CAGE Library Preparation Kit

Version 1.6
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K.K. DNAFORM

Protocol contents

1. Introduction

1) The protocol describes steps to prepare the CAGE library that is applicable to the illumina next-generation sequencers.

Mixed library is strongly recommended to be employed for the CAGE library preparationin order to recover sufficient amounts of sample for sequencing without a PCR amplification step.
Mixed library allows to sequence for multiple samples at a time. The generated cDNA sequences would be able to be re-classified to the original tube using o a linker that incorporates multiple bar code sequences.
The kit contains sufficient reagents to perform mixed library preparation for 8 RNA samples maximum. The protocol describes the steps to prepare library for both 1 and 8 samples.

- 8 samples can be treated in a single CAGE library preparation at maximum.
- For multiple samples preparation, prepare reagent mix for the required number of samples plus 1 extra reaction and dispense it into each reaction tube.
- The tables at each step indicates the required volume of reagents for both 1 and 8 sample (9 samples practically) preparation.

2) The round bracket shows the proper pipette to use for each step.

- Example: (P200 / 8ch-P300)・・・P200 for 1 sample and 8ch-P300 for multiple samples are recommended.

3) The square brackets indicate tables, figures and cautions to be referred.

Example 1: [Table 1]・・・Refer to Table 1 for the operation.
Example 2: [Fig. 2]・・・Refer to Fig. 2 for the operation.
Example 3: [xx in Step 3<a>]・・・Refer to xx in Step 3<a> for the operation.

4) Experiments should be performed with careful attention to the following instructions in each step.

- Be careful about cross contamination when multiple samples are treated.
- Create RNase free environment prior to starting RNA treatment by destroying RNase on equipments (pipettes, ice buckets, tube racks, etc.), benchtops and globes by wiping with RNase Decontamination Solution, such as RNaseZap®, and wearing lab coats and masks. Treat equipments and globes during the experiment as well in case they come to contact with un-treated equipments and areas.
- Fully understand the protocol prior to starting preparation.
- Especially be careful about overlooking small differences between similar steps, for example small difference in reagent volumes.

5) Preferable room temperature to perform the CAGE library preparation is 20 - 25℃. Avoid extremely low room temperature because it may cause the precipitation of chemical substances in the buffer solution.

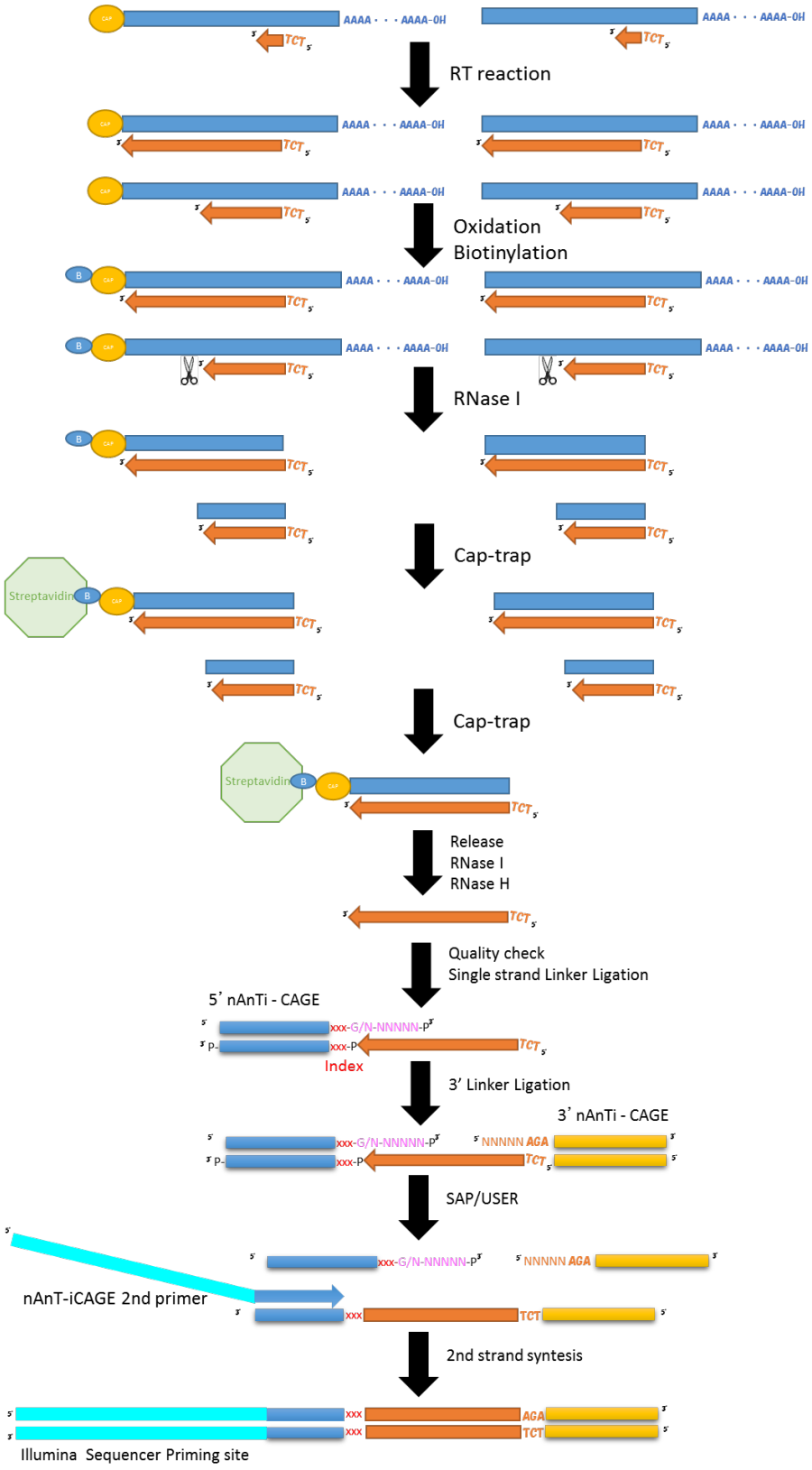
2. Total RNA sample

1) The protocol is optimized to prepare CAGE library using 5 μg/sample of total RNA. Insufficient volume of total RNA may not be able to provide sufficient amount of sample for sequencing.
   - Required concentration of total RNA is ≧1 μg/μl.
   - Concentrate and adjust the concentration of total RNA when its concentration is too low. Do not use a coprecipitating agent for ethanol precipitation.

2) Meeting to the following criteria is strongly recommended for the quality of total RNA to be used for the CAGE library preparation. Total RNA with low quality may not be able to provide sufficient amount of sample for sequencing.
   - ≧1.8 of A260/A230 ratio and ≧1.8 of A260/A280 ratio of spectrophotomater readings.
   - ≧7 of the RNA integrity number (RIN) with Agilant Bioanalyzer or equivalent quality

3) RNA samples must be placed on ice anytime unless there is specific instruction. It must be kept at -80℃ for longer storage.

3. Overview of the CAGE library preparation



4. Required reagents, consumables and equipments for the CAGE library preparation

1) CAGE Library Preparation Kit components

The following table shows CAGE Library Preparation Kit components and labeling. All components labeled with the name of reagents on the sides of the tubes and the numbers on the top of the caps. Ensure the labeling when using the components.

Label Number	Reagents	Amounts (µl)		Storage Condition
		8 sample	48 sample	
RNA	Positive control RNA (1 µg/µl)	7 µl	7 µl	-80°C
P1	RT primer	12 µl	60 µl	-20°C
P2	2nd primer	16 µl	16 µl	-20°C
L1-#1	5' Linker #1 (ACC)	6 µl	36 µl	-20°C
L1-#4	5' Linker #4 (CAC)	6 µl	36 µl	-20°C
L1-#5	5' Linker #5 (AGT)	6 µl	36 µl	-20°C
L1-#7	5' Linker #7 (GCG)	6 µl	36 µl	-20°C
L1-#9	5' Linker #9 (ATG)	6 µl	36 µl	-20°C
L1-#10	5' Linker #10 (TAC)	6 µl	36 µl	-20°C
L1-#15	5' Linker #15 (ACG)	6 µl	36 µl	-20°C
L1-#25	5' Linker #25 (GCT)	6 µl	36 µl	-20°C
L2	3' Linker	40 µl	240 µl	-20°C
R1	H_2O (DNase / RNase Free)	5100 µl	40000 µl	4°C
R2	10 mM dNTP each	150 µl	150 µl	-20°C
R3	Trehalose/Sorbitol	1000 µl	1000 µl	-20°C
R4	1 M NaOAc (pH4.5)	300 µl	300 µl	-20°C
R5	250 mM $NaIO_4$	160 µl	480 µl	-80°C
R6	1 M Tris-HCl (pH8.5)	1700 µl	1700 µl	-20°C
R7	1 M NaOAc (pH6.0)	500 µl	500 µl	-20°C
R8	10 mM Biotin Hydrazaide	160 µl	480 µl	-80°C
R9	10 µg/µl tRNA	20 µl	80 µl	-20°C
R10	10× RNase I buffer	500 µl	500 µl	-20°C
R11	Wash buffer1	5100 µl	25000 µl	4°C
R12	Wash buffer2	1700 µl	18000 µl	4°C
R13	Wash buffer3	1700 µl	18000 µl	4°C
R14	Releasing buffer	1700µl	3400 µl	-20°C
R15	10× SAP buffer	300 µl	300 µl	-20°C
R16	DNA polymerase buffer	150 µl	150 µl	-20°C
E1	RNase I (10 unit/µl)	30 µl	180 µl	-20°C
E2	RNase H (60unit/µl)	6 µl	6 µl	-20°C
E3	Ligation mixture	300 µl	1800 µl	-20°C
E4	SAP (1 unit/µl)	100 µl	100 µl	-20°C
E5	USER (1 unit/µl)	20 µl	110 µl	-20°C
E6	DNA polymerase (2 unit/µl)	16 µl	16 µl	-20°C

E7	Exonuclease I (20 unit/μl)	16 μl	16 μl	-20°C
Op1	40% glycerol	400 μl	400 μl	-20°C

2) Required reagents not included in the kit

The following reagents are required to prepare CAGE Library using the kit.

Manufacturer	Products	Size	Name in this protocol	Cat. No
Agilent Technologies	Agilent High Sensitivity DNA Analysis Kit	10 chips	Highsensitivity DNA kit	5067-4626
Ambion	RNase*Zap*	250 ml	ZAP	AM9780
Beckman coulter	Agencourt RNACleanXP	40 ml	RNACleanXP	A63987
Beckman coulter	Agencourt AMPure XP	5 ml	AMPure XP	A63880
Thermo Fisher Scientific	Dynabeads® M-270 Streptavidin	10 ml	Streptavidin beads	653.06
Invitrogen	Qμant-iT™ Oligreen® ssDNA Reagent and Kit	200-2000 assays	Oligreen	O11492
Invitrogen	PicoGreen dsDNA Quantitation Kit	200-2000 assays	PicoGreen	P7589
Invitrogen	SuperScript®III Reverse Transcriptase	10000 U	SSIII	18080-044
Wako	Ethanol(99.5)	500 ml	EtOH	057-00456
Wako	2-Propanol	500 ml	Isopropanol	166-04836

3) Required materials and equipments

The following materials and equipments or their equivalents are required for CAGE Library preparation using the kit.

Manufacturer	Products	Size	name in protocol	cat. No
Agilent Technologies	Agilent 2100 Bioanalyzer	1	Bioanalyzer	
AXYGEN	1.5 ml SnapLock Microtube, Non-Sterile,MaxyClear, Maxymum Recovery	250 tubes	1.5 ml tube	MCT-150-L-C
AXYGEN	2.0 ml SnapLock Microtube, Non-Sterile,MaxyClear, Maxymum Recovery	250 tubes	2.0 ml tube	MCT-200-L-C
AXYGEN	96 well PCR Plate, 0.2 ml, Non-Sterile, Clear	50 plates	96-well plate	PCR-96-C
AXYGEN	16 well micro PCR plate, clear	1000 plates	16-well plate	PCR-16-C
AXYGEN	0.2 ml 8-Strip PCR Dome Tube Cap	1250strips	PCR Cap	PCR-02CP-C
AXYGEN	0.2 ml Thin Wall Clear PCR Strip Tubes and Clear Strip Caps.	1250strips	8-strip tube	PCR-0208-CP-C
BECKMAN/Kubota	Centrifuge with a rotor for multiple well plates (Allegra 6KR Centrifuge/plateSpin Ⅱ)	1	Plate centrifuge	
BIOHIT	Proline plus multi-channel pipette 8ch 0.5 - 10 µl	1	8ch-P10	728120
BIOHIT	Proline plus multi-channel pipette 8ch 10 - 100 µl	1	8ch-P100	728130
BIOHIT	Proline plus multi-channel pipette 8ch 30 - 300 µl	1	8ch-P300	728140
genevac	Tabletop centrifugal concentrator (miVAC DNA)	1	Speed Vac	
GILSON	PIPETMAN P-2	1	P2	
GILSON	PIPETMAN P-20	1	P20	

GILSON	PIPETMAN P-200	1	P200	
GILSON	PIPETMAN P-1000	1	P1000	
Invitrogen	Dynal MPC-96S		Magnetic bar	DB12027
Invitrogen	Dynal MPC-S		Magnetic stand	
IWAKI	CFM－100	1	Tabletop centrifugal machine	15044
MJ Research	Thermal cycler(for 96 well)PTC-225 Tetrad Thermal Cycler PCR	1	Thermal cycler	
AS ONE	Dry Bath	1	Heat block	EB-303
NIPPON Genetics Co., Ltd	0.1-10 µl MulTI Micro Guard G LowBinding γ-ray sterile, cell rack	96tips× 10 racks	10 µl filter tip	28201T
NIPPON Genetics Co., Ltd	0.1-10 µl(long) LowBinding (Ultra low binding) Long reach γ-ray sterile, cell rack	96 tips× 10 racks	10 µl long filter tip	38001T
NIPPON Genetics Co., Ltd	0.5-20 µl MulTI Guard LowBinding γ-ray sterile, cell rack	96 tips× 10 racks	20 µl filter tip	14211T
NIPPON Genetics Co., Ltd	1-200 µl(long)MulTI Guard 200NX LowBinding γ-ray sterile, cell rack	96 tips× 10 racks	200 µl filter tip	30551T
NIPPON Genetics Co., Ltd	100-1000 µl MulTI Guard LowBinding γ-ray sterile, cell rack	96 tips× 10 racks	1000 µl filter tip	14201T
WAKENYAKU Co., Ltd	Puchihachi PCR tube tabletop centrifugal machine	1	8chTabletop centrifugal machine	2816
ABI	ABI PRISM 384well plate	50 plates		4309849
ABI	ABI PRISM Optical Adhesive covers	100 sheets		4311971
Wallac	ARVO SX 1420 Multilabel counter	1	ARVO	

5. CAGE library preparation protocol

Day1

◆Step 1<a> 1st strand cDNA Synthesis (1hr 30min)

Purpose: Synthesize the 1st strand cDNA by reverse transcription reaction with random primers using total RNA as a template.

