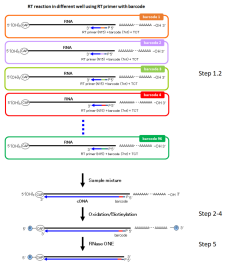
Jan 29, 2020

Low Quantity single strand CAGE protocol



In 1 collection

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

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Keywords: CAGE (Cap Analysis of Gene Expression), Transcription Starting Sites, Gene promoter, enhancer RNA, capped rna, functional annotation of the mammalian genome, low quantity of rna, mapping at single nucleotide resolution, rna amount, gene expression analysis, single strand cage protocol, single nucleotide resolution, mammalian genome, gene expression, rna, measuring transcript level, nuclear rna, large cage dataset, transcription, single strand, cellular compartments like nuclear rna, transcript level, genome, sequencing platform, avoiding pcr amplification, dna, encyclopedia of dna element, active promoter, pcr amplification, dna element,

Abstract

The Cap Analysis 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, is 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)-Illumina / ABI Prism®	KAPA BIOSYSTEMS	KK4835
Sodium periodate ACS Reagent Grade, 25 g	MP Biomedicals	0215257725-
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, 100ML non-sterile; 0.2 µm filtered,	SIGMA-ALDRICH	S2404-
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),	Wako Pure Chemical Industries,	311-90411
1M Tris-HCl (pH 7.5),	Wako Pure Chemical Industries,	316-90221
1M Tris-HCl (pH 8.5),	Wako Pure Chemical Industries,	316-90405
3M Sodium Acetate (pH 5.2),	Wako Pure Chemical Industries,	316-90081
Dimethyl Sulfoxide,	Wako Pure Chemical Industries,	046-21981
Ethanol (99.5),	Wako Pure Chemical Industries,	057-00456
2M NaOH,	Wako Pure Chemical Industries,	194-05631
Hybridization buffer HT1,	Illumina	

<Equipment>

Product name,	Maker	Cat. No.
Axygen 0.2 mL Polypropylene PCR Tube Strips and Domed Cap Strips, 8 Tubes/Strip, 8 Domed Caps/Strip,	CORNING, AXYGEN	PCR-0208-CP-C

Clear, Nonsterile		
Axygen 1.5 mL Maxymum Recovery Snaplock Microcentrifuge Tube, Polypropylene, Clear, Nonsterile, 250 Tubes/Pack, 10 Packs/Case	CORNING, AXYGEN	MCT-150-L-C
Axygen 16 Well Polypropylene PCR Microplate, Clear, Nonsterile	CORNING, AXYGEN	PCR-16-C
Axygen PCR 1 × 8 Strip Domed Caps, Fit 0.2 mL PCR Tube Strips, Clear, Nonsterile,	CORNING, AXYGEN	PCR-02CP-C
X-Pierce Sealing Films, Sterile,	EXCEL Scientific, Inc.	XPS-25
10 µL (0.1 - 10 µL) LongReach Barrier tip,	Sorenson BioScience, Inc.	38000T
10 µL (0.1 - 10 µL) Ultra G Barrier tip,	Sorenson BioScience, Inc.	28200T
1000 µL (100-1000 µl) Barrier tip,	Sorenson BioScience, Inc.	14200T
20 µL (1-20 µL) Barrier tip,	Sorenson BioScience, Inc.	14210T
200 µL (1-200 µL) NX Barrier tip,	Sorenson BioScience, Inc.	30550T
MicroAmp Optical 384-Well Reaction Plate with Barcode,	ThermoFisher SCIENTIFIC	4309849
MicroAmp Optical Adhesive Film,	ThermoFisher SCIENTIFIC	4360954
PIPETMAN Classic, P1000,	GILSON	F123602
PIPETMAN Classic, P2,	GILSON	F144801
PIPETMAN Classic, P20,	GILSON	F123600
PIPETMAN Classic, P200,	GILSON	F123601
MJ Research Tetrad PTC-225 Thermal Cycler,	GMI, MJ Research	8252-30-1004
MINI CENTRIFUGE,	HARMONY (LMS)	CFM-1000
PlateSpin II,	KUBOTA	
Proline Plus Mechanical Pipettes, 8-ch, 0.5 - 10 µL,	sartorius	728120
Proline Plus Mechanical Pipettes, 8-ch, 10 - 100 µL,	sartorius	728130
Proline Plus Mechanical Pipettes, 8-ch, 30 - 300 µL,	sartorius	728140
Vortex-Genie 2,	Scientific Industries, Inc.	SI-0286
miVAC DNA,	SP Scientific, Genevac	DNA-10000-G00
miVac rotor for micro plate,	SP Scientific, Genevac	DRS-00000-200
Dynabeads MPC-S (Magnetic Particle Concentrator),	ThermoFisher SCIENTIFIC	A13346
DynaMag-96 Side Skirted Magnet,	ThermoFisher SCIENTIFIC	12027

