Instruction for Use

AccuNext Single Cell/Low Input cDNA Synthesis & Amplification Kit

AG12501 AG12502
Version.V2E1

Research Use Only
Not For Diagnosis Procedures

1. Description

This product is suitable for high quality cDNA synthesis and amplification from RNA inputs with Poly A tailing, primary cells or Eukaryotic cells without cell walls (e.g. mammalian cells). The kit contains enzymatic components required for cell lysis, Reverse Transcription an PCR amplification.

For Reverse Transcription, input template is recommended to be 1-1000 of cells, or 10 pg ~ 100 ng of total RNA. It is compatible to input volume of 1~9.5 μl, with expected output cDNA product of 2 ~ 20 ng. With utilization of unique template switching capability from the Reverse Transcriptase and LNA (Locked Nucleic Acid) Technology in adapter primers included in the kit, synthesis and amplification efficiency is effectively boosted, and final cDNA library is retained with mRNA 5' end, preserving original genetic expression information of the mRNA transcript. The final output of first-strand cDNA library could be used for Next-Generation Sequencing, and downstream analysis of genetic expression variation, alternative splicing, fusion gene expression and others.

2. Kit Information

Kit Name	Cat. No	Specification
AccuNext Single Cell/Low Input cDNA Synthesis & Amplification Kit	AG 12501	12 rxns
	AG 12502	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 12501 (12 rxns)	AG 12502 (48 rxns)
Control Total RNA* (1μg/μl)	5 μl	5 μl
5' Template Switching Oligo	12 μl	48 μl

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

Package 2-2 (-20°C Storage Component)

Kit Components	AG 12501 (12 rxns)	AG 12502 (48 rxns)
10 X Cell Lysis Buffer	228 μl	920 μl
RNase Inhibitor (40 U/μl)	18 μl	72 μl
3' Oligo (dT) Primer	24 μl	96 μl
5 X First-Strand Synthesis Buffer	48 μl	192 μl
AccuNext Reverse Transcriptase(100U/μl)	24 μl	96 μl
AccuNext DNA Polymerase (1U/μl)	12 μl	48 μl
2 X *AccuNext* PCR Buffer	300 μl	1.2 ml
PCR Primers	12 μl	48 μl
Nuclease Free Water	1 ml	1 ml

