JAN 29, 2020

Low Quantity single strand CAGE protocol

In 1 collection

Hazuki Takahashi¹, Hiromi Nishiyori-Sueki¹, Piero Carninci¹

¹Laboratory for Transcriptome Technology, RIKEN Center for Integrative Medical Sciences, Yokohama, Kanagawa 230-0045, Japan

Hazuki Takahashi

Laboratory for Transcriptome Technology, RIKEN Center for In...

OPEN ACCESS

DOI:
dx.doi.org/10.17504/protocols.io.bbwwkipcw

Protocol Citation: Hazuki Takahashi, Hiromi Nishiyori-Sueki, Piero Carninci 2020. Low Quantity single strand CAGE protocol. **protocols.io** <https://dx.doi.org/10.17504/protocols.io.bbwwkipcw>

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Protocol status: Working
We use this protocol in our group and it is working.

Created: Jan 29, 2020

Last Modified: Jan 29, 2020

PROTOCOL integer ID:
32428

Keywords: CAGE (Cap Analysis of Gene Expression), Transcription Starting Sites, Gene promoter, enhancer RNA

ABSTRACT

The Cap Aalysis of Gene Expression (CAGE) is a powerful method to identify the Transcription Starting Sites (TSSs) of capped RNAs while simultaneously measuring transcript level. CAGE allows mapping at single nucleotide resolution all active promoters and enhancers. Large CAGE datasets have been over the years by individual labs and consortia, including the Encyclopedia of DNA Elements (ENCODE) and Functional Annotation Of the Mammalian Genome (FANTOM). These dataset constitute open resource for TSS annotations and gene expression analysis. Here we provide a protocol for the most recent CAGE version of the method, which is called Low Quantity (LQ) single strand (ss) CAGE (LQ-)ssCAGE. The methods allow to profile samples starting from low quantity of RNAs. Samples may typically include cells cultured in small volumes to maximizing large scale perturbations, cellular compartments like nuclear RNAs or samples from developmental stages. The advantage of (LQ-)ssCAGE is to allow working with small samples and simultaneously avoiding PCR amplification: this step help to preserve exquisite quantification of expression. The protocol makes use of simplified steps, by loading single stranded cap selected cDNA. Further, it allows reducing the RNA amount down to 25 ng and to shorten preparation time (less than 3 days) and to prepare libraries parallel, increasing the number of samples that one operator can reasonably handle (up to 96 samples). By proper organization of the flow of libraries preparation, an operator could further scale up the throughput. The obtained libraries are suitable for sequencing by various Illumina sequencing platforms using indexed reads and original barcode identifiers.

MATERIALS

<Materials and Reagents>

Product name

Maker

Cat. No.

Agencourt AMPure XP

BECKMAN COULTER

A63881	
Agencourt RNAClean XP	BECKMAN COULTER
A63987	
KAPA Library Quantification Kits (500 reactions)-	KAPA BIOSYSTEMS
KK4835	
Illumina / ABI Prism®	
Sodium periodate ACS Reagent Grade,	MP Biomedicals
0215257725-25 g	
5M Sodium Chloride, Molecular Biology Grade,	Promega
V4221	
RNase One Ribonuclease,	Promega
M4261	
Lithium chloride solution 8 M, for molecular biology, ≥99%	SIGMA-ALDRICH
L7026-100ML	
Sodium acetate buffer solution,	
BioXtra, pH 7.0±0.05 (25 °C), for molecular biology, 3 M,	SIGMA-ALDRICH
S2404-100ML	
non-sterile; 0.2 µm filtered,	
Sodium periodate ACS Reagent Grade,	SIGMA-ALDRICH
311448-5G	
DNA Ligation Kit < Mighty Mix > ,	TAKARA BIO INC.
6023	
Ribonuclease H (RNase H) (20 ~ 60 U/µL),	TAKARA BIO INC.
2150A	
10 mM dNTP Mix,	ThermoFisher SCIENTIFIC
18427013	
Dynabeads M-270 Streptavidin,	ThermoFisher SCIENTIFIC
65305	
Ribonuclease H (2 U/µL),	ThermoFisher SCIENTIFIC
18021014	
RNaseZap RNase Decontamination Solution,	ThermoFisher SCIENTIFIC
AM9780	
SuperScript III Reverse Transcriptase,	ThermoFisher SCIENTIFIC
18080044	
UltraPure 0.5 M EDTA, pH 8.0,	ThermoFisher SCIENTIFIC
15575020	
UltraPure DNase/RNase-Free Distilled Water,	ThermoFisher
SCIENTIFIC 10977015	
Biotin (Long Arm) Hydrazide,	Vector Laboratories
SP-1100	
0.5M EDTA (pH 8.0),	Wako Pure Chemical
Industries, 311-90075	
10w/v% Polyoxyethylene(20) Sorbitan Monolaurate Solution,	Wako Pure Chemical
Industries, 161-24801	

1 M Tris-HCl (pH 7.0), Industries, 311-90411	Wako Pure Chemical
1M Tris-HCl (pH 7.5), Industries, 316-90221	Wako Pure Chemical
1M Tris-HCl (pH 8.5), Industries, 316-90405	Wako Pure Chemical
3M Sodium Acetate (pH 5.2), Industries, 316-90081	Wako Pure Chemical
Dimethyl Sulfoxide, Industries, 046-21981	Wako Pure Chemical
Ethanol (99.5), Industries, 057-00456	Wako Pure Chemical
2M NaOH, Industries, 194-05631	Wako Pure Chemical
Hybridization buffer HT1,	Illumina

<Equipment>

Product name, Cat. No.	Maker
Axygen 0.2 mL Polypropylene PCR Tube Strips PCR-0208-CP-C and Domed Cap Strips, 8 Tubes/Strip, 8 Domed Caps/Strip, Clear, Nonsterile	CORNING, AXYGEN
Axygen 1.5 mL Maxymum Recovery Snaplock MCT-150-L-C Microcentrifuge Tube, Polypropylene, Clear, Nonsterile, 250 Tubes/Pack, 10 Packs/Case	CORNING, AXYGEN
Axygen 16 Well Polypropylene PCR Microplate, PCR-16-C Clear, Nonsterile	CORNING, AXYGEN
Axygen PCR 1 x 8 Strip Domed Caps, PCR-02CP-C Fit 0.2 mL PCR Tube Strips, Clear, Nonsterile, X-Pierce Sealing Films, Sterile, XPS-25	CORNING, AXYGEN
10 µL (0.1 - 10 µL) LongReach Barrier tip, 38000T	EXCEL Scientific, Inc.
10 µL (0.1 - 10 µL) Ultra G Barrier tip, 28200T	Sorenson BioScience, Inc.
1000 µL (100-1000 µl) Barrier tip, 14200T	Sorenson BioScience, Inc.
20 µL (1-20 µL) Barrier tip, 14210T	Sorenson BioScience, Inc.
200 µL (1-200 µL) NX Barrier tip,	Sorenson BioScience, Inc.