<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	TCTGATGCTCNNNNNNNNNNNNNNN	25
TCT_7nt_N15_#002	P	TCTTATGAGCNNNNNNNNNNNNNNN	25
TCT_7nt_N15_#003	P	TCTCGACGATNNNNNNNNNNNNNNN	25

TCT_7nt_N15_#004	P	TCTTAGTCACNNNNNNNNNNNNNN		25
TCT_7nt_N15_#005	P	TCTCGTACTGNNNNNNNNNNNNNN		25
TCT_7nt_N15_#006	P	TCTTACGTAGNNNNNNNNNNNNNN		25
TCT_7nt_N15_#007	P	TCTAGACTCGNNNNNNNNNNNNNN		25
TCT_7nt_N15_#008	P	TCTTCGCTGANNNNNNNNNNNNNN		25
TCT_7nt_N15_#009	P	TCTCGATCTGNNNNNNNNNNNNNN		25
TCT_7nt_N15_#010	P	TCTTGTCACGNNNNNNNNNNNNNN	25	
TCT_7nt_N15_#011	P	TCTATGCACTNNNNNNNNNNNNNN	25	
TCT_7nt_N15_#012	P	TCTGACATCGNNNNNNNNNNNNNN	25	
TCT_7nt_N15_#013	P	TCTATCTAGCNNNNNNNNNNNNNN	25	
TCT_7nt_N15_#014	P	TCTTGTCGCANNNNNNNNNNNNNN	25	
TCT_7nt_N15_#015	P	TCTCAGATCTNNNNNNNNNNNNNN	25	
TCT_7nt_N15_#016	P	TCTTCAGAGCNNNNNNNNNNNNNN	25	
TCT_7nt_N15_#017	P	TCTATACTGCNNNNNNNNNNNNNN	25	
TCT_7nt_N15_#018	P	TCTATCGTGANNNNNNNNNNNNNN	25	
TCT_7nt_N15_#019	P	TCTGAGCTCTNNNNNNNNNNNNNN	25	
TCT_7nt_N15_#020	P	TCTTCTCGAGNNNNNNNNNNNNNN		25
TCT_7nt_N15_#021	P	TCTCAGTGTCNNNNNNNNNNNNNN	25	
TCT_7nt_N15_#022	P	TCTGCTACGANNNNNNNNNNNNNN		25
TCT_7nt_N15_#023	P	TCTAGTACGCNNNNNNNNNNNNNN		25
TCT_7nt_N15_#024	P	TCTGACTAGCNNNNNNNNNNNNNN		25
TCT_7nt_N15_#025	P	TCTTACGCTANNNNNNNNNNNNNN		25
TCT_7nt_N15_#026	P	TCTGCTATAGNNNNNNNNNNNNNN		25
TCT_7nt_N15_#027	P	TCTACACTGTNNNNNNNNNNNNNN		25
TCT_7nt_N15_#028	P	TCTACATGTCNNNNNNNNNNNNNN	25	
TCT_7nt_N15_#029	P	TCTGCAGACTNNNNNNNNNNNNNN		25
TCT_7nt_N15_#030	P	TCTTAGCGACNNNNNNNNNNNNNN		25
TCT_7nt_N15_#031	P	TCTACAGTCGNNNNNNNNNNNNNN	25	
TCT_7nt_N15_#032	P	TCTGTATGACNNNNNNNNNNNNNN		25
TCT_7nt_N15_#033	P	TCTCAGCAGTNNNNNNNNNNNNNN		25
TCT_7nt_N15_#034	P	TCTACAGCTGNNNNNNNNNNNNNN		25
TCT_7nt_N15_#035	P	TCTGCTGATANNNNNNNNNNNNNN		25
TCT_7nt_N15_#036	P	TCTTGTCGACNNNNNNNNNNNNNN		25
TCT_7nt_N15_#037	P	TCTTGCGATANNNNNNNNNNNNNN		25
TCT_7nt_N15_#038	P	TCTAGTGTCANNNNNNNNNNNNNN		25
TCT_7nt_N15_#039	P	TCTATACGTCNNNNNNNNNNNNNN	25	
TCT_7nt_N15_#040	P	TCTCACTATGNNNNNNNNNNNNNN		25
TCT_7nt_N15_#041	P	TCTTCACAGTNNNNNNNNNNNNNN	25	
TCT_7nt_N15_#042	P	TCTTACGCGTNNNNNNNNNNNNNN		25
TCT_7nt_N15_#043	P	TCTCGACGTANNNNNNNNNNNNNN		25
TCT_7nt_N15_#044	P	TCTAGCATGCNNNNNNNNNNNNNN		25
TCT_7nt_N15_#045	P	TCTATCGCGTNNNNNNNNNNNNNN		25
TCT_7nt_N15_#046	P	TCTGATATGCNNNNNNNNNNNNNN	25	