5. Experimental Principle and Workflow

Figure (1) cDNA Synthesis Principle of AG12501/AG12502

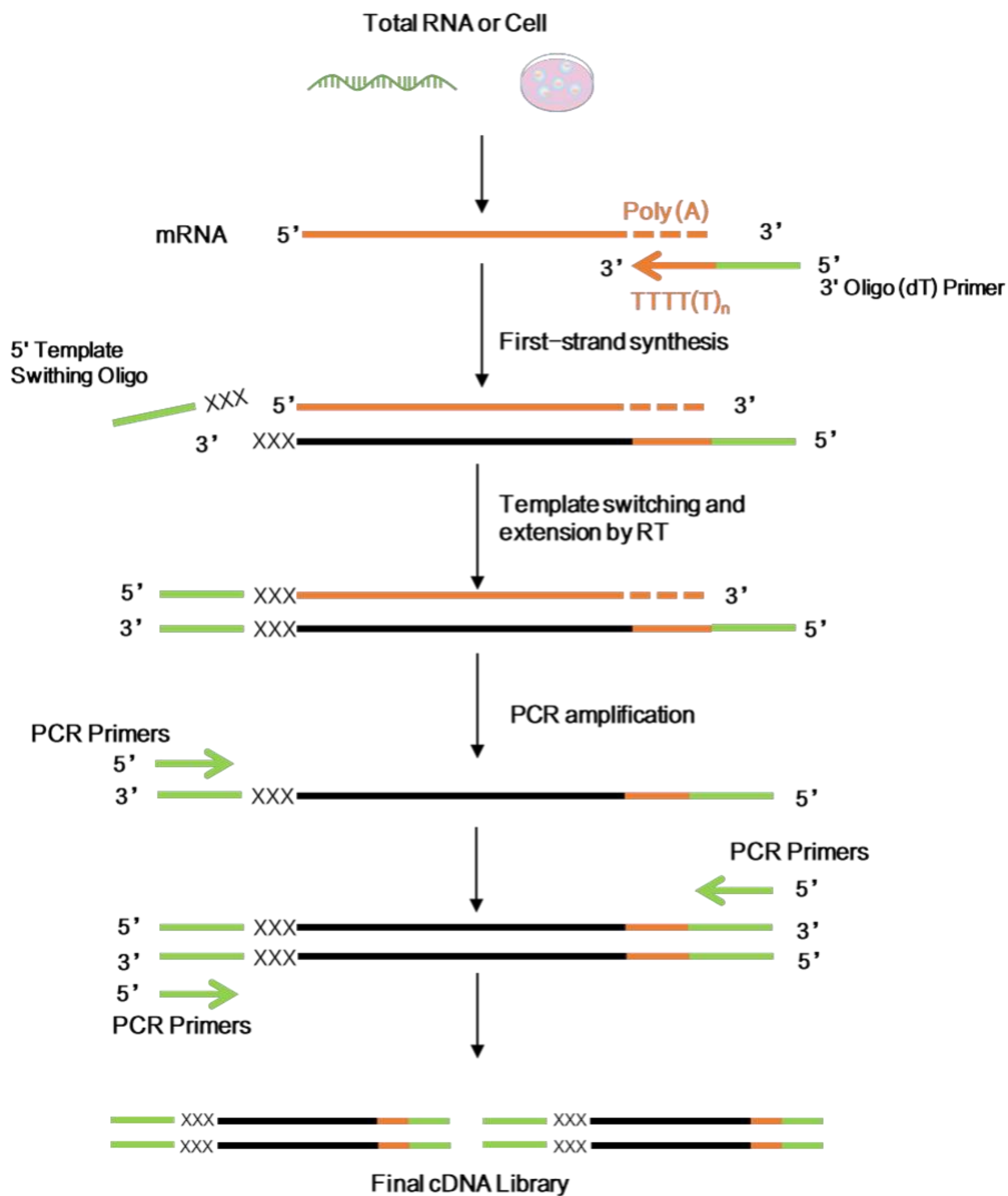
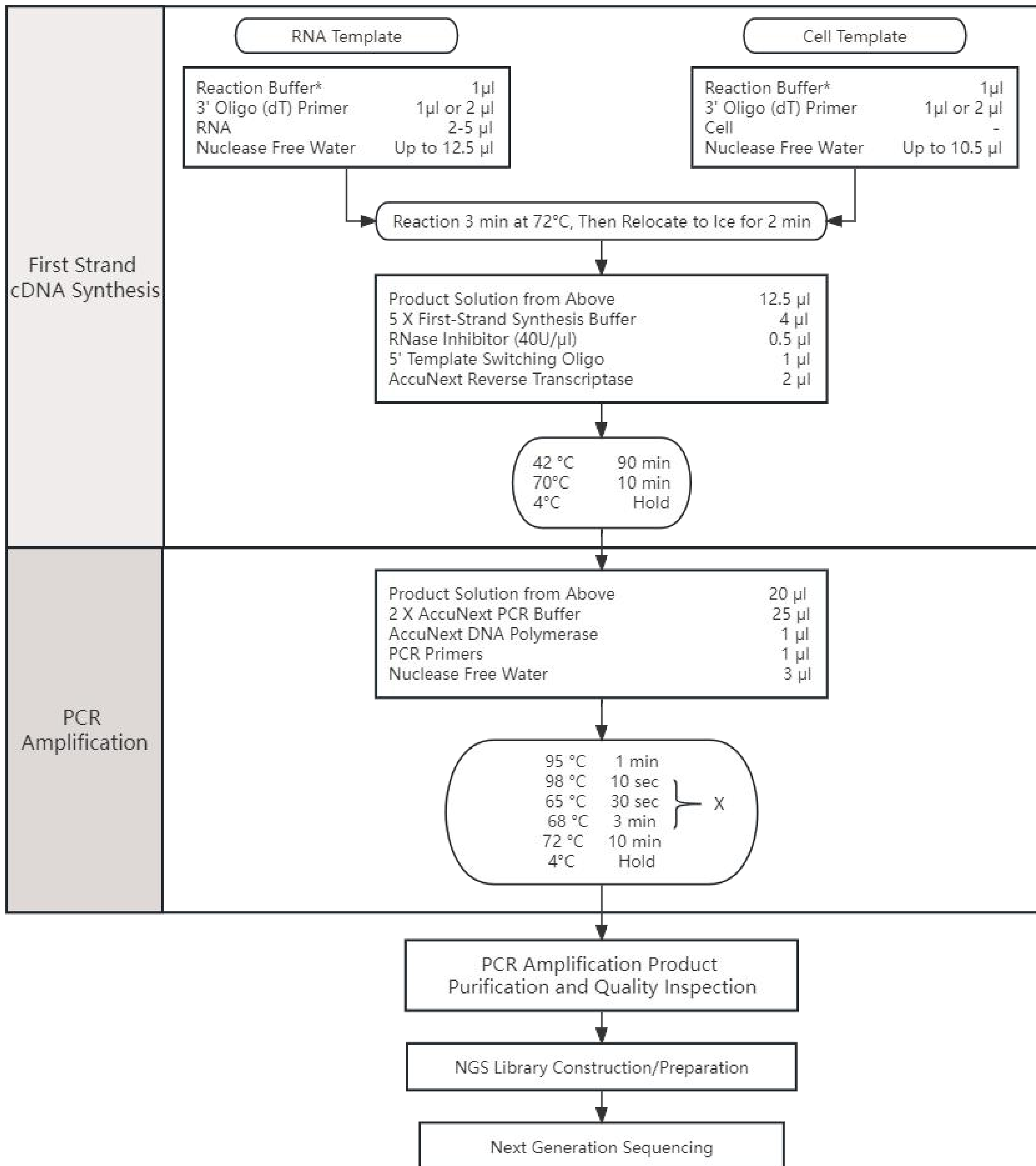