30550T	
MicroAmp Optical 384-Well Reaction Plate with Barcode, SCIENTIFIC 4309849	ThermoFisher
MicroAmp Optical Adhesive Film, 4360954	ThermoFisher SCIENTIFIC
PIPETMAN Classic, P1000, F123602	GILSON
PIPETMAN Classic, P2, F144801	GILSON
PIPETMAN Classic, P20, F123600	GILSON
PIPETMAN Classic, P200, F123601	GILSON
MJ Research Tetrad PTC-225 Thermal Cycler, 8252-30-1004	GMI, MJ Research
MINI CENTRIFUGE, CFM-1000	HARMONY (LMS)
PlateSpin II,	KUBOTA
Proline Plus Mechanical Pipettes, 8-ch, 0.5 - 10 µL, 728120	sartorius
Proline Plus Mechanical Pipettes, 8-ch, 10 - 100 µL, 728130	sartorius
Proline Plus Mechanical Pipettes, 8-ch, 30 - 300 µL, 728140	sartorius
Vortex-Genie 2, SI-0286	Scientific Industries, Inc.
miVAC DNA, DNA-10000-G00	SP Scientific, Genevac
miVac rotor for micro plate, DRS-00000-200	SP Scientific, Genevac
Dynabeads MPC-S (Magnetic Particle Concentrator), SCIENTIFIC A13346	ThermoFisher
DynaMag-96 Side Skirted Magnet, SCIENTIFIC 12027	ThermoFisher

<RT primer (for ssCAGE)>

name	5' modification	Sequence	3' modification base
N6+TCT	P	TCTNNNNNN	9

<RT primers containing barcode (for LQ-ssCAGE)>

name	5' modification	Sequence
Base		

TCT_7nt_N15_#001 P 25	TCTGATGCTCNNNNNNNNNNNNNNNNNN
TCT_7nt_N15_#002 P 25	TCTTATGAGCNNNNNNNNNNNNNNNNNN
TCT_7nt_N15_#003 P 25	TCTCGACGATNNNNNNNNNNNNNNNNNN
TCT_7nt_N15_#004 P 25	TCTTAGTCACNNNNNNNNNNNNNNNNNN
TCT_7nt_N15_#005 P 25	TCTCGTACTGNNNNNNNNNNNNNNNNNN
TCT_7nt_N15_#006 P 25	TCTTACGTAGNNNNNNNNNNNNNNNNNN
TCT_7nt_N15_#007 P 25	TCTAGACTCGNNNNNNNNNNNNNNNNNN
TCT_7nt_N15_#008 P 25	TCTTCGCTGANNNNNNNNNNNNNNNNNNN
TCT_7nt_N15_#009 P 25	TCTCGATCTGNNNNNNNNNNNNNNNNNN
TCT_7nt_N15_#010 P 25	TCTTGTCACGNNNNNNNNNNNNNNNNNN
TCT_7nt_N15_#011 P 25	TCTATGCACTNNNNNNNNNNNNNNNNNN
TCT_7nt_N15_#012 P 25	TCTGACATCGNNNNNNNNNNNNNNNNNN
TCT_7nt_N15_#013 P 25	TCTATCTAGCNNNNNNNNNNNNNNNNNN
TCT_7nt_N15_#014 P 25	TCTTGTCGCANNNNNNNNNNNNNNNNNNN
TCT_7nt_N15_#015 P 25	TCTCAGATCTNNNNNNNNNNNNNNNNNN
TCT_7nt_N15_#016 P 25	TCTTCAGAGCNNNNNNNNNNNNNNNNNN
TCT_7nt_N15_#017 P 25	TCTATACTGCNNNNNNNNNNNNNNNNNN
TCT_7nt_N15_#018 P 25	TCTATCGTGANNNNNNNNNNNNNNNNNNN
TCT_7nt_N15_#019 P 25	TCTGAGCTCTNNNNNNNNNNNNNNNNNN
TCT_7nt_N15_#020 P 25	TCTTCTCGAGNNNNNNNNNNNNNNNNNN
TCT_7nt_N15_#021 P 25	TCTCAGTGTCNNNNNNNNNNNNNNNNNN
TCT_7nt_N15_#022 P 25	TCTGCTACGANNNNNNNNNNNNNNNNNNN

TCT_7nt_N15_#023 P 25	TCTAGTACGCNNNNNNNNNNNNNNNNNN
TCT_7nt_N15_#024 P 25	TCTGACTAGCNNNNNNNNNNNNNNNNNN
TCT_7nt_N15_#025 P 25	TCTTACGCTANNNNNNNNNNNNNNNNNN
TCT_7nt_N15_#026 P 25	TCTGCTATAGNNNNNNNNNNNNNNNNNN
TCT_7nt_N15_#027 P 25	TCTACACTGTNNNNNNNNNNNNNNNNNN
TCT_7nt_N15_#028 P 25	TCTACATGTCNNNNNNNNNNNNNNNNNN
TCT_7nt_N15_#029 P 25	TCTGCAGACTNNNNNNNNNNNNNNNNNN
TCT_7nt_N15_#030 P 25	TCTTAGCGACNNNNNNNNNNNNNNNNNN
TCT_7nt_N15_#031 P 25	TCTACAGTCGNNNNNNNNNNNNNNNNNN
TCT_7nt_N15_#032 P 25	TCTGTATGACNNNNNNNNNNNNNNNNNN
TCT_7nt_N15_#033 P 25	TCTCAGCAGTNNNNNNNNNNNNNNNNNN
TCT_7nt_N15_#034 P 25	TCTACAGCTGNNNNNNNNNNNNNNNNNN
TCT_7nt_N15_#035 P 25	TCTGCTGATANNNNNNNNNNNNNNNNNN
TCT_7nt_N15_#036 P 25	TCTTGTCGACNNNNNNNNNNNNNNNNNN
TCT_7nt_N15_#037 P 25	TCTTGCGATANNNNNNNNNNNNNNNNNN
TCT_7nt_N15_#038 P 25	TCTAGTGTCANNNNNNNNNNNNNNNNNN
TCT_7nt_N15_#039 P 25	TCTATACGTCNNNNNNNNNNNNNNNNNN
TCT_7nt_N15_#040 P 25	TCTCACTATGNNNNNNNNNNNNNNNNNN
TCT_7nt_N15_#041 P 25	TCTTCACAGTNNNNNNNNNNNNNNNNNN
TCT_7nt_N15_#042 P 25	TCTTACGCGTNNNNNNNNNNNNNNNNNN
TCT_7nt_N15_#043 P 25	TCTCGACGTANNNNNNNNNNNNNNNNNN
TCT_7nt_N15_#044 P 25	TCTAGCATGCNNNNNNNNNNNNNNNNNN