TCT_7nt_N15_#047	P	TCTTAGCATCNNNNNNNNNNNNNN		25
TCT_7nt_N15_#048	P	TCTGCTCATGNNNNNNNNNNNNNN		25
TCT_7nt_N15_#049	P	TCTCTGATGCNNNNNNNNNNNNNN		25
TCT_7nt_N15_#050	P	TCTGCATATGNNNNNNNNNNNNNN	25	
TCT_7nt_N15_#051	P	TCTGTCACATNNNNNNNNNNNNNN	25	
TCT_7nt_N15_#052	P	TCTCTAGCATNNNNNNNNNNNNNN		25
TCT_7nt_N15_#053	P	TCTCACGTGTNNNNNNNNNNNNNN		25
TCT_7nt_N15_#054	P	TCTCTAGTACNNNNNNNNNNNNNN		25
TCT_7nt_N15_#055	P	TCTGATCGTANNNNNNNNNNNNNN		25
TCT_7nt_N15_#056	P	TCTCTAGTCGNNNNNNNNNNNNNN		25
TCT_7nt_N15_#057	P	TCTGACACTGNNNNNNNNNNNNNN		25
TCT_7nt_N15_#058	P	TCTAGCTCGANNNNNNNNNNNNNN		25
TCT_7nt_N15_#059	P	TCTGACTGCANNNNNNNNNNNNNN		25
TCT_7nt_N15_#060	P	TCTAGACGCTNNNNNNNNNNNNNN		25
TCT_7nt_N15_#061	P	TCTCAGTCTGNNNNNNNNNNNNNN	25	
TCT_7nt_N15_#062	P	TCTTGACTGCNNNNNNNNNNNNNN		25
TCT_7nt_N15_#063	P	TCTATCAGCGNNNNNNNNNNNNNN		25
TCT_7nt_N15_#064	P	TCTCTCAGAGNNNNNNNNNNNNNN		25
TCT_7nt_N15_#065	P	TCTTATGCACNNNNNNNNNNNNNN		25
TCT_7nt_N15_#066	P	TCTTGCTACANNNNNNNNNNNNNN		25
TCT_7nt_N15_#067	P	TCTATGCGCANNNNNNNNNNNNNN		25
TCT_7nt_N15_#068	P	TCTTCGTGACNNNNNNNNNNNNNN		25
TCT_7nt_N15_#069	P	TCTCGATACGNNNNNNNNNNNNNN		25
TCT_7nt_N15_#070	P	TCTGCTAGCANNNNNNNNNNNNNN		25
TCT_7nt_N15_#071	P	TCTTAGCGTANNNNNNNNNNNNNN	25	
TCT_7nt_N15_#072	P	TCTCTGACGANNNNNNNNNNNNNN		25
TCT_7nt_N15_#073	P	TCTTACGTCANNNNNNNNNNNNNN		25
TCT_7nt_N15_#074	P	TCTCATAGCTNNNNNNNNNNNNNN		25
TCT_7nt_N15_#075	P	TCTTCGATCANNNNNNNNNNNNNN		25
TCT_7nt_N15_#076	P	TCTCATATCGNNNNNNNNNNNNNN	25	
TCT_7nt_N15_#077	P	TCTCTCGATANNNNNNNNNNNNNN		25
TCT_7nt_N15_#078	P	TCTAGTCAGTNNNNNNNNNNNNNN		25
TCT_7nt_N15_#079	P	TCTTCGATAGNNNNNNNNNNNNNN		25
TCT_7nt_N15_#080	P	TCTGCGTATANNNNNNNNNNNNNN		25
TCT_7nt_N15_#081	P	TCTATGTCAGNNNNNNNNNNNNNN	25	
TCT_7nt_N15_#082	P	TCTACGTCGANNNNNNNNNNNNNN		25
TCT_7nt_N15_#083	P	TCTCACTAGTNNNNNNNNNNNNNN		25
TCT_7nt_N15_#084	P	TCTTCACGTGNNNNNNNNNNNNNN		25
TCT_7nt_N15_#085	P	TCTGCTGTACNNNNNNNNNNNNNN		25
TCT_7nt_N15_#086	P	TCTTACTGAGNNNNNNNNNNNNNN		25
TCT_7nt_N15_#087	P	TCTCGATGCANNNNNNNNNNNNNN		25
TCT_7nt_N15_#088	P	TCTTGTCAGANNNNNNNNNNNNNN		25
TCT_7nt_N15_#089	P	TCTCTCTAGANNNNNNNNNNNNNN		25