Figure (2) Experimental Workflow



* Reaction Buffer should be prepared per following protocol.

6. Required Material not Included

Intended Use	Required Materials
RNA Quality Inspection	Agilent RNA 6000 Pico Kit (Agilent，Code. 5067-1513) or other equivalent product
Magnetic Bead Purification	NucleoMag NGS Clean-up and Size Select (Macherey-Nagel， Code.744970.50) 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
DNA Library Construction/Preparation	Nextera XT DNA Library Preparation Kit (Illumina，Code.FC-131-1024) 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, Microcentrifuge, Agilent® Bioanalyzer® or similar fragment analyzer and associated consumables, Qubit 4 Fluorometer

7. Recommendation and Precaution

7.1 General Guideline and Sample Requirement

(1) The protocol is intended for isolated cultured or primary cells, but is not compatible with fixed cells. The protocol is to be used for total RNA.

(2) Cells should be intact and sorted in the 10 X cell lysis buffer provided in this kit. Cells should be washed and resuspended in PBS prior to sorting/isolation. Carryover of media may affect the cDNA synthesis efficiency.

(3) The RNA samples should be free of salts (e.g., Mg2+), divalent cation chelating agents (e.g., EDTA), or organics (e.g., ethanol). If an excess amount of genomic DNA is present in RNA samples, an optional DNase I treatment could be performed. Inactivate/Remove DNase I after treatment. Inspect quality of the input RNA by running it on an Agilent Bioanalyzer to determine the RNA integrity number (RIN).

(4) Keep all buffers and enzymes on ice, unless otherwise listed.

7.2 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.

7.3 Recommendation for PCR Amplification

(1) Select number of cycles per recommendation table in the protocol. The number of cycle is recommended to be less with satisfactory quantity of PCR product.

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

(3) The number of cycle should be able to produce enough quantity for further use.

7.4 Recommendation for PCR Product Purification

(1) Thaw bead to room temperature, 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.

7.5 Recommendation for Library Construction/Preparation

(1) Recommend to use the Nextera XT DNA Library Preparation Kit (Illumina, Code. FC-131-1024) or equivalent product for Illumina platform.

7.6 Recommendation for Sequencing Data Analysis

(1) If use enzymatic or mechanical fragmentation methods to add adapters on both ends of double strand cDNA, Reads 2 data will contain dT30 Reads of 3' Oligo (dT) Primer.

