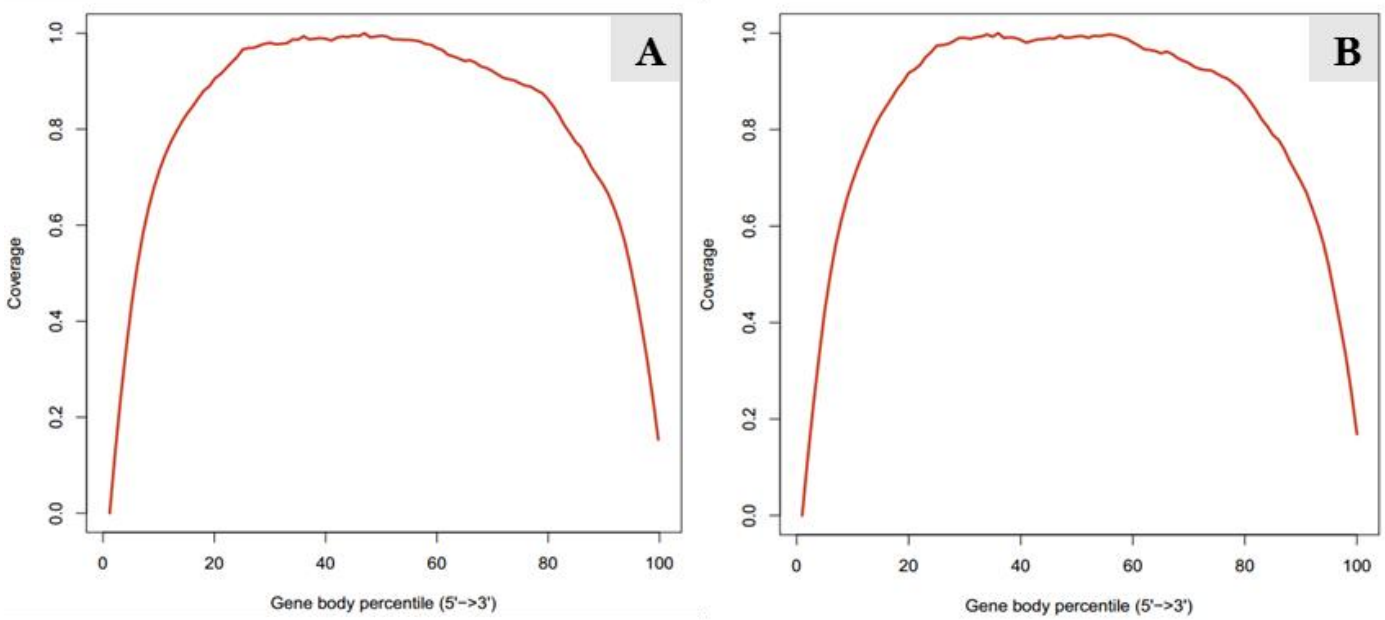
Appendix 3 Consistency Test - Sequencing Date Distribution from two repeat runs

Using 100 pg (A), 10 ng(B) of 293T Cell mRNA



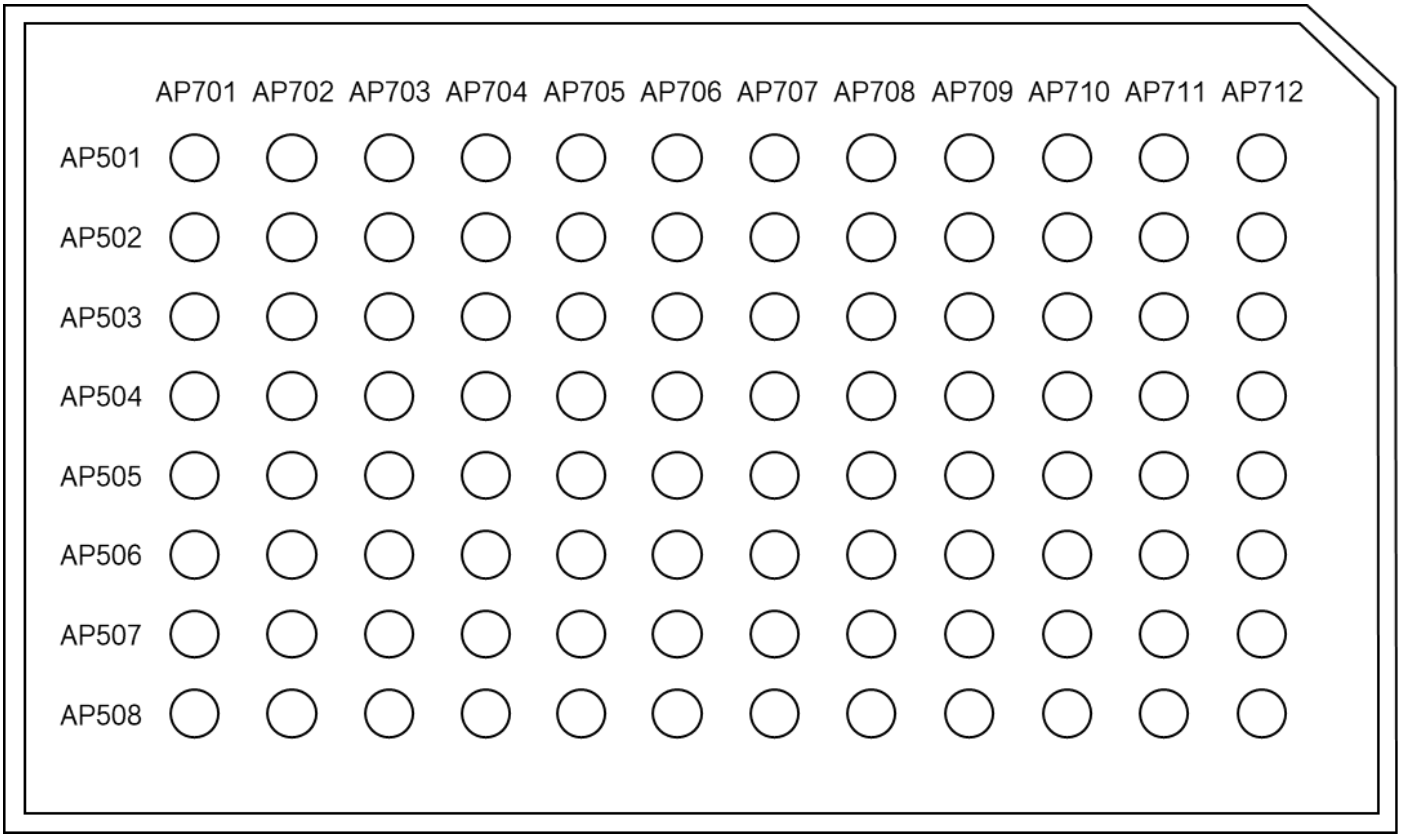
Appendix 4 Uniformity Test - Distribution of Sequencing Data on the Gene

Using 100 pg (A), 10 ng(B) of 293T Cell mRNA



Appendix 5 Recommendation Adapter Pairing - Full Plate Setup

Using AG12507 *AccuNext* Dual Index (CDI) Kit for Illumina



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