TCT_7nt_N15_#090	P	TCTCGTACATNNNNNNNNNNNNNN		25
TCT_7nt_N15_#091	P	TCTCTAGCTGNNNNNNNNNNNNNN	25	
TCT_7nt_N15_#092	P	TCTCGCTATANNNNNNNNNNNNNN		25
TCT_7nt_N15_#093	P	TCTTAGCTCANNNNNNNNNNNNNN		25
TCT_7nt_N15_#094	P	TCTGTATAGCNNNNNNNNNNNNNN	25	
TCT_7nt_N15_#095	P	TCTGTGACTCNNNNNNNNNNNNNN		25
TCT_7nt_N15_#096	P	TCTAGCATCGNNNNNNNNNNNNNN		25
TCT_7nt_N15_#097	P	TCTTGAGCATNNNNNNNNNNNNNN		25
TCT_7nt_N15_#098	P	TCTCGTATAGNNNNNNNNNNNNNN	25	
TCT_7nt_N15_#099	P	TCTACTAGCGNNNNNNNNNNNNNN		25
TCT_7nt_N15_#100	P	TCTACTGATGNNNNNNNNNNNNNN	25	
TCT_7nt_N15_#101	P	TCTGTACATGNNNNNNNNNNNNNN	25	
TCT_7nt_N15_#102	P	TCTTGACTCANNNNNNNNNNNNNN	25	
TCT_7nt_N15_#103	P	TCTGATCTCANNNNNNNNNNNNNN	25	
TCT_7nt_N15_#104	P	TCTCGACTGTNNNNNNNNNNNNNN	25	
TCT_7nt_N15_#105	P	TCTCAGTAGCNNNNNNNNNNNNNN	25	
TCT_7nt_N15_#106	P	TCTACTATGCNNNNNNNNNNNNNN	25	
TCT_7nt_N15_#107	P	TCTACGATCGNNNNNNNNNNNNNN	25	
TCT_7nt_N15_#108	P	TCTGATCACGNNNNNNNNNNNNNN	25	
TCT_7nt_N15_#109	P	TCTTGCATAGNNNNNNNNNNNNNN	25	
TCT_7nt_N15_#110	P	TCTCGTCATANNNNNNNNNNNNNN	25	
TCT_7nt_N15_#111	P	TCTAGCTCAGNNNNNNNNNNNNNN	25	
TCT_7nt_N15_#112	P	TCTGTAGCACNNNNNNNNNNNNNN	25	
TCT_7nt_N15_#113	P	TCTCTATGCGNNNNNNNNNNNNNN	25	
TCT_7nt_N15_#114	P	TCTGACTACTNNNNNNNNNNNNNN	25	
TCT_7nt_N15_#115	P	TCTGATCGCTNNNNNNNNNNNNNN	25	
TCT_7nt_N15_#116	P	TCTCTGATAGNNNNNNNNNNNNNN	25	
TCT_7nt_N15_#117	P	TCTGTGCATANNNNNNNNNNNNNN	25	
TCT_7nt_N15_#118	P	TCTGATGCATNNNNNNNNNNNNNN	25	
TCT_7nt_N15_#119	P	TCTACTGCAGNNNNNNNNNNNNNN	25	
TCT_7nt_N15_#120	P	TCTTGATCACNNNNNNNNNNNNNN	25	

<5′ linkers>

name, 5′ modification, sequence, 3′ modification, base

5′adaptor N6, -, AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNNN, P, 64

5′adaptor GN5, -, AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTGNNNNN, P, 64

5′adaptor down, P, AGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGTAGATCTCGGTGGTCGCCGTATCATT, P, 58