(2) Adapter reads could be edited before analyzing the transciptome, if already sequenced via Read 2.

8. Protocol

8.1 First-Strand cDNA Synthesis

(1) Defrost required reagent, briefly centrifuge and mix well. Keep on ice for further use.

Note: Do not vortex for the 5′ Template Switching Oligo, *AccuNext* Reverse Transcriptase and RNase Inhibitor.

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

(2) Sample Treatment (Cell / RNA)

For Cell Sample

I. Prepare reaction buffer as below table. Mix well and centrifuge.

Components	Volume
10 X Cell Lysis Buffer	19 μL
RNase Inhibitor	1 μL
Total	20 μL

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

Components	Volume
Reaction Buffer (Prepared above)	1 μL
Cell*	-
Nuclease free water	Up to 12.5 μL

For Cell template, recommended input is 1-1000 cells.

*: If input is PBS re-suspended cell, input volume should not be more than 5 μL;

Not recommended to be cleansed repeatedly and re-suspended by 1 X PBS solution with no Mg^{2+} and Ca^{2+}, due to inhibition on PCR reaction by the potential chemical leftovers.

**: If cell input is sorted by the flow cytometer, directly sort cells into the 12.5μL reaction mix, then vortex and mix well.

III. Place sample on ice, add the 3′ Oligo (dT) Primer, then vortex and mix well.

*: If PCR extension cycle < 17 cycles, add 2 μL of 3′ Oligo (dT) Primer;

If input is single cell or PCR extension cycle ≥ 17 cycles, add 1 μL of 3′ Oligo (dT) Primer. Then fill with Nuclease Free Water up to 12.5 μL.

For RNA Template

I. Prepare reaction buffer as below table. Mix well and centrifuge.

Components	Volume
10 X Cell Lysis Buffer	19 μL
RNase Inhibitor	1 μL
Total	20 μL

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

Components	Volume
Reaction Buffer (Prepared above)	1 μL
3' Oligo (dT) Primer	2 μL*
RNA**	-
Nuclease free water	Up to 12.5 μL

*: If PCR extension cycle < 17 cycles, add 2 μL of 3' Oligo (dT) Primer;

If PCR extension cycle ≥ 17 cycles, add 1 μL of 3' Oligo (dT) Primer. Then fill with Nuclease Free Water up to 12.5 μL.

**: Recommended RNA input is 10 pg ~ 100 ng. Component Control RNA included in this kit is 1 μg/μL, dilute to 2ng/μL, then add 5 μL for each use.

(3) PCR Denaturation and Annealing

Use PCR program as below to denature and anneal the Samples.

Temperature	Time
72°C	3 min
4°C	Hold

(4) Prepare the RT Master Mix [As below in step(5)], Immediately start Reverse Transcription reaction after completion of incubation in Step (3) above.

(5) Prepare RT Master Mix and RT Program

I. Prepare RT Master Mix as below.

Components	Volume
Reaction Product Solution from Step (3) above	12.5 μL
5 X First-Strand Synthesis Buffer	4 μL
RNase Inhibitor	0.5 μL
5' Template Switching Oligo	1 μL
AccuNext Reverse Transcriptase	2 μL
Total	20 μL

II. Run PCR Program as below.

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

III. For synthesis product, please keep on ice for further downstream experiment, or store at -20°C for overnight.

SAFE STOPING POINT: Please store at -80°C for long term storage, store at -20°C for overnight.

8.2 PCR Amplification of cDNA

(1) Defrost the 2X *AccuNext* PCR Buffer and PCR Primers, vortex and mix well. Place on ice after centrifuge.
Use pipette to gently mix the *AccuNext* DNA Polymerase and then place on ice for further use.

(2) Prepare PCR Master Mix as below.

Components	Volume
cDNA from **8.1 First Strand cDNA Synthesis** above	20μL
2 X AccuNext PCR Buffer	25 μL
AccuNext DNA Polymerase	1 μL
PCR Primers	1 μL
Nuclease Free Water	Up to 50 μL

(3) Vortex gently and mix well, then centrifuge.

(4) Run PCR Program as below.