TCT_7nt_N15_#045 P 25	TCTATCGCGTNNNNNNNNNNNNNNNNNN
TCT_7nt_N15_#046 P 25	TCTGATATGCNNNNNNNNNNNNNNNNNN
TCT_7nt_N15_#047 P 25	TCTTAGCATCNNNNNNNNNNNNNNNNNN
TCT_7nt_N15_#048 P 25	TCTGCTCATGNNNNNNNNNNNNNNNNNN
TCT_7nt_N15_#049 P 25	TCTCTGATGCNNNNNNNNNNNNNNNNNN
TCT_7nt_N15_#050 P 25	TCTGCATATGNNNNNNNNNNNNNNNNNN
TCT_7nt_N15_#051 P 25	TCTGTCACATNNNNNNNNNNNNNNNNNN
TCT_7nt_N15_#052 P 25	TCTCTAGCATNNNNNNNNNNNNNNNNNN
TCT_7nt_N15_#053 P 25	TCTCACGTGTNNNNNNNNNNNNNNNNNN
TCT_7nt_N15_#054 P 25	TCTCTAGTACNNNNNNNNNNNNNNNNNN
TCT_7nt_N15_#055 P 25	TCTGATCGTANNNNNNNNNNNNNNNNNNN
TCT_7nt_N15_#056 P 25	TCTCTAGTCGNNNNNNNNNNNNNNNNNN
TCT_7nt_N15_#057 P 25	TCTGACACTGNNNNNNNNNNNNNNNNNN
TCT_7nt_N15_#058 P 25	TCTAGCTCGANNNNNNNNNNNNNNNNNNN
TCT_7nt_N15_#059 P 25	TCTGACTGCANNNNNNNNNNNNNNNNNNN
TCT_7nt_N15_#060 P 25	TCTAGACGCTNNNNNNNNNNNNNNNNNN
TCT_7nt_N15_#061 P 25	TCTCAGTCTGNNNNNNNNNNNNNNNNNN
TCT_7nt_N15_#062 P 25	TCTTGACTGCNNNNNNNNNNNNNNNNNN
TCT_7nt_N15_#063 P 25	TCTATCAGCGNNNNNNNNNNNNNNNNNN
TCT_7nt_N15_#064 P 25	TCTCTCAGAGNNNNNNNNNNNNNNNNNN
TCT_7nt_N15_#065 P 25	TCTTATGCACNNNNNNNNNNNNNNNNNN
TCT_7nt_N15_#066 P 25	TCTTGCTACANNNNNNNNNNNNNNNNNNN

TCT_7nt_N15_#067 P 25	TCTATGCGCANNNNNNNNNNNNNNNNNN
TCT_7nt_N15_#068 P 25	TCTTCGTGACNNNNNNNNNNNNNNNNNN
TCT_7nt_N15_#069 P 25	TCTCGATACGNNNNNNNNNNNNNNNNNN
TCT_7nt_N15_#070 P 25	TCTGCTAGCANNNNNNNNNNNNNNNNNN
TCT_7nt_N15_#071 P 25	TCTTAGCGTANNNNNNNNNNNNNNNNNN
TCT_7nt_N15_#072 P 25	TCTCTGACGANNNNNNNNNNNNNNNNNN
TCT_7nt_N15_#073 P 25	TCTTACGTCANNNNNNNNNNNNNNNNNN
TCT_7nt_N15_#074 P 25	TCTCATAGCTNNNNNNNNNNNNNNNNNN
TCT_7nt_N15_#075 P 25	TCTTCGATCANNNNNNNNNNNNNNNNNN
TCT_7nt_N15_#076 P 25	TCTCATATCGNNNNNNNNNNNNNNNNNN
TCT_7nt_N15_#077 P 25	TCTCTCGATANNNNNNNNNNNNNNNNNN
TCT_7nt_N15_#078 P 25	TCTAGTCAGTNNNNNNNNNNNNNNNNNN
TCT_7nt_N15_#079 P 25	TCTTCGATAGNNNNNNNNNNNNNNNNNN
TCT_7nt_N15_#080 P 25	TCTGCGTATANNNNNNNNNNNNNNNNNN
TCT_7nt_N15_#081 P 25	TCTATGTCAGNNNNNNNNNNNNNNNNNN
TCT_7nt_N15_#082 P 25	TCTACGTCGANNNNNNNNNNNNNNNNNN
TCT_7nt_N15_#083 P 25	TCTCACTAGTNNNNNNNNNNNNNNNNNN
TCT_7nt_N15_#084 P 25	TCTTCACGTGNNNNNNNNNNNNNNNNNN
TCT_7nt_N15_#085 P 25	TCTGCTGTACNNNNNNNNNNNNNNNNNN
TCT_7nt_N15_#086 P 25	TCTTACTGAGNNNNNNNNNNNNNNNNNN
TCT_7nt_N15_#087 P 25	TCTCGATGCANNNNNNNNNNNNNNNNNN
TCT_7nt_N15_#088 P 25	TCTTGTCAGANNNNNNNNNNNNNNNNNN