<3′ linkers >

name

sequence, 3′ modification, base

3′end adaptor up 01
NNNAGATCGGAAGAGCACACGTCTGAACTCCAGTCACATCACGATCTCGTATGCCGTCTTCTGCTTG, P, 67
3′end adaptor down 01
CAAGCAGAAGACGGCATACGAGATCGTGATGTGACTGGAGTTCAGACGTGTGCTCTTCCGA, - , 61
3′end adaptor up 02
NNNAGATCGGAAGAGCACACGTCTGAACTCCAGTCACCGATGTATCTCGTATGCCGTCTTCTGCTTG, P, 67
3′end adaptor down 02
CAAGCAGAAGACGGCATACGAGATACATCGGTGACTGGAGTTCAGACGTGTGCTCTTCCGA, - , 61
3′end adaptor up 03
NNNAGATCGGAAGAGCACACGTCTGAACTCCAGTCACTTAGGCATCTCGTATGCCGTCTTCTGCTTG, P, 67
3′end adaptor down 03
CAAGCAGAAGACGGCATACGAGATGCCTAAGTGACTGGAGTTCAGACGTGTGCTCTTCCGA, - , 61
3′end adaptor up 04
NNNAGATCGGAAGAGCACACGTCTGAACTCCAGTCACTGACCAATCTCGTATGCCGTCTTCTGCTTG, P, 67
3′end adaptor down 04
CAAGCAGAAGACGGCATACGAGATTGGTCAGTGACTGGAGTTCAGACGTGTGCTCTTCCGA, - , 61
3′end adaptor up 05
NNNAGATCGGAAGAGCACACGTCTGAACTCCAGTCACACAGTGATCTCGTATGCCGTCTTCTGCTTG, P, 67
3′end adaptor down 05
CAAGCAGAAGACGGCATACGAGATCACTGTGTGACTGGAGTTCAGACGTGTGCTCTTCCGA, - , 61
3′end adaptor up 06
NNNAGATCGGAAGAGCACACGTCTGAACTCCAGTCACGCCAATATCTCGTATGCCGTCTTCTGCTTG, P, 67
3′end adaptor down 06
CAAGCAGAAGACGGCATACGAGATATTGGCGTGACTGGAGTTCAGACGTGTGCTCTTCCGA, - , 61
3′end adaptor up 07
NNNAGATCGGAAGAGCACACGTCTGAACTCCAGTCACCAGATCATCTCGTATGCCGTCTTCTGCTTG, P, 67
3′end adaptor down 07
CAAGCAGAAGACGGCATACGAGATGATCTGGTGACTGGAGTTCAGACGTGTGCTCTTCCGA, - , 61
3′end adaptor up 08
NNNAGATCGGAAGAGCACACGTCTGAACTCCAGTCACACTTGAATCTCGTATGCCGTCTTCTGCTTG, P, 67
3′end adaptor down 08
CAAGCAGAAGACGGCATACGAGATTCAAGTGTGACTGGAGTTCAGACGTGTGCTCTTCCGA, - , 61
3′end adaptor up 09
NNNAGATCGGAAGAGCACACGTCTGAACTCCAGTCACGATCAGATCTCGTATGCCGTCTTCTGCTTG, P, 67
3′end adaptor down 09
CAAGCAGAAGACGGCATACGAGATCTGATCGTGACTGGAGTTCAGACGTGTGCTCTTCCGA, - , 61
3′end adaptor up 10
NNNAGATCGGAAGAGCACACGTCTGAACTCCAGTCACTAGCTTATCTCGTATGCCGTCTTCTGCTTG, P, 67
3′end adaptor down 10
CAAGCAGAAGACGGCATACGAGATAAGCTAGTGACTGGAGTTCAGACGTGTGCTCTTCCGA, - , 61
3′end adaptor up 11
NNNAGATCGGAAGAGCACACGTCTGAACTCCAGTCACGGCTACATCTCGTATGCCGTCTTCTGCTTG, P, 67