Table 1 : Required reagents, consumables and equipment for the 1st strand cDNA synthesis

Reagents included in the kit (tube number)	Consumables	Equipment
☐ Positive control RNA(1 μg/μl) (RNA)	☐ 10 μl filter tip	☐ P2
☐ RT primer (P1)	☐ 20 μl filter tip	☐ P20
☐ H_2O (R1)	☐ 200 μl filter tip	☐ P200
☐ 10 mM dNTP (R2)	☐ 1.5 ml tube	☐ 8ch-P300
☐ Treharose / Sorbitol (R3)	☐ 16-well plate	☐ ice bucket
Reagents not included in the kit		☐ Thermal cycler
SuperScript®III－Reverse Transcriptase ☐ SSIII(200 U/μl) ☐ 5×First-Strand Buffer ☐ 0.1 M DTT		

Preparations

・Dissolve the following reagents on ice, mix by tapping or inverting the tubes and spindown.
  □ RT primer
  □ 5×First-Strand Buffer
  □ 0.1M DTT
  □ 10 mM dNTP
・Set the thermal cycler to 65℃.
・Program CAGE_RT program [Table 4] on the thermal cycler.
・Prepare a 16-well plate (sample plate ①) and label with the sample name or number.

<1st strand cDNA synthesis **>**

Note: In case optional Poly(A) Polymerase reaction (page 62)is performed, start from the step 5 but skip incubation step 6.

1.☐ Dissolve RNA on ice.
2.☐ Dispense 5 μl (5 μg) of total RNA into the sample plate ①. (P20)
Note: Perform ethanol precipitation or SpeedVac concentration in case RNA concentration is too low. However, do not use glycogen for ethanol precipitation (disturbance on biotynilation reaction is expected). When SpeedVac concentration is used, DO NOT dry the sample completely.

3.☐ Prepare RT primer premix [Table 2] in a 1.5 ml tube. Mix well by pipetting slowly for 10 times. Spindown to collect solution to the bottom of the tube in the tabletop centrifuge. Place the tube on ice until just before use. (P2、P20、P200)

Table 2 : RT primer premix

Reagent	1 sample	Equipment	8 samples (Prepare for 9)	Equipment
☐ H_2O (R1)	☐ 4.0 µl	P20	☐ 36.0 µl	P200
☐ RT primer (P1)	☐ 1.0 µl	P2	☐ 9.0 µl	P20
Total volume	5.0 µl		45.0 µl	

4.☐ Dispense 5 µl/sample of RT primer premix into the sample plate ① that contains RNA. Spindown the plate in the plate centrifuge to collect the solution to the bottom. Place the plate on ice until just before use. (P2、P20、P200)

5.☐ Prepare RT enzyme premix [Table 3] in a 1.5 ml tube. Mix well by pipetting slowly for 10 times. Spindown to collect solution to the bottom of the tube in the tabletop centrifuge. Place the tube on ice until just before use. (P2、P20、P200)

Note: Pipet slowly to take Trehalose / Sorbitol because it is high-in viscosity. After dispensing, mix well by pipetting.

Table 3 : RT enzyme premix

Reagent	1 sample	Equipment	8 samples (Prepare for 9)	Equipment
☐ H_2O (R1)	☐ 6.1 µl	P20	☐ 54.9 µl	P200
☐ 5×First-Strand Buffer	☐ 7.6 µl	P20	☐ 68.4 µl	P200
☐ 0.1M DTT	☐ 1.9 µl	P2	☐ 17.1 µl	P20
☐ 10 mM dNTP (R2)	☐ 1.0 µl	P2	☐ 9.0 µl	P20
☐ Trehalose / Sorbitol (R3)	☐ 7.6 µl	P20	☐ 68.4 µl	P200
☐ SSIII(200 U/µl)	☐ 3.8 µl	P20	☐ 34.2 µl	P200
Total volume	28.0 µl		252.0 µl	

6.☐ Set the sample plate ①(RNA/Primer), which is prepared in the step 1<a>4, in thermal cycler that is set at 65°C. Incubate for 5 min. [Table 4 : 65C program]

Table 4 : 65C program

Temperature	Time
65℃	5 min

7.☐ Immediately after incubation in the step 6, place the plate on ice and stand for 1min.

8.☐ Dispense 28 µl/sample of RT enzyme premix into the sample plate ①. (P200)

9.☐ Set the scale of pipette at 38 µl and mix the reaction mixture well by pipetting 10 times. (P200 / 8ch-P300)

10.☐ Spindown the plate in the plate centrifuge to collect the solution to the bottom of the plate.

11.☐ Set the sample plate ① in the thermal cycler and carry out Table 5 : CAGE_RT program.

Table 5 : CAGE_RT program

Temperature	Time
25℃	30 sec
50℃	60 min
4℃	forever

12.☐ After the reaction is completed, spindown the samples in the plate centrifuge to collect the solution to the bottom of the plate.

13.☐ Place the sample on ice or -80℃ until proceeding to the step 1 <b> .

◆Step 1<b> RNAClean XP purification (2hrs)

Propose: Remove unreacted primers and texchange buffer in the Step 1<a> sample.

Table 6: Required reagents, consumables and equipments for RNAClean XP purification

Reagents not included in the kit	Consumable	Equipment
☐ RNAClean XP	☐ 200 µl filter tip ☐ 1000 µl filter tip ☐ 16-well plate ☐ 8-strip tube	☐ P200 ☐ 8ch-P100 ☐ 8ch-P300 ☐ Ice bucket ☐ Thermal cycler ☐ magnetic bar
☐ 70% Ethanol		
☐ Warmed H_2O at 37℃(In the following described as 37℃-H_2O		

Preparation

・Incubate RNAClean XP for 30 min at room temperature. Mix the beads well just before use by inverting the tube, tapping or vortex etc..
・Set the thermal cycler to 37℃.
・【Preparation of 37℃-H_2O】Dispense 200 µl of H_2O into 8-strip tube and incubate at 37℃ in the thermal cycler.
・Prepare a 16-well plate（sample plate②）and label it with sample names or numbers.

< Purification using RNAClean XP>

1.☐ Add 68.4 µl/sample of the RNAClean XP into the sample plate ①. Set the scale of pipette at 86 and mix well the samples by pipetting 10 times. (P200 / 8ch-P300)[Table 7]

Important: (Pipetting)

Be careful to avoid bubbles during pipetting because the RNAClean XP is highly-viscouse reagent. To mix well, which is essential, suck the mixture from the bottom of the well and push it out to the upper area of the mixture.

Use the same way to pipet for RNAClean XP and AMPure XP in all steps below.

Table 7 : RNAClean XP mixture after 1st strand cDNA synthesis

Reagent	1 sample	Equipment
☐ sample plate ①	☐ 38.0 µl	
☐ RNAClean XP	☐ 68.4 µl	P200
Total volume	106.4 µl	

2.☐ Incubate for 30 min at room temperature.
3.☐ After incubation, set the sample plate ① at the magnetic bar and stand for 5min.
4.☐ Prepare 70% ethanol for the washing step referring Table 8.

Note: Prepare 70% ethanol between steps 2 and 3. Prepare excess amount in case of reservoir is used with multi-channel pipette. (Approximately 5,000 µl for 8 samples)

Table 8 : 70% Ethanol for wash

Reagent	1 sample	Equipment	8 samples (use reservoir)	Equipment
☐ H_2O (R1)	☐ 150 µl	P200	☐ 1500 µl	P1000
☐ EtOH	☐ 350 µl	P1000	☐ 3500 µl	P1000
Total volume	500 µl		5000 µl	

5.☐ Remove the supernatant by pipetting. (P200 / 8ch-P300)[Refer to Fig.1]

Note: When removing the supernatant leave small amount of the supernatant (approximately 5 µl), which can be relatively avoid beads intake.

Important: (Treatment of supernatant)

RNAClean XP beads make a pellet on the side of the magnetic bar. Insert the edge of the pipette tip in the solution not touching the beads and slowly pipet the supernatant keeping the edge of the pipette tip at the surface area of the supernatant and moving towards the bottom. [Refer to Fig. 1] Be careful not to pipet the beads.

Use the same way to treat the supernatant for the operations using RNAClean XP and AMPure XP in all steps below.

6.☐ Add 200 µl of 70% ethanol into each well. (P200 / 8ch-P300)

Note: Carry out the ethanol wash setting the sample plate on the magnetic bar.

Note: Pipetting is not required after adding ethanol.

Note: It makes the operation easier to dispense 70% ethanol into clean reservoir in advance when multi-channel pipette is used.

7.☐ Remove the supernatant in the same way as the step 5. (P200 / 8ch-P300)

Note: Remove ethanol as much as possible.

8.☐ Repeat the step 6 and 7. (wash twice total)

9.☐ Remove the sample plate ① from the magnetic bar. Add 42 µl/sample of 37°C-H_2O and suspend the beads by pipetting 60 times. (P200 / 8ch-P300)

10.☐ Set the sample plate ① in the thermal cycler and incubate for 5 min at 37℃.

11.☐ Centrifuge the sample plate ① at 1,000 ×g for 1 min to collect the scattered beads to the bottom.

12.☐ Set the sample plate ① at the magnetic bar and stand for 5 min.

13.☐ Collect the supernatant with a pipette and transfer it to the sample plate ②. (P200 / 8ch-P300)

14.☐ To remove the mixed ethanol in the eluted sample in the washing process, set the sample plate② in the thermal cycler without the cap of the plate and incubate for 10 min at 37℃. [Refer to Fig.2]

Note: Place the lid of tip case that is wiped with Zap on the plate to avoid contamination with dust.

15.☐ Close the cap of the sample plate ②

16.☐ Spindown the plate in the tabletop centrifuge to collect the solution to the bottom.

17.☐ Place the sample on ice or at -80℃ until proceeding to the step 1 <c>.

18.□

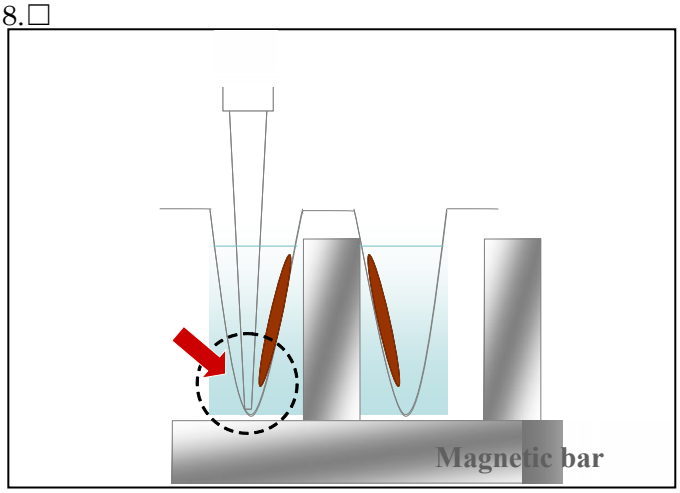


Fig.1: Method to remove the supernatant using magnetic bar

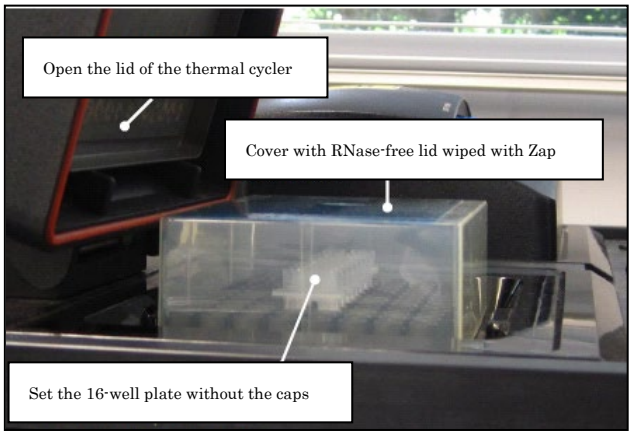


Fig.2: Setup for removing ethanol from the solution

Day2

◆Step 1<c> Oxidation and Biotinylation (Oxidation：3hrs、Biotinylation：4hrs)

Purpose: mRNA contains two diol groups in the cap structure at the 5'-end and ribose at the 3'-end. Oxidize with sodium periodate ($NaIO_4$) and bind Biotin (long arm) hydrazide to it.

Important: Sodium periodate ($NaIO_4$) and biotin (long arm) hydrazide are sensitive to light. Protect them from light by wrapping with aluminum foil and by closing the lid of the thermal cycler during reaction.