TCT_7nt_N15_#089 P 25	TCTCTCTAGANNNNNNNNNNNNNNNNNNN
TCT_7nt_N15_#090 P 25	TCTCGTACATNNNNNNNNNNNNNNNNNN
TCT_7nt_N15_#091 P 25	TCTCTAGCTGNNNNNNNNNNNNNNNNNN
TCT_7nt_N15_#092 P 25	TCTCGCTATANNNNNNNNNNNNNNNNNNN
TCT_7nt_N15_#093 P 25	TCTTAGCTCANNNNNNNNNNNNNNNNNNN
TCT_7nt_N15_#094 P 25	TCTGTATAGCNNNNNNNNNNNNNNNNNN
TCT_7nt_N15_#095 P 25	TCTGTGACTCNNNNNNNNNNNNNNNNNN
TCT_7nt_N15_#096 P 25	TCTAGCATCGNNNNNNNNNNNNNNNNNN
TCT_7nt_N15_#097 P 25	TCTTGAGCATNNNNNNNNNNNNNNNNNN
TCT_7nt_N15_#098 P 25	TCTCGTATAGNNNNNNNNNNNNNNNNNN
TCT_7nt_N15_#099 P 25	TCTACTAGCGNNNNNNNNNNNNNNNNNN
TCT_7nt_N15_#100 P 25	TCTACTGATGNNNNNNNNNNNNNNNNNN
TCT_7nt_N15_#101 P 25	TCTGTACATGNNNNNNNNNNNNNNNNNN
TCT_7nt_N15_#102 P 25	TCTTGACTCANNNNNNNNNNNNNNNNNNN
TCT_7nt_N15_#103 P 25	TCTGATCTCANNNNNNNNNNNNNNNNNNN
TCT_7nt_N15_#104 P 25	TCTCGACTGTNNNNNNNNNNNNNNNNNN
TCT_7nt_N15_#105 P 25	TCTCAGTAGCNNNNNNNNNNNNNNNNNN
TCT_7nt_N15_#106 P 25	TCTACTATGCNNNNNNNNNNNNNNNNNN
TCT_7nt_N15_#107 P 25	TCTACGATCGNNNNNNNNNNNNNNNNNN
TCT_7nt_N15_#108 P 25	TCTGATCACGNNNNNNNNNNNNNNNNNN
TCT_7nt_N15_#109 P 25	TCTTGCATAGNNNNNNNNNNNNNNNNNN
TCT_7nt_N15_#110 P 25	TCTCGTCATANNNNNNNNNNNNNNNNNNN

TCT_7nt_N15_#111 P 25	TCTAGCTCAGNNNNNNNNNNNNNNNNNN
TCT_7nt_N15_#112 P 25	TCTGTAGCACNNNNNNNNNNNNNNNNNN
TCT_7nt_N15_#113 P 25	TCTCTATGCGNNNNNNNNNNNNNNNNNN
TCT_7nt_N15_#114 P 25	TCTGACTACTNNNNNNNNNNNNNNNNNN
TCT_7nt_N15_#115 P 25	TCTGATCGCTNNNNNNNNNNNNNNNNNN
TCT_7nt_N15_#116 P 25	TCTCTGATAGNNNNNNNNNNNNNNNNNN
TCT_7nt_N15_#117 P 25	TCTGTGCATANNNNNNNNNNNNNNNNNN
TCT_7nt_N15_#118 P 25	TCTGATGCATNNNNNNNNNNNNNNNNNN
TCT_7nt_N15_#119 P 25	TCTACTGCAGNNNNNNNNNNNNNNNNNN
TCT_7nt_N15_#120 P 25	TCTTGATCACNNNNNNNNNNNNNNNNNN

<5' linkers>

name, 5' modification, sequence, 3' modification, base

5'adaptor N6, -,

AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATC
TNNNNNN, P, 64

5'adaptor GN5, -,

AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATC
TGNNNNNN, P, 64

5'adaptor down, P,

AGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGTAGATCTCGGTGGTCGCCGTATCAT
T, P, 58

<3' linkers >

name

sequence, 3' modification, base

3'end adaptor up 01

NNNAGATCGGAAGAGCACACGTCTGAACTCCAGTCACATCACGATCTCGTATGCCG
TCTTCTGCTTG, P, 67

3'end adaptor down 01

CAAGCAGAAGACGGCATACGAGATCGTGATGTGACTGGAGTTCAGACGTGTGCTCTT
CCGA, -, 61

3'end adaptor up 02

NNNAGATCGGAAGAGCACACGTCTGAACTCCAGTCACCGATGTATCTCGTATGCCG
TCTTCTGCTTG, P, 67
3'end adaptor down 02
CAAGCAGAAGACGGCATACGAGATACATCGGTGACTGGAGTTCAGACGTGTGCTCTT
CCGA, -, 61
3'end adaptor up 03
NNNAGATCGGAAGAGCACACGTCTGAACTCCAGTCACTTAGGCATCTCGTATGCCG
TCTTCTGCTTG, P, 67
3'end adaptor down 03
CAAGCAGAAGACGGCATACGAGATGCCTAAGTGACTGGAGTTCAGACGTGTGCTCTT
CCGA, -, 61
3'end adaptor up 04
NNNAGATCGGAAGAGCACACGTCTGAACTCCAGTCACTGACCAATCTCGTATGCCG
TCTTCTGCTTG, P, 67
3'end adaptor down 04
CAAGCAGAAGACGGCATACGAGATTGGTCAGTGACTGGAGTTCAGACGTGTGCTCTT
CCGA, -, 61
3'end adaptor up 05
NNNAGATCGGAAGAGCACACGTCTGAACTCCAGTCACACAGTGATCTCGTATGCCG
TCTTCTGCTTG, P, 67
3'end adaptor down 05
CAAGCAGAAGACGGCATACGAGATCACTGTGTGACTGGAGTTCAGACGTGTGCTCTT
CCGA, -, 61
3'end adaptor up 06
NNNAGATCGGAAGAGCACACGTCTGAACTCCAGTCACGCCAATATCTCGTATGCCG
TCTTCTGCTTG, P, 67
3'end adaptor down 06
CAAGCAGAAGACGGCATACGAGATATTGGCGTGACTGGAGTTCAGACGTGTGCTCTT
CCGA, -, 61
3'end adaptor up 07
NNNAGATCGGAAGAGCACACGTCTGAACTCCAGTCACCAGATCATCTCGTATGCCG
TCTTCTGCTTG, P, 67
3'end adaptor down 07
CAAGCAGAAGACGGCATACGAGATGATCTGGTGACTGGAGTTCAGACGTGTGCTCTT
CCGA, -, 61
3'end adaptor up 08
NNNAGATCGGAAGAGCACACGTCTGAACTCCAGTCACACTTGAATCTCGTATGCCG
TCTTCTGCTTG, P, 67
3'end adaptor down 08
CAAGCAGAAGACGGCATACGAGATTCAAGTGTGACTGGAGTTCAGACGTGTGCTCTT
CCGA, -, 61
3'end adaptor up 09
NNNAGATCGGAAGAGCACACGTCTGAACTCCAGTCACGATCAGATCTCGTATGCCG
TCTTCTGCTTG, P, 67