3′end adaptor down 11
CAAGCAGAAGACGGCATACGAGATGTAGCCGTGACTGGAGTTCAGACGTGTGCTCTTCCGA, - , 61
3′end adaptor up 12
NNNAGATCGGAAGAGCACACGTCTGAACTCCAGTCACCTTGTAATCTCGTATGCCGTCTTCTGCTTG, P, 67
3′end adaptor down 12
CAAGCAGAAGACGGCATACGAGATTACAAGGTGACTGGAGTTCAGACGTGTGCTCTTCCGA, - , 61
3′end adaptor up 13
NNNAGATCGGAAGAGCACACGTCTGAACTCCAGTCACAGTCAACAATCTCGTATGCCGTCTTCTGCTTG, P, 69
3′end adaptor down 13
CAAGCAGAAGACGGCATACGAGATTGTTGACTGTGACTGGAGTTCAGACGTGTGCTCTTCCGA, - , 63
3′end adaptor up 14
NNNAGATCGGAAGAGCACACGTCTGAACTCCAGTCACAGTTCCGTATCTCGTATGCCGTCTTCTGCTTG, P, 69
3′end adaptor down 14
CAAGCAGAAGACGGCATACGAGATACGGAACTGTGACTGGAGTTCAGACGTGTGCTCTTCCGA, - , 63
3′end adaptor up 15
NNNAGATCGGAAGAGCACACGTCTGAACTCCAGTCACATGTCAGAATCTCGTATGCCGTCTTCTGCTTG, P, 69
3′end adaptor down 15
CAAGCAGAAGACGGCATACGAGATTCTGACATGTGACTGGAGTTCAGACGTGTGCTCTTCCGA, - , 63
3′end adaptor up 16
NNNAGATCGGAAGAGCACACGTCTGAACTCCAGTCACCCGTCCCGATCTCGTATGCCGTCTTCTGCTTG, P, 69
3′end adaptor down 16
CAAGCAGAAGACGGCATACGAGATCGGGACGGGTGACTGGAGTTCAGACGTGTGCTCTTCCGA, - , 63
3′end adaptor up 18
NNNAGATCGGAAGAGCACACGTCTGAACTCCAGTCACGTCCGCACATCTCGTATGCCGTCTTCTGCTTG, P, 69
3′end adaptor down 18
CAAGCAGAAGACGGCATACGAGATGTGCGGACGTGACTGGAGTTCAGACGTGTGCTCTTCCGA, - , 63
3′end adaptor up 19
NNNAGATCGGAAGAGCACACGTCTGAACTCCAGTCACGTGAAACGATCTCGTATGCCGTCTTCTGCTTG, P, 69
3′end adaptor down 19
CAAGCAGAAGACGGCATACGAGATCGTTTCACGTGACTGGAGTTCAGACGTGTGCTCTTCCGA, - , 63
3′end adaptor up 20
NNNAGATCGGAAGAGCACACGTCTGAACTCCAGTCACGTGGCCTTATCTCGTATGCCGTCTTCTGCTTG, P, 69
3′end adaptor down 20
CAAGCAGAAGACGGCATACGAGATAAGGCCACGTGACTGGAGTTCAGACGTGTGCTCTTCCGA, - , 63
3′end adaptor up 21
NNNAGATCGGAAGAGCACACGTCTGAACTCCAGTCACGTTTCGGAATCTCGTATGCCGTCTTCTGCTTG, P, 69
3′end adaptor down 21
CAAGCAGAAGACGGCATACGAGATTCCGAAACGTGACTGGAGTTCAGACGTGTGCTCTTCCGA, - , 63
3′end adaptor up 22
NNNAGATCGGAAGAGCACACGTCTGAACTCCAGTCACCGTACGTAATCTCGTATGCCGTCTTCTGCTTG, P, 69
3′end adaptor down 22
CAAGCAGAAGACGGCATACGAGATTACGTACGGTGACTGGAGTTCAGACGTGTGCTCTTCCGA, - , 63

3′end adaptor up 23
NNNAGATCGGAAGAGCACACGTCTGAACTCCAGTCACGAGTGGATATCTCGTATGCCGTCTTCTGCTTG, P, 69
3′end adaptor down 23
CAAGCAGAAGACGGCATACGAGATATCCACTCGTGACTGGAGTTCAGACGTGTGCTCTTCCGA, - , 63
3′end adaptor up 25
NNNAGATCGGAAGAGCACACGTCTGAACTCCAGTCACACTGATATATCTCGTATGCCGTCTTCTGCTTG, P, 69
3′end adaptor down 25
CAAGCAGAAGACGGCATACGAGATATATCAGTGTGACTGGAGTTCAGACGTGTGCTCTTCCGA, - , 63
3′end adaptor up 27
NNNAGATCGGAAGAGCACACGTCTGAACTCCAGTCACATTCCTTTATCTCGTATGCCGTCTTCTGCTTG, P, 69
3′end adaptor down 27
CAAGCAGAAGACGGCATACGAGATAAAGGAATGTGACTGGAGTTCAGACGTGTGCTCTTCCGA, - , 63

<Recipes>

1. 250 mM $NaIO_4$

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 µL
0.5 M EDTA (pH 8.0)	100 µL
RNase, DNase-free water	49.4 mL