Table 9：Required reagents, consumables and equipment for Oxidation and Biotinylation

Reagents included in the kit (tube number)	Consumable	Equipment
☐ 1 M NaOAc(pH 4.5) (R4)		
☐ 250 mM $NaIO_4$ (R5)		☐ P2
☐ 40% glycerol (Op1)		☐ P20
☐ 1 M Tris-HCl(pH 8.5) (R6)	☐ 10 µl filter tip	☐ P200
☐ 1 M NaOAc(pH 6.0) (R7)	☐ 20 µl filter tip	☐ 8ch-P10
☐ 10 mM Biotin Hydrazaide (R8)	☐ 200 µl filter tip	☐ 8ch-P100
☐ RNAClean XP	☐ 16-well plate	☐ 8ch-P300
Reagents not included in the kit	☐ 8-strip tube	☐ Ice bucket
☐ 70% Ethanol	☐ 1.5 ml tube	☐ Thermal cycler
☐ 37℃- H_2O		☐ Magnetic bar
☐ Isopropanol		☐ Aluminum foil

Preparation

・Dispense sufficient amount of the following reagents into the 8-strip tube for multi-channel pipette use.
□1 M NaOAc(pH 4.5) (R4)
□250 mM $NaIO_4$ (R5)
Important: Store by wrapping with aluminum foil to keep away light.
□1 M Tris-HCl(pH 8.5) (R6)
(Reagent above can be stored at room temperature after dispensing into the tubes.)
□40% glycerol (Op1)
(Reagent above must be stored at -20℃ after dispensing into the tubes.)

<Oxidization of diol residues>

1.☐ Dissolve the sample plate ② on ice if it is stored in a frozen state. If needed, spindown in the plate centrifuge.

Note: After this step, the addition of reagents to the sample must be done on ice all the time.

2.☐ Add 2 µl/sample of 1 M NaOAc (pH 4.5; R4) to the sample plate ②[Table 10]. Set the scale of the pipette at 40 and mix well by pipetting 10 times. (P200 / 8ch-P300)

Important: This chemical reaction is sensitive to pH. It's important to keep the appropriate pH strictly.

3.☐ Add 2 µl/sample of 250 mM $NaIO_4$ (R5) to the sample plate ②[Table 10]. Set the scale of the pipette at 40 and mix well by pipetting 10 times. (P200 / 8ch-P300)

Table 10 : Oxidation reaction mixture

Reagent	1 sample	Equipment
☐ sample plate ②	☐ 40.0 µl	
☐ 1M NaOAc (pH 4.5) (R4)	☐ 2.0 µl	P2 or8ch-P10
☐ 250 mM $NaIO_4$ (R5)	☐ 2.0 µl	P2 or 8ch-P10
Total volume	44.0 µl	

4.☐ Spindown in the plate centrifuge to collect the solution to the bottom.
5.☐ Incubate for 45 min on ice with a light-blocking.
Important: This reaction must be done under the light-blocking condition by wrapping with aluminum foil.
Important: Strictly keep the reaction time to avoid overreaction.
6.☐ After 45min incubation, add 2 µl/sample of 40% glycerol into the sample plate ②. Set the scale of the pipette at 40 and mix well by pipetting 10 times. (P200 / 8ch-P300) [Table 11]
Note: Slowly pipet 40% glycerol because it is high-viscosity reagent.
7.☐ Add 14 µl/sample of 1 M Tris-HCl(pH 8.5) into the sample plate ②. Set the scale of the pipette at 40 and mix well by pipetting 10 times. (P200 / 8ch-P300) [Table 11]

Table 11 : Reaction mixture after Oxidation

Reagent	1 sample	Equipment
☐ sample plate ② after Oxidation	☐ 44.0 µl	
☐ 40% glycerol (Op1)	☐ 2.0 µl	P2 or 8ch-P10
☐ 1 M Tris-HCl (pH 8.5) (R6)	☐ 14.0 µl	P20 or 8ch-P100
Total volume	60.0 µl	

<RNAClean XP purification>

Important: Refer to [Important (pipetting) in the Step 1<b>] for pipetting.

Important: Refer to [Important (Treatment of supernatant) in the Step 1<b>] to treat the supernatant.

Preparation

・Incubate RNAClean XP for 30 min at room temperature. Mix well by inverting the tube, tapping or vortex etc. just before use it.
・Set the thermal cycler to 37℃.
・【Preparation of 37℃-H_2O】Dispense 200 µl of H_2O into 8-strip tube and incubate at 37℃ in the thermal cycler.
・Prepare a 16-well plate（sample plate③）and label with the sample name or number.

1.☐ Add 108 µl/sample of RNAClean XP to the sample plate ②. Set the scale of the pipette at 148 and mix well by pipetting 10 times. (P200 / 8ch-P300) [Table 12]

Table 12 : RNAClean XP mixture after Oxidation

Reagent	1 sample	Equipment
☐ sample plate ②	☐ 60.0 µl	
☐ RNAClean XP	☐ 108.0 µl	P200
Total volume	168.0 µl	

2.☐ Incubate for 30 min at room temperature.

3.☐ After incubation, set the sample plate ② at the magnetic bar and stand for 5min.

4.☐ Prepare the required amount of 70% ethanol for washing step referring Table 13.

Note: Prepare 70% ethanol between steps 2 and 3. Prepare excess amount in case reservoir is used with multi-channel pipette. (Approximately 5,000 µl for 8 samples)

Table 13 : 70% Ethanol for wash

Reagent	1 sample	Equipment	8 samples (use reservoir)	Equipment
☐ H_2O (R1)	☐ 150 µl	P200	☐ 1500 µl	P1000
☐ EtOH	☐ 350 µl	P1000	☐ 3500 µl	P1000
Total volume	500 µl		5000 µl	

5.☐ Remove the supernatant by pipetting. (P200 / 8ch-P300)[Refer to Fig.1]

Note: When removing the supernatant leave small amount of the supernatant (approximately 5 µl), which can be relatively avoid beads intake.

6.☐ Add 200 µl of 70% ethanol into each well. (P200 / 8ch-P300)

Note: Carry out ethanol wash setting the sample plate on the magnetic bar.

Note: Pipetting is not required after adding ethanol.

Note: It makes the operation easier to dispense 70% ethanol into clean reservoir in advance when multi-channel pipette is used.

7.☐ Remove the supernatant in the same way as the step 5. (P200 / 8ch-P300)

Note: Remove ethanol as much as possible.

8.☐ Repeat the step 6 and 7. (wash twice total)

9.☐ Remove the sample plate ② from the magnetic bar. Add 42 µl/sample of 37℃-H_2O and suspend the beads by pipetting 60 times. (P200 / 8ch-P300)

10.☐ Set the sample plate ② in the thermal cycler and incubate for 5 min at 37℃.

11.☐ Centrifuge the sample plate ② at 1,000 ×g for 1 min to collect the scattered beads to the bottom.

12.☐ Set the sample plate ② at the magnetic bar and stand for 5 min.

13.☐ Collect the supernatant by pipetting and transfer it to the sample plate ③. (P200 / 8ch-P300)

14.☐ To remove ethanol from the sample, which is mixed in the washing process, set the sample plate③ in the thermal cycler without the cap of the plate and incubate for 10 min at 37℃. [Refer to Fig.2]

Note: Place the lid of tip case that is wiped with Zap on the plate to avoid contamination with dust.

15.☐ Close the cap of the sample plate ③.

16.☐ Spindown the plate in the tabletop centrifuge to collect the solution to the bottom.

17.☐ Store the sample on ice until proceeding to the next Biotinylation reaction step.

Note: Continuously proceeding to Biotinylation reaction after oxidation reaction is preferable for the sample. However, the sample can be stored for 3 days at -80℃.

<Biotinylation of Oxidized diol residues>

Preparation

・Dissolve 10 mM Biotin Hydrazaide (R8) (stored at -20℃) at room temperature under light-blocking condition.

・Set the thermal cycler to 23℃.

・Dispense the following reagents into each well of 8-strip tube for multi-channel pipette use.

□1 M NaOAc(pH 6.0) (R7)
□Isopropanol
(Reagents above can be stored at room temperature after dispensing)
□10 mM Biotin Hydrazaide (R8)
Important: Store by wrapping with aluminum foil to keep away light.
(Reagent above must be stored at -20℃)

1.☐ Add 4 µl/sample of 1 M NaOAc(pH 6.0) to the sample plate ③. Set the scale of the pipette at 40 and mix well by pipetting 10 times. (P200 / 8ch-P300) [Table 14]
2.☐ Add 4 µl/sample of 10 mM Biotin Hydrazaide (R8) to the sample plate ③. Set the scale of the pipette at 40 and mix well by pipetting 10 times. (P200 / 8ch-P300) [Table 14]

Table 14 : Biotinylation reaction mixture

Reagent	1 sample	Equipment
☐ sample plate ③	☐ 40.0 µl	
☐ 1 M NaOAc(pH 6.0) (R7)	☐ 4.0 µl	P20 or 8ch-P10
☐ 10 mM Biotin Hydrazaide (R8)	☐ 4.0 µl	P20 or 8ch-P10
Total volume	48.0 µl	

3.☐ Spindown in the plate centrifuge to collect the solution to the bottom.
4.☐ Set the plate in the thermal cycler. Incubate for 2 hrs at 23℃ under light-blocking condition. [Table 15 : 23℃ program]

Table 15 : 23℃ program

Temperature	Time
23℃	2 hrs

<RNAClean XP purification>

Important: Refer to [Important (pipetting) in the Step 1<b>] for pipetting.

Important: Refer to [Important (Treatment of supernatant) in the Step 1<b>] to treat the supernatant.

Preparation

・Incubate RNAClean XP for 30 min at room temperature. Mix well by inverting the tube, tapping or vortex etc. just before use it.
・Set the thermal cycler to 37℃.
・【Preparation of 37℃-H_2O】Dispense 200 µl of H_2O into 8-strip tube and incubate at 37℃ in the thermal cycler.
・Prepare a 16-well plate（sample plate④） and label with the sample name or number.

1.☐ Add 108 µl/sample of RNAClean XP to the sample plate ③. Set the scale of the pipette at 136 and mix well by pipetting 10 times. (P200 / 8ch-P300) [Table 16]
2.☐ Add 12 µl/sample of isopropanol to the sample plate ③. Set the scale of the pipette at 148 and mix well by pipetting 10 times. (P200 / 8ch-P300) [Table 16]

Table 16 : RNAclean mixture after Biotinylation

Reagent	1 sample	Equipment
☐ sample plate ③	☐ 48.0 µl	
☐ RNAClean XP	☐ 108.0 µl	P200
☐ Isopropanol	☐ 12.0 µl	P20 or 8ch-P100
Total volume	168.0 µl	

3.☐ Incubate for 30 min at room temperature.
4.☐ After incubation, set the sample plate ③ at the magnetic bar and stand for 5min.
5.☐ Prepare required amount of 70% ethanol for washing step referring Table 17.
Note: Prepare 70% ethanol between steps 3 and 4. Prepare excess amount in case a reservoir is used with multi-channel pipette. (Approximately 5,000 µl for 8 samples)

Table 17 : 70% Ethanol for wash

Reagent	1 sample	Equipment	8 samples (use reservoir)	Equipment
☐ H_2O (R1)	☐ 150 µl	P200	☐ 1500 µl	P1000
☐ EtOH	☐ 350 µl	P1000	☐ 3500 µl	P1000
Total volume	500 µl		5000 µl	

6.☐ Remove the supernatant by pipetting. (P200 / 8ch-P300)[Refer to Fig.1]
Note: When removing the supernatant leave small amount of the supernatant (approximately 5 µl), which can be relatively avoid beads intake.
7.☐ Add 200 µl of 70% ethanol into each well. (P200 / 8ch-P300)
Note: Carry out ethanol wash setting the sample plate on the magnetic bar.
Note: Pipetting is not required after adding ethanol.
Note: It makes the operation easier to dispense 70% ethanol into clean reservoir in advance when multi-channel pipette is used.
8.☐ Remove the supernatant in the same way as the step 6. (P200 / 8ch-P300)
Note: Remove ethanol as much as possible.
9.☐ Repeat the step 7 and 8. (wash twice total)
10.☐ Remove the sample plate ③ from the magnetic bar. Add 42 µl/sample of 37°C-H2O and suspend the beads by pipetting 60 times. (P200 / 8ch-P300)
11.☐ Set the sample plate ③ in the thermal cycler and incubate for 5 min at 37℃.
12.☐ Centrifuge the sample plate ③ at 1,000 ×g for 1 min to collect the scattered beads to the bottom.
13.☐ Set the sample plate ③ at the magnetic bar and stand for 5 min.
14.☐ Collect the supernatant by pipetting and transfer it to the sample plate ④. (P200 / 8ch-P300)
15.☐ To remove ethanol from the sample, which is mixed in the washing process, set the sample plate ④ in the thermal cycler without the cap of the plate and incubate for 10 min at 37℃. [Refer to Fig.2]
Note: Place the lid of tip case that is wiped with Zap on the plate to avoid contamination with dust.
16.☐ Close the cap of the sample plate ④.
17.☐ Spindown the plate in the tabletop centrifuge to collect the solution to the bottom.
18.☐ Store the sample at -80℃ until proceeding to the step 1 <d>.

Day3

◆Step 1<d> Preparation of Streptavidin beads (1hr)

Purpose: To avoid unspecific biotin-streptavidin binding, coat Streptavidin beads beads with tRNA.

Important: Refer to [Important (pipetting) in the Step 1<b>] for pipetting.
Important: Refer to [Important (Treatment of supernatant) in the Step 1<b>] to treat the supernatant.

Table 18 : Required reagents, consumables and equipment for preparation of Streptavidin beads

Reagents included in the kit (tube number)	Consumable	Equipment
☐ 10 µg/µl tRNA (R9)	☐ 10 µl filter tip	☐ P2
☐ Wash Buffer 1 (R11)	☐ 20 µl filter tip	☐ P20
Reagents not included in the kit	☐ 200 µl filter tip	☐ P200
☐ Streptavidin beads	☐ 1000µl filter tip	☐ P1000
	☐ 8-strip tube	☐ Ice bucket
	☐ 2.0 ml tube	☐ Magnetic stand

<Preparation of tRNA-Streptavidin beads>

1.☐ Prior to starting the Step 1<e> RNase I digestion, coat Streptavidin beads with tRNA according to step2.-15. below

Note: For multiple samples, prepare reagent mix all together in a 2.0 ml tube.