3'end adaptor down 09
CAAGCAGAAGACGGCATACGAGATCTGATCGTGACTGGAGTTCAGACGTGTGCTCTT
CCGA, -, 61

3'end adaptor up 10
NNNAGATCGGAAGAGCACACGTCTGAACTCCAGTCACTAGCTTATCTCGTATGCCG
TCTTCTGCTTG, P, 67

3'end adaptor down 10
CAAGCAGAAGACGGCATACGAGATAAGCTAGTGACTGGAGTTCAGACGTGTGCTCTT
CCGA, -, 61

3'end adaptor up 11
NNNAGATCGGAAGAGCACACGTCTGAACTCCAGTCACGGCTACATCTCGTATGCCG
TCTTCTGCTTG, P, 67

3'end adaptor down 11
CAAGCAGAAGACGGCATACGAGATGTAGCCGTGACTGGAGTTCAGACGTGTGCTCTT
CCGA, -, 61

3'end adaptor up 12
NNNAGATCGGAAGAGCACACGTCTGAACTCCAGTCACCTTGTAATCTCGTATGCCG
TCTTCTGCTTG, P, 67

3'end adaptor down 12
CAAGCAGAAGACGGCATACGAGATTACAAGGTGACTGGAGTTCAGACGTGTGCTCTT
CCGA, -, 61

3'end adaptor up 13
NNNAGATCGGAAGAGCACACGTCTGAACTCCAGTCACAGTCAACAATCTCGTATGC
CGTCTTCTGCTTG, P, 69

3'end adaptor down 13
CAAGCAGAAGACGGCATACGAGATTGTTGACTGTGACTGGAGTTCAGACGTGTGCTC
TTCCGA, -, 63

3'end adaptor up 14
NNNAGATCGGAAGAGCACACGTCTGAACTCCAGTCACAGTTCCGTATCTCGTATGC
CGTCTTCTGCTTG, P, 69

3'end adaptor down 14
CAAGCAGAAGACGGCATACGAGATACGGAAGTGTGACTGGAGTTCAGACGTGTGCT
CTTCCGA, -, 63

3'end adaptor up 15
NNNAGATCGGAAGAGCACACGTCTGAACTCCAGTCACATGTCAGAATCTCGTATGC
CGTCTTCTGCTTG, P, 69

3'end adaptor down 15
CAAGCAGAAGACGGCATACGAGATTCTGACATGTGACTGGAGTTCAGACGTGTGCTC
TTCCGA, -, 63

3'end adaptor up 16
NNNAGATCGGAAGAGCACACGTCTGAACTCCAGTCACCCGTCCCGATCTCGTATGC
CGTCTTCTGCTTG, P, 69

3'end adaptor down 16
CAAGCAGAAGACGGCATACGAGATCGGGACGGGTGACTGGAGTTCAGACGTGTGCT

CTTCCGA, -, 63
 3'end adaptor up 18
 NNNAGATCGGAAGAGCACACGTCTGAACTCCAGTCACGTCCGCACATCTCGTATGC
 CGTCTTCTGCTTG, P, 69
 3'end adaptor down 18
 CAAGCAGAAGACGGCATACGAGATGTGCGGACGTGACTGGAGTTCAGACGTGTGCT
 CTTCCGA, -, 63
 3'end adaptor up 19
 NNNAGATCGGAAGAGCACACGTCTGAACTCCAGTCACGTGAAACGATCTCGTATGC
 CGTCTTCTGCTTG, P, 69
 3'end adaptor down 19
 CAAGCAGAAGACGGCATACGAGATCGTTTCACGTGACTGGAGTTCAGACGTGTGCTC
 TTCCGA, -, 63
 3'end adaptor up 20
 NNNAGATCGGAAGAGCACACGTCTGAACTCCAGTCACGTGGCCTTATCTCGTATGC
 CGTCTTCTGCTTG, P, 69
 3'end adaptor down 20
 CAAGCAGAAGACGGCATACGAGATAAGGCCACGTGACTGGAGTTCAGACGTGTGCT
 CTTCCGA, -, 63
 3'end adaptor up 21
 NNNAGATCGGAAGAGCACACGTCTGAACTCCAGTCACGTTTCGGAATCTCGTATGC
 CGTCTTCTGCTTG, P, 69
 3'end adaptor down 21
 CAAGCAGAAGACGGCATACGAGATTCCGAAACGTGACTGGAGTTCAGACGTGTGCT
 CTTCCGA, -, 63
 3'end adaptor up 22
 NNNAGATCGGAAGAGCACACGTCTGAACTCCAGTCACCGTACGTAATCTCGTATGC
 CGTCTTCTGCTTG, P, 69
 3'end adaptor down 22
 CAAGCAGAAGACGGCATACGAGATTACGTACGGTGACTGGAGTTCAGACGTGTGCTC
 TTCCGA, -, 63
 3'end adaptor up 23
 NNNAGATCGGAAGAGCACACGTCTGAACTCCAGTCACGAGTGGATATCTCGTATGC
 CGTCTTCTGCTTG, P, 69
 3'end adaptor down 23
 CAAGCAGAAGACGGCATACGAGATATCCACTCGTGACTGGAGTTCAGACGTGTGCTC
 TTCCGA, -, 63
 3'end adaptor up 25
 NNNAGATCGGAAGAGCACACGTCTGAACTCCAGTCACACTGATATATCTCGTATGC
 CGTCTTCTGCTTG, P, 69
 3'end adaptor down 25
 CAAGCAGAAGACGGCATACGAGATATATCAGTGTGACTGGAGTTCAGACGTGTGCTC
 TTCCGA, -, 63
 3'end adaptor up 27

NNNAGATCGGAAGAGCACACGTCTGAACTCCAGTCACATTCCTTTATCTCGTATGCC
 GTCTTCTGCTTG, P, 69
 3'end adaptor down 27
 CAAGCAGAAGACGGCATACGAGATAAAGGAATGTGACTGGAGTTCAGACGTGTGCT
 CTTCCGA, -, 63

<Recipes>

1. 250 mM NaIO₄

Dissolve 1 mg of Sodium periodate (ACS Reagent Grade) in 18.7 µL of RNase/DNase free water and keep in the dark. NOTE: The solution can be aliquoted to 50 µL and stored at 80 °C.