7. 0.1 M NaCl/TE buffer

1 M NaCl	500 µL
1 x TE buffer	4.5 mL

8. 2.5 µM 5′linker
a. Mix following solution and carry out the annealing reaction to generate 100 µM GN5 linker

1 mM 5′adaptor GN5	4 µL
1 mM 5′adaptor down	4 µL
1 M NaCl	4 µL
1 x TE buffer	28 µL

<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 µM N6 linker

1 mM 5′adaptor N6	1 µL
1 mM 5′adaptor down	1 µL
1M NaCl	1 µL
1 x TE buffer	7 µL

c. Mix 40 µL of 100 µM GN5 linker from step a. and 10 µL of 100 µM N6 linker from step b.
d. Dilute 100 µM of mixed 5′linkers to 2.5 µM in 0.1 M NaCl/TE buffer. For instance, add 2.5 µL of 100 µM mixed linkers from step c to 97.5 µL of 0.1 M NaCl/TE buffer for 25 samples.

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

9. 2.5 µM 3′ linker

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

1 mM 3′end adaptor up	1 µL
1 mM 3′end adaptor down	1 µL
1 M NaCl	1 µL
1 x TE buffer	7 µL

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

Troubleshooting

Before start

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**).

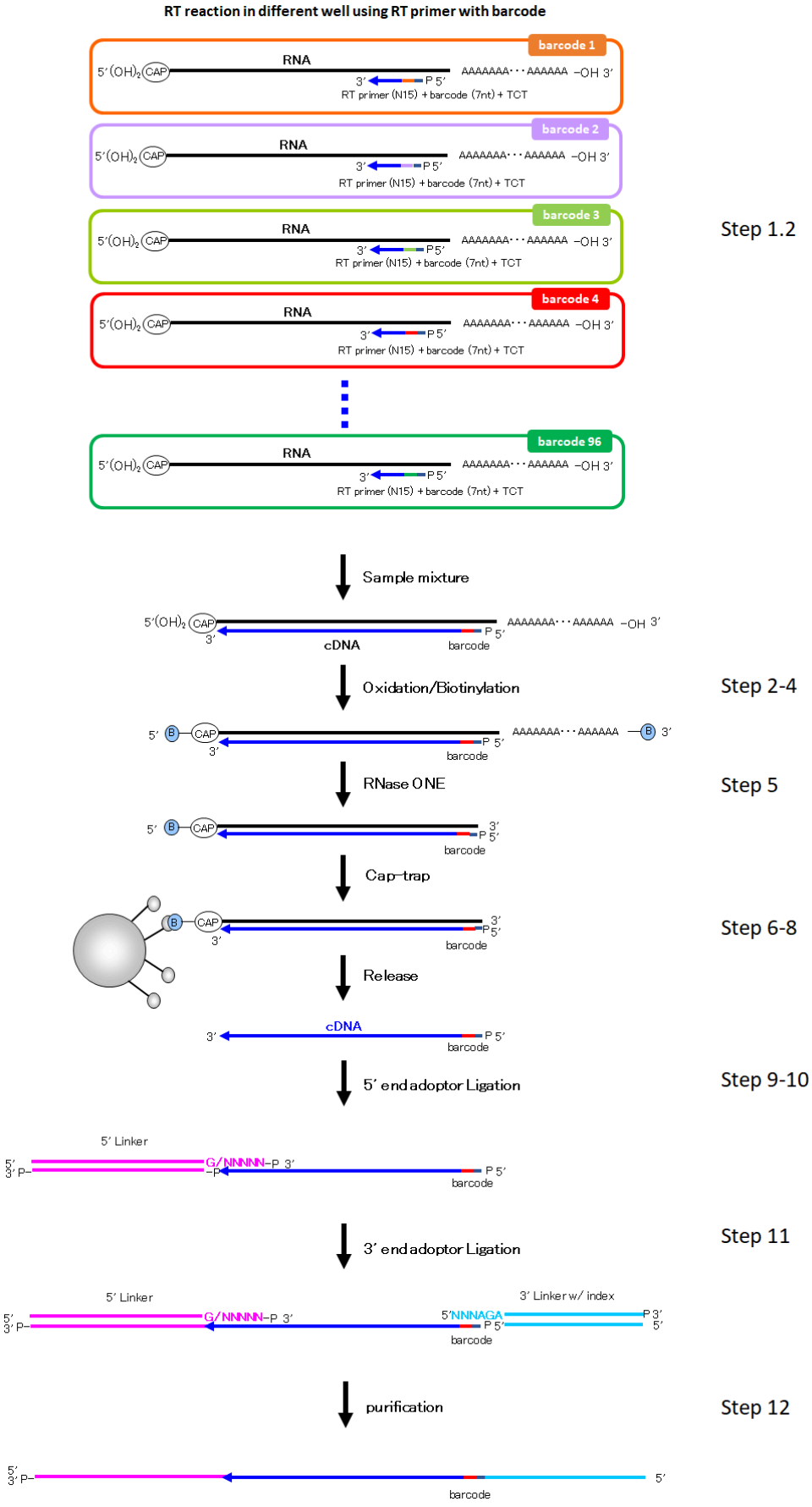
RT reaction in different well using RT primer with barcode
barcode 1
RNA
5′(OH)2 CAP
AAAAAAA···AAAAAA –OH 3′
3′ P 5′
RT primer (N15) + barcode (7nt) + TCT
barcode 2
barcode 3
barcode 4
barcode 96
Sample mixture
cDNA
barcode
Oxidation/Biotinylation
Step 2-4
RNase ONE
Step 5
Cap-trap
Step 6-8
Release
5′ end adoptor Ligation
Step 9-10
5′ Linker
G/NNNNN–P 3′
3′ end adoptor Ligation
Step 11
3′ Linker w/ index
5′NNNAGA
purification
Step 12