2.☐ Streptavidin beads are precipitated. Mix well to diffuse them by inverting the tube.
3.☐ Dispense 50 µl/sample of Streptavidin beads into a 2.0 ml tube.
4.☐ Add 0.625 µl/sample of 10 µg/µl tRNA and mix by tapping the tube [Table 19].
Note: In the following, this mixture will be described as 'tRNA-Streptavidin beads' .

Table 19 : tRNA-Streptavidin beads mixture

Reagent	1 sample	Equipment	8 samples (Prepare for 9)	Equipment
☐ Streptavidin beads	☐ 30µl	P200	☐ 450 µl	P1000
☐ 10 µg/µl tRNA (R9)	☐ 0.625 µl	P2	☐ 5.63 µl	P20
Total volume	50.625 µl		455.63 µl	

5.☐ Incubate for 30 min on ice (tap the tube every 5 min to diffuse the tRNA-Streptavidin beads).
6.☐ After the incubation at the step 5, centrifuge for 5 sec in the tabletop centrifuge to collect the solution and the tRNA-Streptavidin beads to the bottom of the tube.
7.☐ Set the tube from the step 6 at the magnetic stand and stand for 3min to allow tRNA-Streptavidin beads to adhere to the wall of the tube.
8.☐ Remove the supernatant by pipetting. (P200 / P1000)
9.☐ Add 50 µl/sample (50 µl x 9 = 450µl for 8 samples) of the washing buffer 1 and diffuse the tRNA-Streptavidin beads by tapping the tube. (P200 / P1000)
10.☐ Centrifuge the tube to collect the solution and the tRNA-Streptavidin beads to the bottom in the tabletop centrifuge.
11.☐ Set the tube at the magnetic stand and stand for 3min to allow tRNA-Streptavidin beads to adhere to the wall of the tube.
12.☐ Remove the supernatant by pipetting. (P200 / P1000)
13.☐ Repeat the steps 9-12. (Wash with the wash buffer 1 twice)
14.☐ Add 105 µl/sample of Wash Buffer 1 and 0.625 µl/sample of 10 µg/µl tRNA and diffuse the

tRNA-Streptavidin beads **by pipetting 10 times** [Table 20]. (P200 / P1000)

Table 20 : tRNA-Streptavidin beads mixture

Reagent	1 sample	Equipment	8 samples (Prepare for 9)	Equipment
☐ Wash Buffer 1(R11)	☐ 105 µl	P200	☐ 945 µl	P1000
☐ 10 µg/µl tRNA (R9)	☐ 0.625 µl	P2	☐ 5.63 µl	P20
Total volume	105.625 µl		950.63 µl	

15.☐ Dispense 110 µl of the mixture into 8-strip tubes for preparation of 8 samples.
16.☐ Place on ice until just before use.
Important: Use the samples within 1 hr after preparation because Streptavidin on the tRNA-Streptavidin beads is unstable in high salt concentration.

◆Step 1<e> RNase I　digestion (1hr)

Purpose: Digest single strand RNA using RNase I . This step allows to select cDNA that is elongated to the 5'-end of Capped RNA by Cap-Trapping method.

Table 21 : Required reagents, consumables and equipment for RNase I　digestion

Reagents not included in the kit	Consumable	Equipment
☐　RNase I (10 U/μl; E1)	☐　10 μl filter tip ☐　20 μl filter tip ☐　200 μl filter tip ☐　8-strip tube ☐　1.5 ml tube	☐　P2 ☐　P20 ☐　P200 ☐　8ch-P10 ☐　8ch-P300 ☐　Ice bucket ☐　Thermal cycler
☐　10× RNase I buffer (R10)		

Preparation

・Dissolve the sample plate ④ on ice. If needed, spindown in the plate centrifuge.
・Dissolve 10× RNase I buffer on ice.
・Set the thermal cycler to 37℃.

<RNase I　digestion>

1.☐ Prepare the RNase I　pre-mixture according to the Table 22. Place it on ice until just before use.
Note: For multi-channel pipette use, prepare required amount of RNase I pre-mixture in a 1.5 ml tube and dispense into 8-strip tubes.

Table 22 : RNase I　pre-mixture

Reagent	1 sample	Equipment	8 samples (Prepare for 9)	Equipment
☐　10× RNase I　buffer (R10)	☐　4.5 μl	P20	☐　40.5 μl	P200
☐　RNase I (10 U/μl; E1)	☐　0.5 μl	P2	☐　4.5 μl	P20
Total volume	5.0 μl		45.0 μl	

2.☐ Add 5 μl/sample of RNase I pre-mixture prepared in the step 1 to the sample plate ④. Set the scale of the pipette at 40 and mix well by pipetting 10 times. (P200 / 8ch-P300) [Table 23]

Table 23 : RNase I reaction mixture

Reagent	1 sample	Equipment
☐　sample plate ④	☐　40 μl	
☐　RNase I pre-mixture(Table 22)	☐　5 μl	P20 or 8ch-P10
Total volume	45.0 μl	

3.☐ Spindown in the plate centrifuge to collect the solution to the bottom.
4.☐ Set the sample plate ④ in the thermal cycler that is set to 37℃ and incubate for 30 min. [Table 24 : 37C program]

Table 24 : 37C program

Temperature	Time
37 ℃	30 min

5.☐ Spindown in the plate centrifuge to collect the solution to the bottom.
6.☐ Place on ice until proceeding to the Step 1<f>.
Note: Continuously proceeding to Cap-trapping after RNase I reaction is preferable for the sample. However, the sample can be stored for one night at -80℃.

◆Step 1<f> Cap-trapping and Releasing cDNA (2hr 30min)

Purpose: Bind biotinylated Capped RNA to the Streptavidin beads after RNase I reaction and remove unbind substances by washing. Break RNA with RNase I and RNaseH and select cDNA that derive from Capped RNA.

Important: Refer to [Important (pipetting) in the Step 1<b>] for pipetting.
Important: Refer to [Important (Treatment of supernatant) in the Step 1<b>] to treat the supernatant.

<Cap-trapping>

Table 25 : Required reagents, consumables and equipment for Cap-trapping

Reagents included in the kit (tube number)	Consumable	Equipment
☐ Wash Buffer 1 (R11)	☐ 200 µl filter tip ☐ 8-strip tube	☐ P200 ☐ 8ch-P300 ☐ Magnetic bar ☐ Thermal cycler
☐ Wash Buffer 2 (R12)		
☐ Wash Buffer 3 (R13)		
Reagents not included in the kit		
☐ tRNA-Streptavidin beads Prepared in the Step 1<d>		

Preparation

・Set the thermal cycler to 37℃.
・Place the Wash Buffer 1, 2, 3 at room temperature until they become the room temperature. Dispense required amount of the following reagents into each well for multi-channel pipette use.

1.☐ Add 105 µl/sample of tRNA-Streptavidin beads to the sample plate ④ after RNase I treatment. Set the scale of the pipette at 130 and mix well by pipetting 10 times. (P200 / 8ch-P300) [Table 26]

Note: The tRNA-Streptavidin beads tend to be precipitated. Add the tRNA-Streptavidin beads to the sample just after diffusing them by pipetting.

Table 26 : tRNA-Streptavidin beads mixture for Cap-trapping

Reagent	1 sample	Equipment
☐ sample plate ④ after RNase I digestion	☐ 45 µl	
☐ tRNA-Streptavidin beads	☐ 105 µl	P200 or 8ch-P300
Total volume	150 µl	

2.☐ Set the plate without the cap in the thermal cycler that is set to 37℃ and incubate for 30 min at 37℃. [Refer to the Step 1<b> Fig.2]

Note: Set the scale of the pipette at 130 and pipet 10 times every 5min to diffuse tRNA-Streptavidin beads. (P200 / 8ch-P300)

3.☐ After incubation, set the sample plate ④ at the magnetic bar and stand for 5min.

4.☐ Remove the supernatant by pipetting. (P200 / 8ch-P300)

5.☐ Remove the sample plate ④ from the magnetic bar. Add 150 µl/sample of the Wash Buffer 1 and diffuse the tRNA-Streptavidin beads by pipetting 10 times. (P200 / 8ch-P300)

Note: The Wash Buffer contains Tween20. Avoid bubbles during pipetting.

6.☐ Set the sample plate ④ at the magnetic bar and stand for 3min.

7.☐ Remove the supernatant by pipetting. (P200 / 8ch-P300)(wash with the washing buffer 1 once)

(option) Repeat the steps 5-7. (Wash with the Wash Buffer 1 twice)

8.☐ Remove the sample plate ④ from the magnetic bar. Add 150 µl/sample of the Wash Buffer 2 and diffuse the tRNA-Streptavidin beads by pipetting 10 times. (P200 / 8ch-P300)

9.☐ Set the sample plate ④ at the magnetic bar and stand for 3min.

10.☐ Remove the supernatant by pipetting. (P200 / 8ch-P300)(Wash with the washing buffer 2 once)

11.☐ Remove the sample plate ④ from the magnetic bar. Add 150 µl/sample of the Wash Buffer 3 and diffuse the tRNA-Streptavidin beads by pipetting 20 times. (P200 / 8ch-P300)

12.☐ Set the sample plate ④ at the magnetic bar and stand for 3min.

13.☐ Remove the supernatant by pipetting. (P200 / 8ch-P300)

<Releasing captured cDNA>

Table 27 : Required reagents, consumables and equipment for releasing cDNA and purification

	Consumable	Equipment
☐ Releasing Buffer (R14)	☐ 10 µl filter tip	☐ P2
☐ 10× RNase I buffer (R10)	☐ 20 µl filter tip	☐ P20
☐ H2O (R1)	☐ 200 µl filter tip	☐ P200
☐ 37℃- H_2O	☐ 1000 µl filter tip	☐ P1000
☐ RNase I (E1)	☐ 16-well plate	☐ 8ch-P10
☐ RNaseH (E2)	☐ 8-strip tube	☐ 8ch-P300
Reagents not included in the kit	☐ 1.5 ml tube	☐ Ice bucket
☐ AMPure XP		☐ Magnetic bar

Preparation

・Dissolve the Releasing Buffer on ice.
・Dissolve the 10× RNase I buffer on ice.
・Set the thermal cycler to 95℃.
・Set the thermal cycler to 37℃.

1.☐ Add 35 µl/sample of the Releasing Buffer to the sample plate ④(beads only) and diffuse the tRNA-Streptavidin beads by pipetting 10 times. (P200 / 8ch-P300)
2.☐ Set the sample plate ④ in the thermal cycler that is set to 95℃ and incubate for 5 min. [Table 28 : 95℃ program]

Note: After incubation at 95℃, the tubes and caps become very hot. Remove them from the thermal cycler when it comes to around 60℃ to avoid opening the caps and getting burn.

Table 28 : 95C program

Temperature	Time
95 ℃	5 min
4 ℃	Forever

3.☐ After incubation, transfer the plate to ice to rapidly cool it.
4.☐ Spindown in the plate centrifuge to collect the solution to the bottom on the plate.
5.☐ Set the sample plate ④ at the magnetic bar and stand for 3 min.
6.☐ Transfer the supernatant to the sample plate ⑤ by pipetting. (P200 / 8ch-P300)
7.☐ Remove the sample plate ④ from the magnetic bar.
8.☐ Add 30 µl/sample of the 1× RNase I buffer to the sample plate ④ and diffuse the tRNA-Streptavidin beads by pipetting 10 times. (P200 / 8ch-P300)
9.☐ Set the sample plate ④ at the magnetic bar and stand for 3 min.
10.☐ Transfer the supernatant to the sample plate ⑤ by pipetting. (P200 / 8ch-P300)
Note: Total volume will be 65 µl. It can be placed on ice about for 1 hr.
11.☐ Prepare the RNase mix according to the Table 29.

Table 29 : RNase mix

Reagent	1 sample	Equipment	8 samples (Prepare for 9)	Equipment
☐ H_2O(R1)	☐ 2.4 µl	P20	☐ 21.6 µl	P200
☐ 10×RNase I Buffer	☐ 0.5 µl	P2	☐ 4.5 µl	P20
☐ RNaseH(E2)	☐ 0.1 µl	P2	☐ 0.9 µl	P200
☐ RNase I(E1)	☐ 2.0 µl	P2	☐ 18.0 µl	P200
Total volume	5.0 µl		45.0 µl	

12.☐ Add 5 µl/sample of RNase mix prepared with Table 30 to the sample plate ⑤. Set the scale of the pipette at 50 and mix well by pipetting 10 times. (P200 / 8ch-P300) [Table 30]

Table 30 : RNase reaction mixture

Reagent	1 sample	Equipment
☐ sample plate ⑤	☐ 65 µl	
☐ RNase mix	☐ 5 µl	P20
Total volume	70 µl	

13.☐ Spindown in the plate centrifuge to collect the solution to the bottom of the plate.
14.☐ Set the sample plate ⑤ in the thermal cycler that is set to 37℃ and incubate for 15 min. [Table 31 : 37C program]

Table 31 : 37C program

Temperature	Time
37 ℃	15 min

15.☐ Spindown in the plate centrifuge to collect the solution to the bottom of the plate.
16.☐ Place on ice until proceeding to the next step.

<AMPure XP purification>

Important: Refer to [Important (pipetting) in the Step 1<b>] for pipetting.

Important: Refer to [Important (Treatment of supernatant) in the Step 1<b>] to treat the supernatant.

Preparation

・Incubate AMPure XP for 30 min at room temperature. Mix well by inverting the tube, tapping or vortex etc. just before use it.
・Set the thermal cycler to 37℃.
・【Preparation of 37℃-H_2O】Dispense 200 µl of H_2O into 8-strip tube and incubate at 37℃ in the thermal cycler.
・Prepare a 16-well plate（sample plate⑥）and label with the sample name or number.