Step	Temperature	Time	Number of Cycles
Initial-Denaturation	95°C	1 min	1
Denaturation	98°C	10 sec	
Annealing	65°C	30 sec	X*
Extension	68°C	3 min	
Final Extension	72°C	10 min	1
End	4 °C	Hold	-

*:Please select adequate number of running cycles per following recommendation table.

Initial RNA Input	Initial Cell Input	Recommended Number of Cycles
100 ng	-	4 - 5
10 ng	1000 Cells	7 - 8
1 ng	100 Cells	10 - 11
100 pg	10 Cells	14 - 15
10 pg	1 Cell	17 - 18

(5) Place reaction product on ice for further downstream experiment of DNA Purification.
SAFE STOPING POINT: Please store at -80°C for long term storage, store at -20°C for overnight.

8.3 Purification of PCR Amplification Product

Purify the PCR product using magnetic beads. Recommend to use the NucleoMag kit for clean up and size selection of NGS library prep reactions (Macherey-Nagel, Code. 744970.50) or equivalent products.

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 PCR products from upstream steps, vortex and mix well, then centrifuge briefly.
(3) Incubate the mix at room temperature for 10 mins.
(4) Place the mix on the magnetic stand 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) (Keep the mix tube on the magnetic stand) Keep caps open for 5 ~ 10 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 the supernatant carefully, and relocate to new tubes, store at -20°C. Discard the leftover and beads.

SAFE STOPING POINT: Please store at -80°C for long term storage, store at -20°C for overnight.

8.4 Quality Inspection of Amplification Product

(1) Take 2 μL of retrieved product from above, to be tested for Nucleic Acid 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 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 concentration of retrieved product is 2~20 ng. Length of fragment is expected to be 400 ~ 10000 bp, with peak on 1000 ~ 2000 bp. Not amplification product should be observed for negative controls.

8.5 Library Construction/Preparation

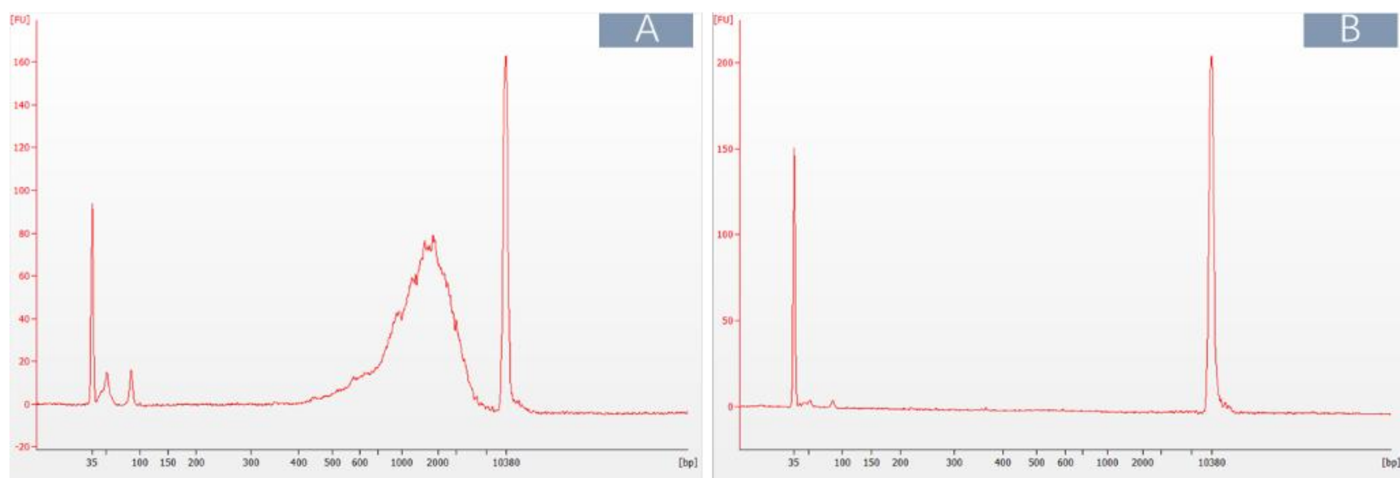
The product could be then used for downstream experiment of library construction/preparation.