2. 100 mM Biotin (long arm) Hydrazide

Dissolve 50 mg of Biotin (long arm) Hydrazide in 1.345 mL of DMSO 1.345ml. Biotin is packed in 50mg/tube.
 NOTE: The solution can be aliquoted to 50 µL and stored at 80 °C.

3. LiCl buffer

Mix following materials and store at room temperature.

8M Lithium chloride solution	3.64 mL
1 M Tris-HCl (pH 7.5)	0.80 mL
10w/v% Polyoxyethylene (20) Sorbitan Monolaurate Solution	0.40 mL
0.5 M EDTA (pH 8.0)	0.16 mL
RNase, DNase –free water	3.64 mL

4. TE wash buffer

Mix following materials and store at room temperature.

RNase, DNase –free water	9.12 mL
1 M Tris-HCl (pH 7.5)	0.40 mL
10w/v% Polyoxyethylene (20) Sorbitan Monolaurate Solution	0.40 mL
0.5 M EDTA (pH 8.0)	0.08 mL

5. Release buffer

Mix following materials and store at room temperature.

RNase ONE 10x Reaction Buffer	100 µL
10w/v% Polyoxyethylene (20) Sorbitan Monolaurate Solution	1.0 µL
RNase, DNase –free water	899 µL

6. 1 x TE buffer

Mix following materials and store at room temperature.

1 M Tris-HCl (pH 8.0) 500 μ L
0.5 M EDTA (pH 8.0) 100 μ L
RNase, DNase-free water 49.4 mL

7. 0.1 M NaCl/TE buffer

1 M NaCl 500 μ L
1 x TE buffer 4.5 mL

8. 2.5 μ M 5'linker

a. Mix following solution and carry out the annealing reaction to generate 100 μ M GN5 linker

1 mM 5'adaptor GN5 4 μ L
1 mM 5'adaptor down 4 μ L
1 M NaCl 4 μ L
1 x TE buffer 28 μ L

<Annealing condition>

95 °C, 5 min: - 0.1 °C sec⁻¹ down to 83 °C; 5 min at 83 °C; - 0.1 °C sec⁻¹ down to 71 °C; 5 min at 71 °C; - 0.1 °C sec⁻¹ down to 59 °C; 5 min at 59 °C; -0.1 °C sec⁻¹, to 47 °C; 5 min at 47 °C; -0.1 °C sec⁻¹, to 35 °C; 5 min at 35 °C; -0.1 °C s⁻¹ to 23 °C; 5 min at 23 °C; -0.1 °C sec⁻¹ to 11 °C, and then hold at 11 °C.

b. Mix following solutions and repeat the annealing reaction at step **a.** to generate 100 μ M N6 linker

1 mM 5'adaptor N6 1 μ L
1 mM 5'adaptor down 1 μ L
1M NaCl 1 μ L
1 x TE buffer 7 μ L

c. Mix 40 μ L of 100 μ M GN5 linker from step a. and 10 μ L of 100 μ M N6 linker from step b.

d. Dilute 100 μ M of mixed 5'linkers to 2.5 μ M in 0.1 M NaCl/TE buffer. For instance, add 2.5 μ L of 100 μ M mixed linkers from step c to 97.5 μ L of 0.1 M NaCl/TE buffer for 25 samples.

NOTE: 2.5 μ M and 100 M mixed 5'linkers can be stored at -20°C.

9. 2.5 μ M 3' linker

a. Mix following solutions and carry out the annealing reaction at the step **8.a.** to generate 100 μ M of 3' linker.

1 mM 3'end adaptor up 1 μ L

1 mM 3'end adaptor down 1 μ L
 1 M NaCl 1 μ L
 1 x TE buffer 7 μ L

b. Dilute 100 μ M mixed linker from step **a.** to 2.5 μ M in 0.1 M NaCl/TE buffer (see details at the step **8.d.**).

BEFORE START INSTRUCTIONS

RNAs should be treated in the RNase free solutions, benches and equipments. We highly recommend to perform RNA quality check before starting (see detail at previously published paper [RNA stability check](#)).

Figure.1 Protocol Flow

1 Reverse Transcription (RT) to generate RNA/cDNA hybrids

1.1 single strand CAGE (ssCAGE)

When the amount of RNAs is more than 1 μg , choose this RT protocol. Here is an example of 5 μg starting RNAs.

When the amount of RNAs is less than 1 μg , go to step **1.2**.

a. Mix 5 μL of RNAs (1 $\mu\text{g}/\mu\text{L}$), 1 μL of RT primer (N6+TCT) and 4 μL of RNase free water in a tube.

Note

NOTE: Use 1.5 mL tube, 8 strip PCR tube or 16-96 wells plate depending on numbers of samples.

b. Incubate the RNA/primer mix from step **a.** at 65 $^{\circ}\text{C}$ for 5 min and immediately put on ice for 2 min.

c. Add the following components to the solution from step **b.** and carefully mix by pipetting on ice.

5 x First-Strand buffer	4 μL
-------------------------	-----------------

10 mM dNTPs	1 μ L
0.1 M DTT	1 μ L
RNase free water	2 μ L
SuperScript III Reverse Transcriptase	2 μ L

- d.** Incubate the mixed solution (total 20 μ L) at 25 °C for 30 sec, 55 °C for 30 min and keep 4 °C to generate RNA/cDNA hybrids.
- e.** Add 36 μ L (1.8-folds) of RNACleanXP beads to 20 μ L of RNA/cDNA hybrids from step **d.** and mix well by pipetting.
- ①. Incubate at room temperature for 5 min. Spin down and set the tube on magnetic bar for 5 min.
 - ②. Discard the supernatant by pipette aspiration.
 - ③. Wash the beads with 200 μ L of 70% EtOH.
 - ④. Set the tube on magnetic bar for 5 min.
 - ⑤. Discard the supernatant by pipette aspiration.
 - ⑥. Repeat step ③ ~ ⑤.
 - ⑦. Discard the 70% EtOH completely by pipette aspiration.
 - ⑧. Add 42 μ L of RNase free water and mix by pipetting extensively (more than 60 times) to elute RNA/cDNA hybrids.
 - ⑨. Incubate at room temperature for 5 min.
 - ⑩. Spin down and set the tube on magnetic bar for 5min.
 - ⑪. Transfer supernatant (~ 40 μ L of the cDNA/RNA hybrids) to new tube.

1.2

Low Quantity single strand CAGE (LQ-ssCAGE)

When the amount of starting RNAs is less than 1 μ g, choose this protocol.