1.☐ Add 126 µl/sample of AMPure XP to the sample plate ⑤. Set the scale of the pipette at 176 and mix well by pipetting 10 times. (P200 / 8ch-P300) [Table 32]

Table 32：AMPure XP mixture after release

Reagent	1 sample	Equipment
☐ sample plate ⑤	☐ 70 µl	
☐ AMPure XP	☐ 126 µl	P200
Total volume	196 µl	

2.☐ Incubate sample plate ⑤ for 30 min at room temperature.
3.☐ Set the sample plate ⑤ at the magnetic bar and stand for 5min.
4.☐ Prepare required amount of 70% ethanol for washing step referring Table 33.
Note: Prepare 70% ethanol between steps 2 and 3. Prepare excess amount in case of reservoir is used with multi-channel pipette. (Approximately 5,000 µl for 8 samples)

Table 33：70% Ethanol for wash

Reagent	1 sample	Equipment	8 samples (use reservoir)	Equipment
☐ H_2O (R1)	☐ 150 µl	P200	☐ 1500 µl	P1000
☐ EtOH	☐ 350 µl	P1000	☐ 3500 µl	P1000
Total volume	500 µl		5000 µl	

5.☐ Remove the supernatant by pipetting. (P200 / 8ch-P300)
Note: When removing the supernatant leave small amount of the supernatant (approximately 5 µl), which can be relatively avoid beads intake.
6.☐ Add 200 µl of 70% ethanol into each well to wash. (P200 / 8ch-P300)
Note: Carry out ethanol wash setting the sample plate on the magnetic bar.
Note: Pipetting is not required after adding ethanol.
Note: It makes the operation easier to dispense 70% ethanol into clean reservoir in advance when multi-channel pipette is used.
7.☐ Remove the supernatant in the same way as the step 5. (P200 / 8ch-P300)
Note: Remove ethanol as much as possible.
8.☐ Repeat the step 6 and 7. (wash twice total)
9.☐ Remove the sample plate ⑤ from the magnetic bar. Add 42 µl/sample of 37°C-H2O and suspend the beads by pipetting 60 times. (P200 / 8ch-P300)
10.☐ Set the sample plate ⑤ in the thermal cycler and incubate for 5 min at 37℃.

11.☐ Centrifuge the sample plate ⑤ at 1,000 ×g for 1 min to collect the scattered beads to the bottom.
12.☐ Set the sample plate ⑤ at the magnetic bar and stand for 5 min.
13.☐ Collect the supernatant by pipetting and transfer it to the sample plate ⑥. (P200 / 8ch-P300)
14.☐ To remove ethanol from the sample, which is mixed in the washing process, set the sample plate ⑥ in the thermal cycler without the cap of the plate and incubate for 10 min at 37℃. [Refer to Fig.2]
Note: Place the lid of tip case that is wiped with Zap on the plate to avoid contamination with dust.
15.☐ Close the cap of the sample plate ⑥.
16.☐ Spindown the plate in the tabletop centrifuge to collect the solution to the bottom.
17.☐ Store the sample at -20℃ until proceeding to the step 1 <g>.

Day4

◆Step 1<g> RNase I (2hr30min)

Purpose: Digest remained RNA with RNase I.

Table 34 : Required reagents, consumables and equipment for RNase I digestion and purification

Reagents not included in the kit	Consumable	Equipment
☐ RNase I(10 U/µl; E1)	☐ 10 µl filter tip ☐ 20 µl filter tip ☐ 200 µl filter tip ☐ 16-well plate ☐ 8-strip tube ☐ 1.5 ml tube	☐ P2 ☐ P20 ☐ P200 ☐ 8ch-P10 ☐ 8ch-P300 ☐ Ice bucket ☐ Thermal cycler ☐ Magnetic bar
☐ 10× RNase I buffer (R10)		
☐ AMPure XP		
☐ 37℃- H_2O		

Preparation

・Dissolve the sample plate ⑥ on ice. If needed, spindown in the plate centrifuge.
・Dissolve 10× RNase I buffer on ice.
・Set the thermal cycler to 37℃.

<RNase I digestion>

1.☐ Prepare the RNase I pre-mixture and place it on ice until just before use. [Table 35]
Note: For multi-channel pipette use, prepare required amount of the buffer in a 1.5 ml tube and dispense into 8-strip tubes.

Table 35 : RNase I pre-mixture

Reagent	1 sample	Equipment	8 samples (Prepare for 9)	Equipment
☐ 10× RNase I buffer (R10)	☐ 4.5 µl	P20	☐ 40.5 µl	P200
☐ RNase I(10 U/µl; E1)	☐ 0.5 µl	P2	☐ 4.5 µl	P20
Total volume	5.0 µl		45.0 µl	

2.☐ Add 5 µl/sample of RNase I pre-mixture prepared in the step 1 to the sample plate ⑥. Set the scale of the pipette at 40 and mix well by pipetting 10 times. (P200 / 8ch-P300) [Table 36]

Table 36 : RNase I reaction mixture

Reagent	1 sample	Equipment
☐ sample plate ⑥	☐ 40 µl	
☐ RNase I pre-mixture(Table 36)	☐ 5 µl	P20 or 8ch-P10
Total volume	45.0 µl	

3.☐ Spindown in the plate centrifuge to collect the solution to the bottom of the plate.
4.☐ Set the sample plate ⑥ in the thermal cycler that is set to 37℃ and incubate for 30 min. [Table 37]

Table 37 : 37C program

Temperature	Time
37 ℃	30 min

<AMPure XP purification>

Important: Refer to [Important (pipetting) in the Step 1<b>] for pipetting.

Important: Refer to [Important (Treatment of supernatant) in the Step 1<b>] to treat the supernatant.

Preparation

・Incubate AMPure XP for 30 min at room temperature. Mix well by inverting the tube, tapping or vortex etc. just before use it.
・Set the thermal cycler to 37℃.
・【Preparation of 37℃-H_2O】Dispense 200 µl of H_2O into 8-strip tube and incubate at 37℃ in the thermal cycler.
・Prepare a 16-well plate（sample plate⑦）and label with the sample name or number.
・Prepare 8-strip tube for cDNA Q.C. Prepare 2 tubes/sample for Oligreen assay and qPCR assay.

1.☐ Add 81 µl/sample of AMPure XP to the sample plate ⑥. Set the scale of the pipette at 106 and mix well by pipetting 10 times. (P200 / 8ch-P300) [Table 38]

Table 38 : AMPure XP mixture after release

Reagent	1 sample	Equipment
☐ sample plate ⑥	☐ 45 µl	
☐ AMPure XP	☐ 81 µl	P200
Total volume	126 µl	

2.☐ Incubate the sample plate ⑥ for 30 min at room temperature.
3.☐ Set the sample plate ⑥ at the magnetic bar and stand for 5min.
4.☐ Prepare required amount of 70% ethanol for washing step referring Table 39.
Note: Prepare 70% ethanol between steps 2 and 3. Prepare excess amount in case of reservoir is used with multi-channel pipette. (Approximately 5,000 µl for 8 samples)

Table 39 : 70% Ethanol for wash

Reagent	1 sample	Equipment	8 samples (use reservoir)	Equipment
☐ H_2O (R1)	☐ 150 µl	P200	☐ 1500 µl	P1000
☐ EtOH	☐ 350 µl	P1000	☐ 3500 µl	P1000
Total volume	500 µl		5000 µl	

5.☐ Remove the supernatant by pipetting. (P200 / 8ch-P300)
Note: When removing the supernatant leave small amount of the supernatant (approximately 5 µl), which can be relatively avoid beads intake.
6.☐ Add 200 µl of 70% ethanol into each well. (P200 / 8ch-P300)

Note: Carry out ethanol wash setting the sample plate on the magnetic bar.

Note: Pipetting is not required after adding ethanol.

Note: It makes the operation easier to dispense 70% ethanol into clean reservoir in advance when multi-channel pipette is used.

7.☐ Remove the supernatant in the same way as the step 5. (P200 / 8ch-P300)

Note: Remove ethanol as much as possible.

8.☐ Repeat the step 6 and 7. (wash twice total)

9.☐ Remove the sample plate ⑥ from the magnetic bar. Add **50 µl**/sample of 37°C-H2O and suspend the beads by pipetting 60 times. (P200 / 8ch-P300)

10.☐ Set the sample plate ⑥ in the thermal cycler and incubate for 5 min at 37℃.

11.☐ Centrifuge the sample plate ⑥ at 1,000 ×g for 1 min to collect the scattered beads to the bottom.

12.☐ Set the sample plate ⑥ at the magnetic bar and stand for 5 min.

13.☐ Collect the supernatant by pipetting and transfer it to the sample plate ⑦. (P200 / 8ch-P300)

14.☐ To remove ethanol from the sample, which is mixed in the washing process, set the sample plate ⑦ in the thermal cycler without the cap of the plate and incubate for 10 min at 37℃. [Refer to Fig.2]

Note: Place the lid of tip case that is wiped with Zap on the plate to avoid contamination with dust.

15.☐ Close the cap of the sample plate ⑦.

16.☐ Spindown the plate in the tabletop centrifuge to collect the solution to the bottom of the plate.

17.☐ Dispense 4 µl each of sample for Oligreen assay and qPCR. Store at -20℃ until proceeding to the Step1<h>cDNA Q.C

18.☐ Store the sample at -20℃ until proceeding to the step 1 <i>.

◆Step 1<h> cDNA Q.C (4hrs)

<QC1> Checking concentration by Oligreen assay

Measure the amount of single strand cDNA by the Oligreen assay.
The protocol that comes with Oligreen or the protocol that is made by the laboratory can be used.

The amount of single strand cDNA may be different depending on the type of the RNA sample. As reference, a few to a few dozens ng of single strand cDNA is applicable.

Select a method to mix cDNA from four options below according to the purpose, in order to determine the amount of cDNA to be used for the following operations based on the results of the measurement,
1. Mix whole amount.
2. Mix a half (20μl) amount.
3. Mix equal weight based on the Oligreen assay
4. Others

<QC2> Check by qPCR

Measure the amount of the target gene single strand cDNA by qPCR.
The protocol made by the laboratory can be used.
When the primer or conditions for qPCR are not set up, this step can be skipped.

Recommendation : qPCR primer

【Human】

Oligo Name	sequence(5'→3')
ACTB Forward	GGCATGGGTCAGAAGGATT
ACTB Reverse	AGGTGTGGTGCCAGATTTTC
rRNA 18S Forward	CTGGTTGATCCTGCCAGTAG
rRNA 18S Reverse	TCTAGAGTCACCAAAGCCGC

【Mouse】

Oligo Name	sequence(5'→3')
ACTB Forward	TATCGCTGCGCTGGTCGTCG
ACTB Reverse	TAGGGCGGCCCACGATGGAG
rRNA 18S Forward	GCCATGCATGTCTAAGTACGCACG
rRNA 18S Reverse	TCAGCGCCCGTCGGCATGTA

【qPCR cycle】

Temperature	Time	Number of Cycles
95℃	30sec	
94℃	15sec	40 cycles
60℃	30sec	
72℃	30sec	
65℃	5sec	
95℃	5sec	

Day5

◆ Step 1<i> SpeedVac for volume down (1hr30min)

Purpose: Concentrate the sample to reduce the volume for reaction in the Step 2<a> Single Strand Linker Ligation.

Table 40 : Required reagents, consumables and equipment for SpeedVac

Reagents not included in the kit	Consumable	Equipment
☐ H_2O (R1)	☐ 10 µl filter tip ☐ 20 µl filter tip ☐ 16-well plate	☐ P20 ☐ 8ch-P10 ☐ 96-plate rack×2 ☐ Syringe needle ☐ SpeedVac＜miVAC DNA＞

Preparation

・Set the rotor of Swing Rotor for Microtiter Plates in the SpeedVac.
・Pre-heat the SpeedVac at 37℃.
・Prepare a 16-well plate and label with sample names（sample plate⑦’）.

1.☐ Dispense the required amount of sample to prepare library, which is calculated based on the <QC1> Oligreen assay, into the sample plate ⑦'.
2.☐ Set the sample plate⑦' in a proper 96-plate rack and wrap with parafilm. [Refer to Fig.3]
3.☐ Make 3 holes / well in parafilm over each well with a syringe needle. [Refer to Fig.3]
Important: To avoid contamination, wrap the plate with parafilm and make 3 holes in it.
4.☐ Set the sample in the pre-heated SpeedVac at 37℃.
Note: Prepare balance prior to setting the sample in the SpeedVac. Measure the weight of the plate stand in which the sample is set and balance every time.
5.☐ Set the timer at 1 : 15 (1 hr 15 min) and push the AUTO button to start the SpeedVac. [Refer to Fig.4]
6.☐ After the SpeedVac stopped, ensure that no solution is in the sample plate⑦’. White precipitation will appear to the bottom of each well when it's completely dried.
7.☐ Dispense 4 µl/sample of H2O into each well and pipetting 60 times. Store the samples at -20℃ until proceeding to the Step2<a>. (P20 / 8ch-P10)

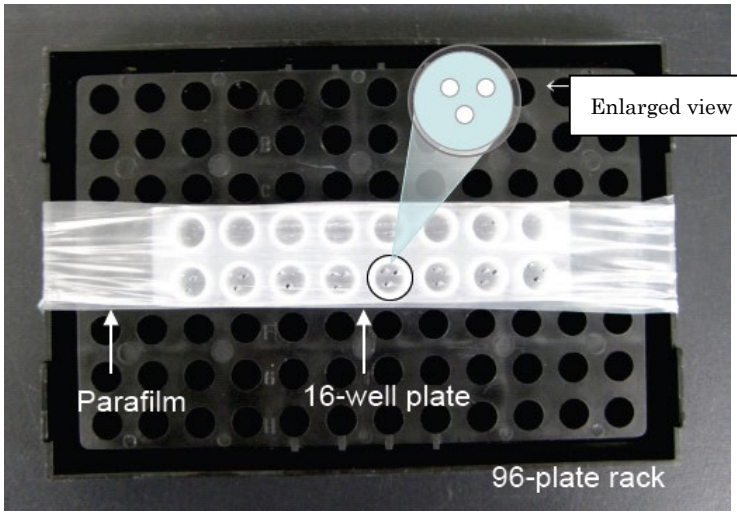


Fig.3： Sample treatment with SpeedVac

Fig.4：Set up of Speed Vac＜miVAC DNA＞

◆Step 2<a> Single Strand Linker Ligation (17hrs)

Purpose: Bind the Single Strand Linker (5' Linker), which is required for sequencing, to the 3'-end of cDNA. The Linker incorporates barcodes that enables to identify total RNA originated from.