If for Illumina platform, recommend to use the Nextera XT DNA Library Preparation Kit (Illumina, Code.FC-131-1024) or equivalent products.

Appendix Experimental Sample Result

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

10 pg of Total RNA used and amplified using 17 cycles



Appendix 2 Example Sequencing Data Analysis

Using 293T Cell

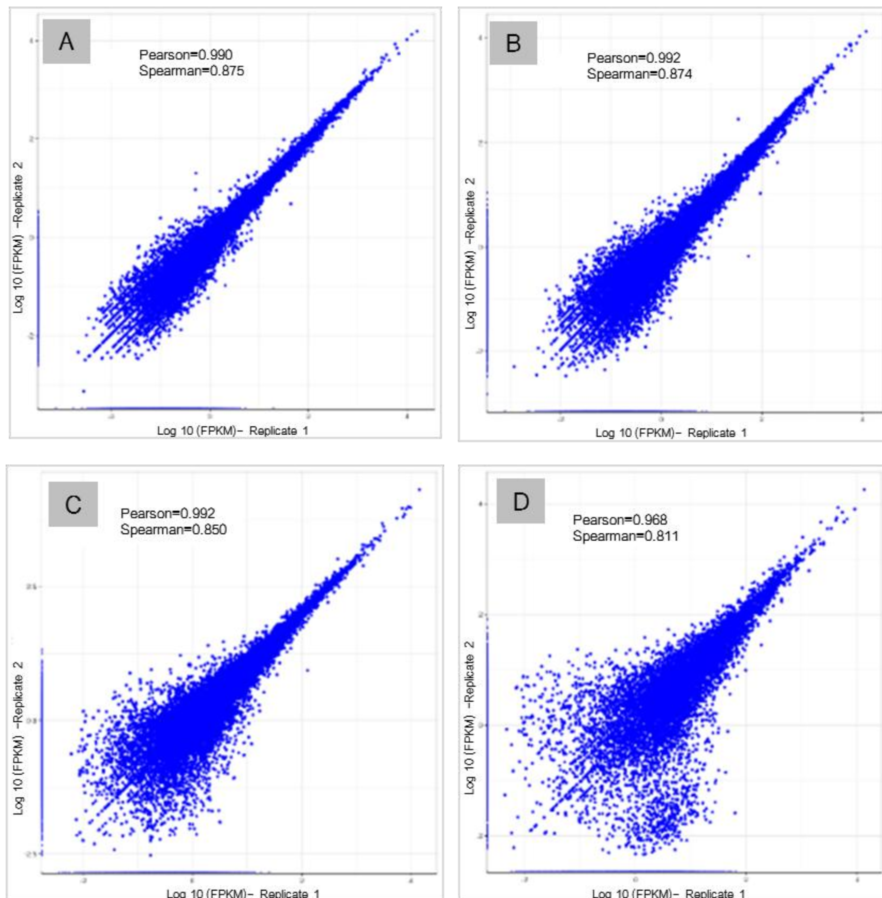
Cell source	293T Cell							
Input amount	1000 cells		100 cells		10 cells		1 cell	
Replicate	1	2	1	2	1	2	1	2
Number of reads (millions)	44.2	42.2	40.5	38.5	42.6	38.9	44.3	30.4
Q30 bases rate(%)	88.9	89.4	89.9	89.4	89.9	90.0	90.4	89.0
Number of transcripts > 0.1 FPKM	19343	21002	18565	18499	15261	15248	9860	9282
Number of transcripts > 1 FPKM	10268	9665	10324	10584	10226	10177	8185	7700
Percentage of reads(%):								
Mapped to genome	82.6	84.1	86	85.8	86.6	86.3	86.0	80.5
Mapped uniquely to genome	75.1	76.4	78.1	78.0	78.4	78.6	78.9	73.9
Exonic	94.8	94.1	94.6	94.0	95.7	95.9	96.6	93.6
Intronic	4.6	5.1	4.9	5.5	3.9	3.7	2.9	5.7
Intergenic	0.6	0.8	0.5	0.5	0.4	0.4	0.4	0.7