Note

NOTE: The amount of RNAs in one tube should be 25 ng ~ 100 ng for the RT reaction. After RT, samples have each barcode and can be mixed up to 5 μ g. For instance, if the starting RNA amount is 50 ng, 100 samples can be mixed. The number of mixed samples is depending on how much samples you have and how deep the samples should be sequenced. Here is an example of 48 mix samples from each 50 ng starting RNA. CRITICAL: The mixed solution should be less than 5 μ g. Otherwise the following linkers at step **9** and **11** are not enough for the linker ligation.

- a.** Mix 4 μ L of 50 ng RNAs (12.5 ng/ μ L) and 1 μ L of 1.25 mM RT primer containing barcode in a 96 wells plate by pipetting.
- b.** Incubate the RNA/primer mix from step **a.** at 65 °C for 5 min and immediately put on ice for 2 min.
- c.** Mix the following components (enzyme mix) on ice:

5 x First Strand buffer	2.0 μ L
0.1 M DTT	0.5 μ L
10 mM dNTPs	0.5 μ L
RNase free water	1.0 μ L
SuperScript III Reverse Transcriptase	1.0 μ L
Total volume	5.0 μ L

Note

NOTE: Make premix solution for 48 samples x 1.1 samples.

- d.** Add 5 μ L of enzyme mix from premix solution at step **c.** to RNA/primer mix from step **b.** and carefully mix 10 times by pipetting on ice.
- e.** Incubate at 25 °C for 30 sec, 50 °C for 30 min and keep 4 °C to generate RNA/cDNA hybrids.
- f.** Mix samples by following steps.
 - ①. Transfer each 10 μ L of RNA/cDNA hybrids from step **e.** to one of new 1.5 mL tube (total volume is 480 μ L).
 - ②. Add 15 μ L of RNase free water to the first 8 wells at the 96 wells plate from step ①, wash wells by pipetting, and transfer the 15 μ L of the solutions to the next 8 wells.
 - ③. Wash the 8 wells by pipetting and transfer 15 μ L of the solutions to the next 8 wells.
 - ④. Repeat step ② three times until the end of 8 wells and transfer all solutions (total volume is 120 μ L (15 μ L x 8 wells)) to the 1.5 mL tube from step ① (final volume is 600 μ L).
 - ⑤. Mix 600 μ L of the solution from step ④ by vortex, spin down and aliquot 200 μ L in new two tubes of 1.5 mL tube (total 200 μ L in 3 tubes of 1.5 mL tube).

Note

NOTE: Step **f.** is for the collection of remaining molecules to avoid losing any RNA/cDNA hybrids in the wells. We recommend to aliquot less than 200 μ L per each 1.5 mL tube at step **f.** ⑤ due to the volume limitations of next RNAClean XP purification step.

- g.** Add 300 μ L (1.8 -folds) of RNAClean XP beads to the 48 mixed RNA/cDNA hybrids in a 1.5 mL tube from step **f.** ⑤, mix well by pipetting and then elute the mixed RNA/cDNA hybrids by following steps.
 - ①. Incubate at room temperature for 10 min, spin down and set the 1.5 mL tube on magnetic bar for 5 min.
 - ②. Discard the supernatant by pipette aspiration.
 - ③. Wash the beads with 1.2 mL of 70% EtOH.
 - ④. Set the 1.5 mL tube on magnetic bar for 5 min.
 - ⑤. Discard the supernatant by pipette aspiration.
 - ⑥. Repeat step ③~ ⑤ twice.

- ⑦. Discard the 70% EtOH completely by pipette aspiration.
- ⑧. Add 100 μ L RNase free water and mix by pipetting extensively (more than 60 times) to elute RNA/cDNA hybrids.
- ⑨. Incubate at room temperature for 5 min.
- ⑩. Spin down and set the tube on magnetic bar for 5min.
- ⑪. Transfer 100 μ L of the cDNA/RNA hybrids to new 1.5 mL tube.
- ⑫. Repeat step ⑧ ~ ⑪ twice (final volume is 200 μ L).
- ⑬. Concentrate 200 μ L of the cDNA/RNA hybrids solutions from step ⑫ to around 40 μ L by SpeedVac vacuum concentrator at 37 °C, and collect the solutions from 3 of 1.5 mL tubes to 1 of 1.5 mL tube (total volume is around 120 μ L) and concentrate to 40 μ L in the 1.5 mL tube at 37°C. The timing is around 2 h.

Note

CRITICAL: DO NOT DRY UP the cDNA/RNA hybrids solutions by the concentrating step ⑬. Check the sample volume during the concentration several times and adjust the final volume to 40 μ L with RNase/DNase-free when the volume become less than 40 μ L.

2 Oxidation to modify diol group of cap structure

- a. Mix 40 μ L of RNA/cDNA hybrid from step 1.1e. or 1.2g., 2 μ L of 1 M NaOAc (pH 4.5) and 2 μ L of 250 mM NaIO₄ in a tube by 10 times pipetting.

Note

NOTE: 1.5 mL tube, 8-Strip PCR tube or 16 wells plate can be used depending on how many samples should be prepared.

- b. Incubate for 5 min on ice in dark by aluminum foil wrapping.
- c. Add 16 μ L of 1 M Tris-HCl (pH 8.5) to neutralize the solution and mix well by pipetting.

3 Purification

Add 108 μ L (1.8 -folds) of RNACleanXP beads to 60 μ L of oxidated RNA/cDNA hybrid from step 2, mix well by pipetting and then elute the 48 samples mixed RNA/cDNA hybrids by following steps.

- ①. Incubate at room temperature for 5min.
- ②. Spin down and set the tube on magnetic bar for 5 min.
- ③. Discard the supernatant by pipette aspiration.
- ④. Wash the beads with 200 μ L of 70% EtOH
- ⑤. Discard the 70% EtOH.
- ⑥. Repeat step ④~ ⑤twice and discarded 70% EtOH completely.
- ⑦. Add 42 μ L of RNase free water and mix by pipetting extensively (more than 60 times) to elute

supernatant.

- ⑧. Incubate at room temperature for 5min.
- ⑨. Spin down and set the tube on magnetic bar for 5 min.
- ⑩. Collect 40 µL of the supernatant to new tube.