Note: This step describes how to make a single 8 mixed library.

Table 41 : Required reagents, consumables and equipment for single strand linker ligation

Reagents included in the kit (tube number)	Consumable	Equipment
☐ 5' Linker (L1-#1~L1-#25)	☐ 10 µl filter tip ☐ 20 µl filter tip ☐ 8-strip tube	☐ P2 ☐ P20 ☐ 8ch-P10 ☐ Ice bucket ☐ Thermal cycler
Reagents not included in the kit		
☐ DNA DNA Ligation mixture (E3)		

Preparation

・Dissolve the 5' Linker to be used on ice.

・Dissolve the required amount of DNA DNA Ligation mixture on ice and mix by pipetting 10 times. (P200) Place it on ice until just before use.

・Set the thermal cycler to 55℃, 95℃ and 16℃.

<Single Strand Linker Ligation>

1.☐ Dispense 5 µl of each 5' Linker into 8-strip tubes according to [Fig.5]. (In the following, it will be discribed as 'Linker plate'.)
2.☐ Incubate the sample plate⑦' that is concentrated in the step 1<i> for 5 min at 95°C. Immediately transfer to ice and stand for 2 min. At the same time, incubate the Linker plate that is prepared in the step 1 for 5 min at 55℃. Immediately transfer to ice and stand for 2 min.
3.☐ Spindown the sample plate⑦' and the linker plate in the plate centrifuge to collect the solution to the bottom of the plate.
4.☐ Dispense 4 µl/sample of 5'Linker into the sample plate⑦'. Refer to [Table43] and [Fig.5]
Note : Pay careful attention to the mixed-up of 5' likers when dispensing them.
Note : Single pipette is more controlable to dispense 10µl of the 5' Linker.
5.☐ Add 16 µl/sample of DNA DNA Ligation mixture to the sample plate ⑦' . Set the scale of the pipette at 15 and mix well by pipetting more than 20 times. (P20 / 8ch-P100) [Table 42][Refer to Fig. 5]
Note: Ligation mix is very viscous and difficult to mix. Ensure that it is mixed well.

Table 42 : single strand linker DNA Ligation mixture

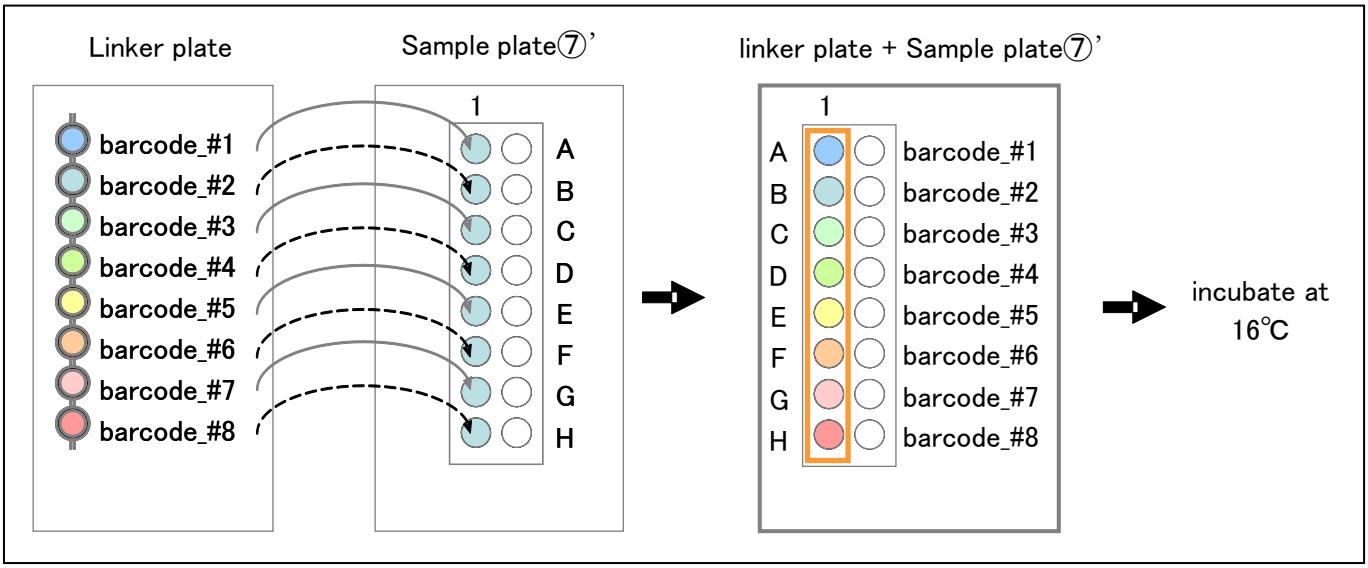
Reagent	1 sample	Equipment
☐ sample plate⑦'	☐ 4 µl	
☐ each single strand linker	☐ 4 µl	P2 or 8ch-P10
☐ DNA DNA Ligation mixture (E3)	☐ 16 µl	P20
Total volume	24 µl	

6.☐ Set the sample plate⑦'in the thermal cycler that is set at 16°C and incubate for 16 hrs. [Table43]
Note : Immediately proceed to the next step after the incubation. When it's impossible, store the sample plate at -20°C.

Table 43 : 16C program

Temperature	Time
16 ℃	16 hrs
4 ℃	forever

Fig.5 : chart of Single Strand Linker Ligation sample plate



Day6

◆Step 2<b> AMPure XP purification (1st and 2nd AMPure XP purification) (4hrs)

<1st AMPure XP purification>
Purpose: Remove single strand liker which is not reacted.

Important: Refer to [Important (pipetting) in the Step 1<b>] for pipetting.
Important: Refer to [Important (Treatment of supernatant) in the Step 1<b>] to treat the supernatant.

Table 44 : Required reagents, consumables and equipment for 1st AMPure XP purification

Reagents not included in the kit	Consumable	Equipment
☐ AMPure XP	☐ 200 µl filter tip ☐ 16-well plate ☐ 8-strip tube	☐ P200 ☐ 8ch-P300 ☐ Thermal cycler ☐ Magnetic bar
☐ 70%Ethanol		
☐ 37℃-H_2O		

Preparation
・Incubate AMPure XP for 30 min at room temperature. Mix well by inverting the tube, tapping or vortex etc. just before use it.
・Set the thermal cycler to 37℃.
・【Preparation of 37℃-H_2O】Dispense 200 µl of H_2O into 8-strip tube and incubate at 37℃ in the thermal cycler.
・Prepare a 16-well plate（sample plate⑧）and label with the sample name or number.

1.☐ Add 43.2 µl/sample of AMPure XP to the sample plate ⑦'. Set the scale of the pipette at 30 µl and mix well by pipetting 10 times. (P200 / 8ch-P300) [Table 45]

Table 45 : AMPure XP mixture after SSLL

Reagent	1 sample	Equipment
☐ sample plate ⑦'	☐ 24.0 µl	
☐ AMPure XP	☐ 43.2 µl	P200
Total volume	67.2 µl	

2.☐ Incubate for 30 min at room temperature.
3.☐ Set the sample plate ⑦' at the magnetic bar and stand for 5min.
4.☐ Prepare required amount of 70% ethanol for washing step referring Table 46.
Note: Prepare 70% ethanol between steps 2 and 3. Prepare excess amount in case a reservoir is used with multi-channel pipette. (Approximately 5,000 µl for 8 samples)

Table 46 : 70% Ethanol for wash

Reagent	1 sample	Equipment	8 samples (use reservoir)	Equipment
☐ H_2O (R1)	☐ 150 µl	P200	☐ 1500 µl	P1000
☐ EtOH	☐ 350 µl	P1000	☐ 3500 µl	P1000
Total volume	500 µl		5000 µl	

5.☐ Remove the supernatant by pipetting. (P200 / 8ch-P300)
Note: When removing the supernatant leave small amount of the supernatant (approximately 5 µl), which can be relatively avoid beads intake.
6.☐ Add 200 µl of 70% ethanol into each well. (P200 / 8ch-P300)
Note: Carry out ethanol wash setting the sample plate on the magnetic bar.
Note: Pipetting is not required after adding ethanol.
Note: It makes the operation easier to dispense 70% ethanol into clean reservoir in advance when multi-channel pipette is used.
7.☐ Remove the supernatant in the same way as the step 5. (P200 / 8ch-P300)
8.☐ Repeat the step 6 and 7. (wash twice total)
9.☐ Remove the sample plate ⑦' from the magnetic bar. Add 42 µl/sample of 37°C-H2O and suspend the beads by pipetting 60 times. (P200 / 8ch-P300)
10.☐ Set the sample plate ⑦' in the thermal cycler and incubate for 5 min at 37℃.
11.☐ Centrifuge the sample plate ⑦' at 1,000 ×g for 1 min to collect the scattered beads to the bottom of the tube.
12.☐ Set the sample plate ⑦' at the magnetic bar and stand for 5 min.
13.☐ Collect the supernatant by pipetting and transfer it to the sample plate ⑧. (P200 / 8ch-P300)
14.☐ To remove ethanol from the sample, which is mixed in the washing process, set the sample plate ⑧ in the thermal cycler without the cap of the plate and incubate for 10 min at 37℃. [Refer to Fig.2]
Note: Place the lid of tip case that is wiped with Zap on the plate to avoid contamination with dust.
15.☐ Close the cap of the sample plate ⑧.
16.☐ Spindown the plate in the plate centrifuge to collect the solution to the bottom of the plate.
17.☐ Place the sample on ice until proceeding to the next step.

<2nd AMPure XP purification>

Purpose: Remove unreacted single strand linker that is remained after the 1st AMPure XP purification

Important: Refer to [Important (pipetting) in the Step 1<b>] for pipetting.
Important: Refer to [Important (Treatment of supernatant) in the Step 1<b>] to treat the supernatant.

Table 47 : Required reagents, consumables and equipment for 2nd AMPure XP purification

Reagents not included in the kit	Consumable	Equipment
☐ AMPure XP	☐ 200 µl filter tip ☐ 16-well plate ☐ 8-strip tube	☐ P200 ☐ 8ch-P300 ☐ Thermal cycler ☐ Magnetic bar
☐ 70%Ethanol		
☐ 37℃-H_2O		

Preparation

・Incubate AMPure XP for 30 min at room temperature. Mix well by inverting the tube, tapping or vortex etc. just before use it.
・Set the thermal cycler to 37℃.
・【Preparation of 37℃-H_2O】Dispense 200 µl of H_2O into 8-strip tube and incubate at 37℃ in the thermal cycler.
・Prepare a 16-well plate（sample plate⑨）and label with the sample name or number.

1.☐ Add 72 µl/sample of AMPure XP to the sample plate ⑧. Set the scale of the pipette at 92 and mix well by pipetting 10 times. (P200 / 8ch-P300) [Table 48]

Table 48 : AMPure XP mixture after SSLL

Reagent	1 sample	Equipment
☐ sample plate ⑧	☐ 40 µl	
☐ AMPure XP	☐ 72 µl	P200
Total volume	112 µl	

2.☐ Incubate for 30 min at room temperature.
3.☐ Set the sample plate ⑧ at the magnetic bar and stand for 5min.
4.☐ Prepare required amount of 70% ethanol for washing step referring Table 49.
Note: Prepare 70% ethanol between steps 2 and 3. Prepare excess amount in case a reservoir is used with multi-channel pipette. (Approximately 5,000 µl for 8 samples)

Table 49 : 70% Ethanol for wash

Reagent	1 sample	Equipment	8 samples (use reservoir)	Equipment
☐ H_2O (R1)	☐ 150 µl	P200	☐ 1500 µl	P1000
☐ EtOH	☐ 350 µl	P1000	☐ 3500 µl	P1000
Total volume	500 µl		5000 µl	

5.☐ Remove the supernatant by pipetting. (P200 / 8ch-P300)
Note: When removing the supernatant leave small amount of the supernatant (approximately 5 µl), which can be relatively avoid beads intake.
6.☐ Add 200 µl of 70% ethanol into each well. (P200 / 8ch-P300)
Note: Carry out ethanol wash setting the sample plate on the magnetic bar.
Note: Pipetting is not required after adding ethanol.
Note: It makes the operation easier to dispense 70% ethanol into clean reservoir in advance when multi-channel pipette is used.
7.☐ Remove the supernatant in the same way as the step 5. (P200 / 8ch-P300)
8.☐ Repeat the step 6 and 7. (wash twice total)
9.☐ Remove the sample plate ⑧ from the magnetic bar. Add 42 µl/sample of 37°C-H2O and suspend the beads by pipetting 60 times. (P200 / 8ch-P300)
10.☐ Set the sample plate ⑧ in the thermal cycler and incubate for 5 min at 37℃.
11.☐ Centrifuge the sample plate ⑧ at 1,000 ×g for 1 min to collect the scattered beads to the bottom.
12.☐ Set the sample plate ⑧ at the magnetic bar and stand for 5 min.
13.☐ Collect the supernatant by pipetting and transfer it to the sample plate ⑨. (P200 / 8ch-P300)
14.☐ Close the cap of the sample plate ⑨.
15.☐ Spindown the plate in the plate centrifuge to collect the solution to the bottom of the plate.
16.☐ Place the sample on ice or at -20℃ until proceeding to the step 2<c>.

◆Step 2<c> SpeedVac for volume down (1hr 30min)

Purpose: Concentrate the sample to reduce the volume for reaction in the Step 2<d> 3'Linker Ligation.