Using 293T Cell Total RNA

RNA source	293T Cell Total RNA											
Input amount	10ng			1ng			100pg			10pg		
Replicate	1	2	3	1	2	3	1	2	3	1	2	3
Number of reads (millions)	46.3	43.7	45.9	45.9	44.1	39.8	39.7	36.8	45.9	42.4	43.9	45.2
Q30 bases rate(%)	91.4	91.4	91.7	91.9	91.5	91.3	91.3	91.5	91.4	91.7	91.7	91.9
Number of transcripts > 0.1 FPKM	22499	22487	22616	21384	21267	21302	16467	16871	16844	9854	9842	9965
Number of transcripts > 1 FPKM	13355	13486	13400	13297	13292	13291	12981	13152	13092	8689	8687	8779
Percentage of reads(%):												
Mapped to genome	91.1	91.4	91.5	90.8	90.7	90.2	91.1	91.2	90.7	91.7	91.6	91.5
Mapped uniquely to genome	79.4	80.2	80.3	82.2	82.2	82.0	83.1	83.1	82.8	84.8	84.6	84.3
Exonic	92.8	92.65	92.75	93.34	93.39	93.19	93.21	93.06	92.98	93.56	93.38	93.36
Intronic	6.7	6.86	6.77	6.18	6.14	6.34	6.29	6.44	6.52	5.96	6.16	6.16
Intergenic	0.49	0.49	0.48	0.48	0.47	0.47	0.5	0.5	0.5	0.48	0.46	0.48

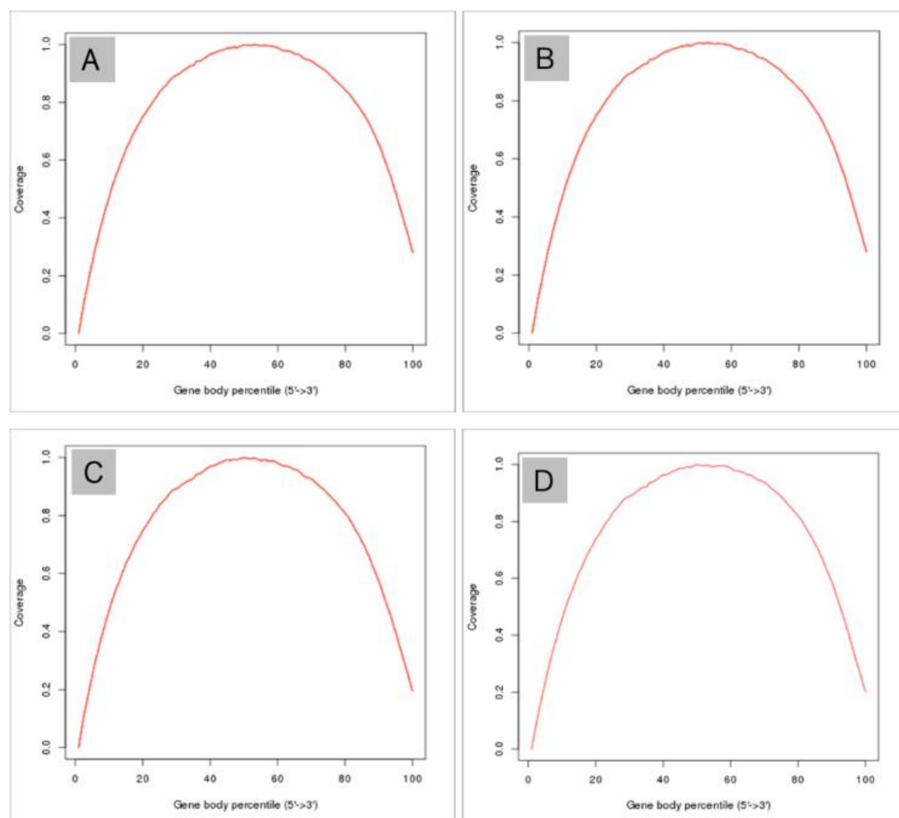
Appendix 3 Consistency Test - Sequencing Date Distribution from two repeat runs

Using 1000 (A), 100(B), 10(C), 1(D) counts of 293T Cell



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

Using 1000 (A), 100(B), 10(C), 1(D) counts of 293T Cell



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