4 Biotinylation by the coupling reaction to the oxidized RNA/cDNA hybrids

II

- a. Mix 40 µL of purified oxidized RNA/cDNA hybrid from step 3, 4 µL of 1M NaOAc (pH 6.3) and 4 µL of 100 mM Biotin (long arm) hydrazide by 10 times pipetting.
- b. Incubate for 30 min at 40 °C.
- c. Add 86.4 µL of RNACleanXP (1.8 -folds) to 44 µL of solution from step b, and perform purification by following step 3 ① ~ ⑩.

Note

NOTE: Biotinylation happens both 5'end of capped RNAs and 3'end of RNA ([see Figure 2 at published protocol](#)).

5 RNaseONE treatment to digest RNA of RNA/cDNA hybrids

II

- a. Add 4.5 µL of 10 x RNaseONE buffer and 0.5 µL of RNaseONE to 40 µL of purified biotinylated RNA/cDNA hybrids from step 4 and mix 10 times by pipetting.
- b. Incubate for 30 min at 37 °C.
- c. Add 81 µL of RNACleanXP (1.8 -folds) to the solution from step b. and purify by the following step 3 ①~ ⑩.

Note

CRITICAL: This step is critical to digest uncompleted cDNA synthesis to the 5' end of capped RNAs ([See Figure 2 at published protocol](#)).

6 Dynabeads M-270 Streptavidin beads preparation

- a. Add 30 µL of Dynabeads M-270 Streptavidin to new 1.5ml tube, set on the magnetic bar for 5 min, and discard supernatant.
- b. Wash the beads with 30 µL of LiCl buffer and discard supernatant.
- c. Repeat step b. for 2 times.
- d. Resuspend the beads in 35 µL of LiCl buffer.

7 CapTrap reaction

- a. Add 35 µL of beads from step 6 to 40 µL of RNA/cDNA hybrids from step 5 and mix well by pipetting.
- b. Incubate for 15 minutes at 37 °C and mix by pipetting at the time of 7 min.
- c. Spin down and set the plate on the magnetic bar for 2 min and discard the supernatant by the pipette aspiration.
- d. Add 150 µL of TE wash buffer and mix 60 times by pipetting. Spin down and set the plate on the magnetic bar for 2 min and discard the supernatant.
- e. Repeat step d. for 3 times.
- f. Add 35 µL of Release buffer to the beads and mix 60 times by pipetting.
- g. Incubate for 5 min at 95°C for 5min and on ice for 1 min.
- h. Spin down and set the tube on the magnetic bar for 2 min.
- i. Transfer the supernatant containing CapTrapped cDNA from step h. to new 16 well plate.
- j. Add 30 µL of Release buffer to the beads and mix well by pipetting, spin down, and set the tube on the magnetic stand for 2 min.
- k. Transfer 30 µL of supernatant containing CapTrapped cDNA to new tube.

8 RNaseONE and RNaseH reaction to remaining RNAs

-
- a. Add 2.9 µL of release buffer, 2 µL of RNaseONE and 0.1 µL of RNase H to 30 µL of CapTrapped cDNA from step 7 and mix 10 times by pipetting.
 - b. Incubate at 37°C for 30 min.
 - c. Add 126 µL of AMPureXP (1.8 -folds) to 33 µL of cDNA from step b. and perform purification by following step 3 ① ~ ⑩.
 - d. Dry up 40 µL of purified CapTrapped cDNA by the SpeedVac concentrator at 37°C for around 75 min.
 - e. Add 4 µL of RNase free water to the dried pellet.

9 5' Single Strand Linker Ligation

- a. Incubate 4 µL of cDNA at 95 °C for 5 min and put on ice for 2 min.
- b. Incubate 4 µL of 5' linker at 55 °C for 5 min and put on ice for 2 min.
- c. Mix 4 µL of 5' linker, 4 µL of CapTrapped cDNA from step a. and 16 µL of Mighty Mix by pipetting (total volume is 24 µL).
- d. Incubate at 16 °C for 16 h (overnight).

10 Purification to remove excess 5' linkers and linker dimers

-
- a. Add 46 µL of RNase free water and 126 µL of AMPureXP (1.8 -folds) to 24 µL of cDNA from step 9, mix well by pipetting.
 - b. Purify the solution by following step 3 ①~ ⑩.
 - c. Incubate at 95 °C for 5 min and immediately put on ice for 2 min.
 - d. Add 48 µL of AMPureXP beads (1.2 -folds) to 40 µL of cDNA and mix well by pipetting.
 - e. Repeat purification by following step 3 ① ~ ⑩.
 - f. Dry up 40 µL of cDNA by SpeedVac concentrator at 80°C for around 35 min.
 - g. Add 4 µL of RNase free water to the dried pellet.

Note

CRITICAL: It is important to perform heat inactivation and repeat purification step to remove non-ligated excess linkers during the purification step. Otherwise, these excess linkers can disturb library sequencing quality; excess linkers compete with the library to anneal Illumina flow-cells. In addition, 5'linker-3'linker dimers can be created at the next step **11**.

11 3' Single Strand Linker Ligation

- a. Incubate 4 µL of cDNA at 95 °C for 5 min and put on ice for 2 min.
- b. Incubate 4 µL of 3' linker at 65 °C for 5 min and put on ice for 2 min.
- c. Mix 4 µL of 3'linker, 4 µL of cDNA from step **a**. and 16 µL of Mighty Mix by pipetting.
- d. Incubate at 30 °C for 4 h.

Note

NOTE: When you have more than 2 libraries with same barcode at RT primer and plan to mix libraries at the sequencing step **14**, use different Index at the 3'linker.

12 Purification to remove excess 3' linkers and linker dimers (final library)

- a. Purify the solution from step **11** by following step **11a.-f**.
- b. Dissolve the pellet with 10 µL of RNase, DNase-free water.

13 Library QC

Quantify the concentration of library by KAPA Library Quantification Kit.

14 Sequencing

- a. Mix the following components

Library (2250 amol)	X µL
RNase, DNase-free water (19-X) µL	
2N NaOH	1 µL
Total	20 µL

- b. Incubate at room temperature for 5 min.
- c. Put the tube on ice and add 20 µL of 1 M Tris-HCl (pH 7.0, pre-chilled) to neutralize.
- d. Add 110 µL of HT1 buffer.

e. Transfer 120 μ L of the denatured and diluted library to HiSeq2500.

Sequencing kit

- ssCAGE: 50 bp Single Read sequencing.
- LQ-ssCAGE: 50 bp Paired-End sequencing (see Figure 1 for the library structure).
 - Read 1: to read sequence information of cDNA
 - Read 2: to read sequence information of barcode in RT primer
 - Index 1: to read sequence information of index at the 3'linker