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 than1 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 µg/µL), 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 °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 µL
0.1 M DTT	1 µL
RNase free water	2 µL
SuperScript III Reverse Transcriptase	2 µL

d. Incubate the mixed solution (total 20 µL) at 25 °C for 30 sec, 55 °C for 30 min and keep 4 °C to generate RNA/cDNA hybrids.
e. Add 36 µL (1.8-folds) of RNACleanXP beads to 20 µL 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 µL 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 µL 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 µL 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 µg, 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 µg. 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 µg. Otherwise the following linkers at step **9** and **11** are not enough for the linker ligation.

a. Mix 4 µL of 50 ng RNAs (12.5 ng/µL) and 1 µL of 1.25 mM RT primer containing barcode in a 96 wells plate by pipetting.
b. Incubate the RNA/primer mix form 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 µL
0.1 M DTT	0.5 µL
10 mM dNTPs	0.5 µL
RNase free water	1.0 µL
SuperScript III Reverse Transcriptase	1.0 µL
Total volume	5.0 µL

Note

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

d. Add 5 µL 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 µL of RNA/cDNA hybrids from step **e.** to one of new 1.5 mL tube (total volume is 480 µL).
②. Add 15 µL 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 µL of the solutions to the next 8 wells.
③. Wash the 8 wells by pipetting and transfer 15 µL of the solutions to the next 8 wells.
④. Repeat step ②three times until the end of 8 wells and transfer all solutions (total volume is 120 µL (15 µL x 8 wells)) to the 1.5 mL tube from step ①(final volume is 600 µL).
⑤. Mix 600 µL of the solution from step ④by vortex, spin down and aliquot 200 µL in new two tubes of 1.5 mL tube (total 200 µL 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 µL per each 1.5 mL tube at step **f.**⑤due to the volume limitations of next RNAClean XP purification step.

g. Add 300 µL (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 µL 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 µL of the cDNA/RNA hybrids to new 1.5 mL tube.
⑫. Repeat step ⑧ ~ ⑪ twice (final volume is 200 µL).
⑬. Concentrate 200 µL of the cDNA/RNA hybrids solutions from step ⑫ to around 40 µL 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 µL) and concentrate to 40 µL 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 µL with RNase/DNase-free when the volume become less than 40 µL.

2 **Oxidation to modify diol group of cap structure**

a. Mix 40 µL of RNA/cDNA hybrid from step **1.1e.** or **1.2g.**, 2 µL of 1 M NaOAc (pH 4.5) and 2 µL of 250 mM $NaIO_4$ in a tube by 10 times pipetting.

Note

NOTE: 1.5 mL tube, 8-Strip PCR tube or16 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 µL of 1 M Tris-HCl (pH 8.5) to neutralize the solution and mix well by pipetting.

3 **Purification**

Add 108 µL (1.8 -folds) of RNACleanXP beads to 60 µL 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 µL of 70% EtOH
⑤. Discard the 70% EtOH.
⑥. Repeat step **④~ ⑤**twice and discarded 70% EtOH completely.
⑦. Add 42 µL 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**

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**

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 µLof 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 µL 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