Table 50 : Required reagents, consumables and equipment for SpeedVac

Reagents not included in the kit	Consumable	Equipment
☐ H_2O (R1)	☐ 10 µl filter tip ☐ 20 µl filter tip ☐ 16-well plate	☐ P20 ☐ 8ch-P10 ☐ 96-plate rack×2 ☐ Syringe needle ☐ SpeedVac＜miVAC DNA＞

Preparation

・Set the rotor of Swing Rotor for Microtiter Plates in the SpeedVac.
・Pre-heat the SpeedVac at 37℃.

1.☐ Set the sample plate⑨ in the proper 96-plate rack and wrap with parafilm. [Refer to Fig.3]]
2.☐ Make 3 holes / well in parafilm over each well with a syringe needle. [Refer to Fig.3]
Important: To avoid contamination, wrap the plate with parafilm and make 3 holes in it
3.☐ Set the sample in the pre-heated SpeedVac at 37℃.
Note: Prepare balance prior to setting the sample in the SpeedVac. Measure the weight of the plate stand in which the sample is set and balance every time.
4.☐ Set the timer at 1 : 15 (1 hr 15 min) and push the AUTO button to start the SpeedVac. [Refer to Fig.4]
5.☐ After the SpeedVac stopped, ensure that no solution is in the sample plate⑨. White precipitation will appear to the bottom of each well when it's completely dried.
6.☐ Dispense 4 µl/sample of H_2O into each well and pipetting 60 times. (P20 / 8ch-P10)
7.☐ Place the samples on ice or at -20℃ until proceeding to the Step2<d>.

◆Step 2<d> 3'Linker Ligation (17hrs)

Purpose: Bind 3' linker that is required for sequencing to cDNA.

Table 51 : Required reagents, consumables and equipment for 3'Linker Ligation

Reagents included in the kit (tube number)	Consumable	Equipment
☐ 3' linker (L2)	☐ 10 µl filter tip ☐ 20 µl filter tip ☐ 200 µl filter tip ☐ 8-strip tube	☐ P2 ☐ P20 ☐ P200 ☐ 8ch-P10 ☐ Ice bucket ☐ Thermal cycler
☐ DNA DNA Ligation mixture (E3)		

Preparation

・Dissolve the 3' Linker to be used on ice.

・Dissolve the required amount of DNA DNA Ligation mixture on ice and mix by pipetting 10 times. (P200) Place it on ice until just before use.

・Set the thermal cycler to 65℃, 95℃ and 16℃.

<3' Linker ligation>

1.☐ Dispense 40µl of 3' linker into only 1 well of a 8-strip tube (Example: The leftmost well). (In the following, it will be discribed as '3'Linker plate'.)

Note : For multi-channel pipette use, 5 µl of 3'linker can be dispensed into each well of 8-strip tube.

2.☐ Incubate the sample plate⑨ that is concentrated in the step 2<c> for 5 min at 95°C. Immediately transfer to ice and stand for 2 min. At the same time, incubate the 3'linker plate that is preprared in the step 1 for 5 min at 65℃. Immediately transfer to ice and stand for 2 min.

3.☐ Spindown the sample plate⑨ and the 3'linker plate in the plate centrifuge to collect the solution to the bottom of the plate.

4.☐ Dispense 4 µl/sample of 3'linker into the sample plate⑨. (P2/8ch-P10) [Table52]

Important : To avoid dispensing errors into wrong wells, check the operation.

5.☐ Add 16 µl/sample of DNA DNA Ligation mixture to the sample plate ⑨. Set the scale of the pipette at 15 and mix well by pipetting more than 20 times. (P200 / 8ch-P100) [Table 53]

Table 52 : 3'Linker DNA Ligation mixture

Reagent	1 sample	Equipment
☐ sample plate⑨	☐ 4 µl	
☐ 3' linker (L2)	☐ 4 µl	P2
☐ DNA DNA Ligation mixture (E3)	☐ 16 µl	P20
Total volume	24 µl	

6.☐ Set the sample plate⑨ in the thermal cycler that is set to 16°C and incubate for 16 hrs.

Note : Immediately proceed to the next step after the incubation. When it's impossible, store the sample plate at -20°C.

Day7

◆Step 2<e> AMPure XP purification (2hrs)
Purpose: Remove 3' linker which is not reacted.

<AMPure XP purification>

Important: Refer to [Important (pipetting) in the Step 1<b>] for pipetting.
Important: Refer to [Important (Treatment of supernatant) in the Step 1<b>] to treat the supernatant.

Table 53 : Required reagents, consumables and equipment for AMPure XP purification

Reagents not included in the kit	Consumable	Equipment
☐ AMPure XP	☐ 200 µl filter tip ☐ 16-well plate ☐ 8-strip tube	☐ P200 ☐ 8ch-P300 ☐ Thermal cycler ☐ Magnetic bar
☐ 70%EtOH		
☐ 37℃-H_2O		

Preparation
・Incubate AMPure XP for 30 min at room temperature. Mix well by inverting the tube, tapping or vortex etc. just before use it.
・Set the thermal cycler to 37℃.
・【Preparation of 37℃-H_2O】Dispense 200 µl of H_2O into 8-strip tube and incubate at 37℃ in the thermal cycler.
・Prepare a 16-well plate（sample plate⑩）and label with the sample name or number.

1.☐ Add 43.2 µl/sample of AMPure XP to the sample plate ⑨. Set the scale of the pipette at 30 and mix well by pipetting 10 times. (P200 / 8ch-P300) [Table 54]

Table 54 : AMPure XP mixture after 3'Linker Ligation

Reagent	1 sample	Equipment
☐ sample plate ⑨	☐ 24.0 µl	
☐ AMPure XP	☐ 43.2 µl	P200
Total volume	67.2 µl	

2.☐ Incubate for 30 min at room temperature.
3.☐ Set the sample plate ⑨ at the magnetic bar and stand for 5min.
4.☐ Prepare required amount of 70% ethanol for washing step referring Table 55.
Note: Prepare 70% ethanol between steps 2 and 3. Prepare excess amount in case of reservoir is used with multi-channel pipette. (Approximately 5,000 µl for 8 samples)

Table 55 : 70% Ethanol for wash

Reagent	1 sample	Equipment	8 samples (use reservoir)	Equipment
☐ H_2O (R1)	☐ 150 µl	P200	☐ 1500 µl	P1000
☐ EtOH	☐ 350 µl	P1000	☐ 3500 µl	P1000
Total volume	500 µl		5000 µl	

5.☐ Remove the supernatant by pipetting. (P200 / 8ch-P300)
Note: When removing the supernatant leave small amount of the supernatant (approximately 5 µl), which can be relatively avoid beads intake.
6.☐ Add 200 µl of 70% ethanol into each well. (P200 / 8ch-P300)
Note: Carry out ethanol wash setting the sample plate on the magnetic bar.
Note: Pipetting is not required after adding ethanol.
Note: It makes the operation easier to dispense 70% ethanol into clean reservoir in advance when multi-channel pipette is used.
7.☐ Remove the supernatant in the same way as the step 5. (P200 / 8ch-P300)
Note: Remove ethanol as much as possible.
8.☐ Repeat the step 6 and 7. (wash twice total)
9.☐ Remove the sample plate ⑨ from the magnetic bar. Add 42 µl/sample of 37°C-H2O and suspend the beads by pipetting 60 times. (P200 / 8ch-P300)
10.☐ Set the sample plate ⑨ in the thermal cycler and incubate for 5 min at 37℃.
11.☐ Centrifuge the sample plate ⑨ at 1,000 ×g for 1 min to collect the scattered beads to the bottom of the tube.
12.☐ Set the sample plate ⑨ at the magnetic bar and stand for 5 min.
13.☐ Collect the supernatant by pipetting and transfer it to the sample plate ⑩. (P200 / 8ch-P300)
14.☐ To remove ethanol from the sample, which is mixed in the washing process, set the sample plate ⑩ in the thermal cycler without the cap of the plate and incubate for 10 min at 37℃. [Refer to Fig.2]
Note: Place the lid of tip case that is wiped with Zap on the plate to avoid contamination with dust.
15.☐ Close the cap of the sample plate ⑩.
16.☐ Spindown the plate in the plate centrifuge to collect the solution to the bottom of the plate.
17.☐ Place the sample on ice or at -20℃ until proceeding to the step 2<f>.

◆Step 2<f> Shrimp Alkaline Phosphatase(SAP) and USER treatment(4hrs)

Purpose: Remove phosphate group of linkers and digest dUTP contained in 3'linker up strand.

Table 56 : Required reagents, consumables and equipment for SAP and USER

Reagents not included in the kit	Consumable	Equipment
☐ Shrimp Alkaline Phosphatase (E4)	☐ 10 µl filter tip ☐ 10 µl long filter tip ☐ 20 µl filter tip ☐ 200 µl filter tip ☐ 16-well plate ☐ 1.5 ml tube	☐ P2 ☐ P20 ☐ P200 ☐ 8ch-P300 ☐ Ice bucket ☐ Thermal cycler ☐ Magnetic bar
☐ 10×SAP buffer (R15)		
☐ AMPure XP		
☐ H_2O (R1)		
☐ USER (E5)		

Preparation

・Dissolve 10× SAP buffer on ice.

・Set the SAP program (Table 59) in the thermal cycler.

・Set the USER program (Table 61) in the thermal cycler.

<SAP>

1.☐ Prepare the SAP pre-mixture. Place on ice until just before use. [Table 57]

Table 57 : SAP pre-mixture

Reagent	1 sample	Equipment	8 samples (Prepare for 9)	Equipment
☐ H_2O (R1)	☐ 4 µl	P20	☐ 36.0 µl	P200
☐ 10× SAP buffer (R15)	☐ 5 µl	P20	☐ 45.0 µl	P200
☐ SAP(1 U/µl; E4)	☐ 1 µl	P2	☐ 9.0 µl	P20
Total volume	10 µl		90.0 µl	

2.☐ Add 10 µl/sample of SAP pre-mixture prepared in the step 1 to the sample plate ⑩. Set the scale of the pipette at 40 and mix well by pipetting 10 times. (P200 / 8ch-P300) [Table 58]

Table 58 : SAP mixture

Reagent	1 sample	Equipment
☐ sample plate⑩	☐ 40 µl	
☐ SAP pre-mixture(Table 58)	☐ 10 µl	P20
Total volume	50 µl	

3.☐ Spindown in the plate centrifuge to collect the solution to the bottom of the plate.

4.☐ Set the sample plate ⑩ in the thermal cycler that is set for SAP program and start incubation.

Table 59 : SAP program

Temperature	Time
37℃	30 min
65℃	15 min
4℃	Forever

5.☐ After the incubation, place the sample on ice until USER treatment starts.

<USER>

1.☐ Add 2 µl/sample of USER enzyme to the sample plate ⑩ after SAP treatment. Set the scale of the pipette at 40 and mix well by pipetting 10 times. (P200 / 8ch-P300) [Table 60]

Table 60 : USER mixture

Reagent	1 sample	Equipment
☐ sample plate ⑩ after SAP treatment	☐ 50 µl	
☐ USER	☐ 2 µl	P2
Total volume	52 µl	

2.☐ Spindown in the plate centrifuge to collect the solution to the bottom of the plate.

3.☐ Set the sample plate ⑩ in the thermal cycler that is set for USER program and start incubation. [Table 61]

Note: After the step at 95℃ for 5min, transfer the plate to ice to rapidly cool it.

Note: After incubation at 95℃, the tubes and caps become very hot. Remove them from the thermal cycler when it comes to around 60℃ to avoid opening the caps and getting burn.

Table 61 : USER program

Temperature	Time
37℃	30 min
95℃	5 min
On ice	2 min

<AMPure XP purification>

Important: Refer to [Important (pipetting) in the Step 1<b>] for pipetting.

Important: Refer to [Important (Treatment of supernatant) in the Step 1<b>] to treat the supernatant.

Table 62 : Required reagents, consumables and equipment for AMPure XP purification

Reagents not included in the kit	Consumable	Equipment
☐ AMPure XP	☐ 200 µl filter tip ☐ 16-well plate ☐ 8-strip tube	☐ P200 ☐ 8ch-P300 ☐ Thermal cycler ☐ Magnetic bar
☐ 70%EtOH		
☐ 37℃-H_2O		

Preparation

・Incubate AMPure XP for 30 min at room temperature. Mix well by inverting the tube, tapping or vortex etc. just before use it.

・Set the thermal cycler to 37℃.

・【Preparation of 37℃-H_2O】Dispense 200 µl of H_2O into 8-strip tube and incubate at 37℃ in the thermal cycler.

・Prepare a 16-well plate（sample plate⑪）and label with the sample name or number.

1.☐ Add 93.6 µl/sample of AMPure XP to the sample plate ⑩. Set the scale of the pipette at 126 and mix well by pipetting 10 times. (P200 / 8ch-P300) [Table 63]

Table 63 : AMPure XP mixture after USER treatment

Reagent	1 sample	Equipment
☐ sample plate ⑩ after USER treatment	☐ 52.0 µl	
☐ AMPure XP	☐ 93.6 µl	P200
Total volume	145.6 µl	

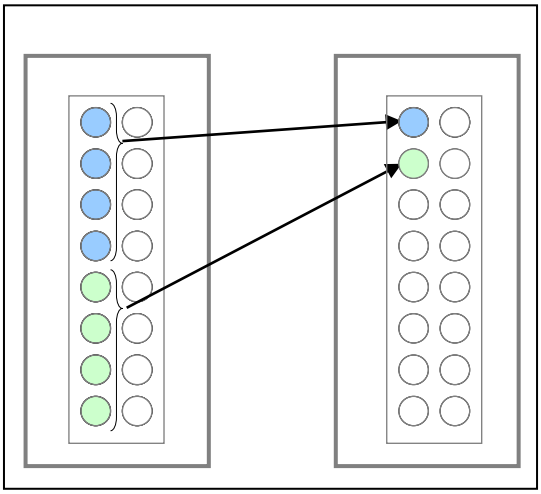
2.☐ Incubate for 30 min at room temperature.
3.☐ Set the sample plate ⑩ at the magnetic bar and stand for 5min.
4.☐ Prepare required amount of 70% ethanol for washing step referring Table 64.
Note: Prepare 70% ethanol between steps 2 and 3. Prepare excess amount in case a reservoir is used with multi-channel pipette. (Approximately 5,000 µl for 8 samples)

Table 64 : 70% Ethanol for wash

Reagent	1 sample	Equipment	8 samples (use reservoir)	Equipment
☐ H_2O	☐ 150 µl	P200	☐ 1500 µl	P1000
☐ EtOH	☐ 350 µl	P1000	☐ 3500 µl	P1000
Total volume	500 µl		5000 µl	

5.☐ Remove the supernatant by pipetting. (P200 / 8ch-P300)
Note: When removing the supernatant leave small amount of the supernatant (approximately 5 µl), which can be relatively avoid beads intake.
6.☐ Add 200 µl of 70% ethanol into each well. (P200 / 8ch-P300)
Note: Carry out ethanol wash setting the sample plate on the magnetic bar.
Note: Pipetting is not required after adding ethanol.
Note: It makes the operation easier to dispense 70% ethanol into clean reservoir in advance when multi-channel pipette is used.
7.☐ Remove the supernatant in the same way as the step 5. (P200 / 8ch-P300)
Note: Remove ethanol as much as possible.
8.☐ Repeat the step 6 and 7. (wash twice total)
9.☐ Remove the sample plate ⑩ from the magnetic bar. Add 42 µl/sample of 37°C-H2O and suspend the beads by pipetting 60 times. (P200 / 8ch-P300)
10.☐ Set the sample plate ⑩ in the thermal cycler and incubate for 5 min at 37℃.
11.☐ Centrifuge the sample plate ⑩ at 1,000 ×g for 1 min to collect the scattered beads to the bottom of the tube.
12.☐ Set the sample plate ⑩ at the magnetic bar and stand for 5 min.
13.☐ Collect the supernatant with a single pipette according to Fig.6 : sample mix and transfer it to the sample plate ⑪. Each well will contain 160 µl.
14.☐ Close the cap of the sample plate ⑪.
15.☐ Spindown the plate in the plate centrifuge to collect the solution to the bottom of the plate.
16.☐ Place the sample on ice or at -20℃ until proceeding to the step 2<g>.

Fig.6 : sample mix



40 µl/sample × 4well = 160 µl / well

◆Step 2<g> SpeedVac for volume down (2hr 30min)

Purpose: Concentrate the sample to reduce the volume of the sample for the 2nd strand cDNA synthesis reaction in the Step 2<h>.

Table 65 : Required reagents, consumables and equipment for SpeedVac

Reagents not included in the kit	Consumable	Equipment
☐ H_2O (R1)	☐ 20 µl filter tip	☐ P20 ☐ 96-plate rack × 2 ☐ Syringe needle ☐ SpeedVac＜miVAC DNA＞

Preparation

・Set the rotor of Swing Rotor for Microtiter Plates in the SpeedVac.

・Pre-heat the SpeedVac at 37℃.

1.☐ Set the sample plate ⑪ in the proper 96-plate rack and wrap with parafilm. [Refer to Fig.3]

2.☐ Make 3 holes / well in parafilm over each well with a syringe needle. [Refer to Fig.3]
Important: To avoid contamination, wrap the plate with parafilm and make 3 holes in it.

3.☐ Set the sample in the pre-heated SpeedVac at 37℃.

Note: Prepare balance prior to setting the sample in the SpeedVac. Measure the weight of the plate stand in which the sample is set and balance every time.

4.☐ Set the timer at 2 : 30 (2 hr 30 min) and push the AUTO button to start the SpeedVac. [Refer to Fig.4]

5.☐ After the SpeedVac stopped, ensure that no solution is in the sample plate ⑪. White precipitation will appear to the bottom of each well when it's completely dried.

6.☐ Dispense 20 µl/sample of H2O into B well and pipetting 60 times. (P20)

7.☐ Transfer 20 µl of the eluted solution in the step 6 to the A well according to [Fig.7] and pipette 60 times. (P20)

8.☐ Dispense 20 µl/sample of H2O into B well and pipetting 60 times again. (P20)

9.☐ Transfer 20 µl of the eluted solution in the step 8 to the A well and pipette 60 times. (P20)
Note: 8 mixed libraries will be produced. The solution volume will be 40 µl.

10.☐ Place the samples on ice or at -20℃ until proceeding to the Step2<h>.

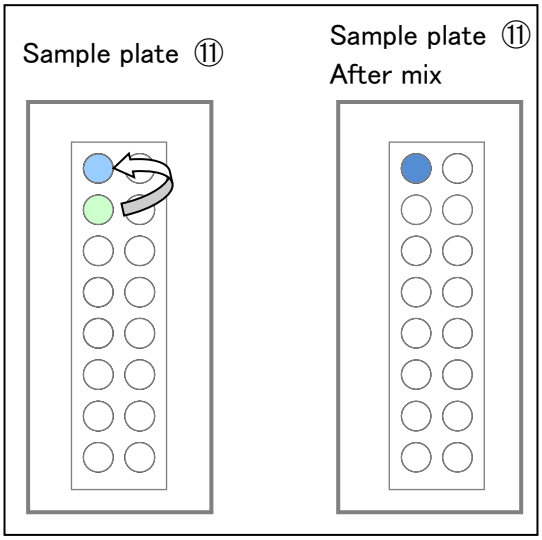


Fig.7 : sample mix

Day8

◆Step 2<h> 2nd strand cDNA synthesis and purification (3hrs 30min)

Purpose: Synthesis of ds cDNA using primers for the 2nd synthesis and purification.

Table 66 : Required reagents, consumables and equipment for 2nd strand cDNA synthesis

Reagent	Consumable	Equipment
☐ 2nd primer (P2)	☐ 10 µl filter tip	☐ P2
☐ DNA polymerase (2unit/ul ; E6)	☐ 10 µl long filter tip	☐ P20
☐ DNA polymerase buffer (10×; R16)	☐ 20 µl filter tip	☐ P200
☐ 10mM dNTPs(R2)	☐ 200 µl filter tip	☐ 8chP-300
☐ H_2O(R1)	☐ 1.5 ml tubes	☐ ice bucket
		☐ Thermal cycler

Preparation

・Dissolve, tap or invert the tubes and spindown the following reagents.

□ ThermoPol Reaction Buffer Pack (10×)

□ 10 mM dNTP

□ 2nd primer

・Set the thermal cycler for the pre-2nd synthesis[Table70] and the 2nd synthesis[Table72].

<2nd strand synthesis>

1.☐ Prepare the 2nd strand synthesis pre-mixture. Place on ice until just before use. [Table 67]

Table 67 : 2nd strand synthesis pre-mixture

Reagent	1 sample	Equipment
☐ H_2O (R1)	☐ 2 µl	P20
☐ DNA polymerase buffer (10×; R16)	☐ 5 µl	P2
☐ 10 mM dNTP (R2)	☐ 1 µl	P2
☐ 2nd Primer (P2)	☐ 1 µl	P2
Total volume	9 µl	

2.☐ Add 9 µl/sample of the 2nd strand synthesis pre-mixture prepared in the step 1. Set the scale of the pipette at 40 and mix well by pipetting 10 times. (P200 / 8ch-P300) [Table 68]

Table 68 : 2nd strand synthesis mixture

Reagent	1 sample	Equipment
☐ sample plate ⑪	☐ 40 µl	
☐ 2nd strand synthesis pre-mixture[Table67]	☐ 9 µl	P20
Total volume	49 µl	

3.☐ Spindown in the plate centrifuge to collect the solution to the bottom of the plate.

4.☐ Set the Sample plate ⑪ in the thermal cycler and perform the pre-2nd synthesis. [Table69]

Table 69 : pre-2nd synthesis

Temperature	Time
95℃	5 min
55℃	5 min
On ice	1min

5.☐ After the reaction, spindown the sample plate ⑪ in the plate centrifuge to collect the solution to the bottom of the plate.
6.☐ Transfer the sample plate ⑪ to the ice bucket. Add 1 µl/sample of DNA polymerase(E6). Set the scale of the pipette at 40 and mix well by pipetting 10 times. (P200 / 8ch-P300) [Table 70]

Table 70 : DNA polymerase(E6) mixture

Reagent	1 sample	Equipment
☐ sample plate ⑪	☐ 49 µl	
☐ DNA polymerase (2unit/ul; E6)	☐ 1 µl	P2
Total volume	50 µl	

7.☐ Spindown in the plate centrifuge to collect the solution to the bottom of the plate.
8.☐ Set the Sample plate ⑪ in the thermal cycler that is set for the 2nd synthesis and perform the program. [Table71]

Table 71 : 2nd synthesis

Temperature	Time
55℃	5 min
72℃	30 min
4℃	∞

9.☐ After completing the 2nd synthesis, spindown the sample plate ⑪ in the plate centrifuge to collect the solution to the bottom of the plate.
10.☐ Place on ice until proceeding to the next step.

<Exonuclease I treatment >

Table 72 : Required reagents, consumables and equipment for Exonuclease I

Reagent	Consumable	Equipment
☐ Exonuclease I (E7)	☐ 10 µl filter tip ☐ 10 µl long filter tip ☐ 200 µl filter tip	☐ P2 ☐ 8chP-300 ☐ Ice bucket ☐ Thermal cycler

1.☐ Add 1 µl/sample of Exonuclease I to the sample plate ⑪. Set the scale of the pipette at 40 and mix well by pipetting 10 times. (P200 / 8ch-P300) [Table 73]

Table 73 : Exonuclease I reaction mixture

Reagent	1 sample	Equipment
☐ sample plate ⑪	☐ 50 µl	
☐ Exonuclease I (E7)	☐ 1 µl	P2
Total volume	51 µl	

2.☐ Spindown in the plate centrifuge to collect the solution to the bottom of the plate.
3.☐ Incubate the sample plate ⑪ for 30 min at 37℃. [Table 75]

Table 74 : Exonuclease I treatment

Temperature	Time
37℃	30 min
4℃	∞

<AMPure XP purification>

Important: Refer to [Important (pipetting) in the Step 1<b>] for pipetting.
Important: Refer to [Important (Treatment of supernatant) in the Step 1<b>] to treat the supernatant.

Table 75 : Required reagent, consumables and equipment for AMPure XP purification

Reagents not included in the kit	Consumable	Equipment
☐ AMPure XP	☐ 200 µl filter tip	☐ P200
☐ 70%EtOH	☐ 16-well plate	☐ 8ch-P300
☐ 37℃-H_2O	☐ 8-strip tube	☐ Thermal cycler
	☐ 1.5 ml tube	☐ Magnetic bar

Preparation

・Incubate AMPure XP for 30 min at room temperature. Mix well by inverting the tube, tapping or vortex etc. just before use it.
・Set the thermal cycler to 37℃.
・【Preparation of 37℃-H_2O】Dispense 200 µl of H_2O into 8-strip tube and incubate at 37℃ in the thermal cycler.
・Prepare a new 1.5ml tube and label with sample names (sample tube⑫).

1.☐ Add 91.8 µl/sample of AMPure XP to the sample plate ⑪. Set the scale of the pipette at 123 and mix well by pipetting 10 times. (P200 / 8ch-P300) [Table 76]

Table 76 : AMPure XP mixture after Exonuclease I treatment

Reagent	1 sample	Equipment
☐ sample plate ⑪	☐ 51.0 µl	
☐ AMPure XP	☐ 91.8 µl	P200
Total volume	142.8 µl	

2.☐ Incubate for 30 min at room temperature.
3.☐ Set the sample plate ⑪ at the magnetic bar and stand for 5min.
4.☐ Prepare required amount of 70% ethanol for washing step referring Table 78.
Note: Prepare 70% ethanol between steps 2 and 3.

Table 77 : 70% Ethanol for wash

Reagent	1 sample	Equipment
☐ H_2O (R1)	☐ 150 µl	P200
☐ EtOH	☐ 350 µl	P1000
Total volume	500 µl	

5.☐ Remove the supernatant by pipetting. (P200 / 8ch-P300)
Note: When removing the supernatant leave small amount of the supernatant (approximately 5 µl), which can be relatively avoid beads intake.
6.☐ Add 200 µl of 70% ethanol into each well. (P200 / 8ch-P300)
Note: Carry out ethanol wash setting the sample plate on the magnetic bar.
Note: Pipetting is not required after adding ethanol.
7.☐ Remove the supernatant in the same way as the step 5. (P200 / 8ch-P300)
8.☐ Repeat the step 6 and 7. (wash twice total)
9.☐ Remove the sample plate ⑪ from the magnetic bar. Add 42 µl/sample of 37°C-H2O and suspend the beads by pipetting 60 times. (P200 / 8ch-P300)
10.☐ Set the sample plate ⑪in the thermal cycler and incubate for 5 min at 37℃.
11.☐ Centrifuge the sample plate⑪ at 1,000 ×g for 1 min to collect the scattered beads to the bottom.
12.☐ Set the sample plate ⑪ at the magnetic bar and stand for 5 min.
13.☐ Collect the supernatant and transfer it to the sample tube ⑫. (P200)
14.☐ Spindown the tube in the tabletop centrifuge to collect the solution to the bottom of the tube.
15.☐ Place the sample on ice or at -20℃ until proceeding to the step 2<i>.

◆Step 2<i> SpeedVac for volume down(1hr)

Purpose: Concentrate the sample for the final Q.C. in the Step 2<j>

Table 78 : Required reagents, consumables and equipment for SpeedVac

Reagents not included in the kit	Consumable	Equipment
☐ H_2O (R1)	☐ 20 µl filter tip ☐ 1.5 ml tube	☐ P20 ☐ Syringe needle ☐ SpeedVac＜miVAC DNA＞

Preparation

・Set a sample holder for 1.5ml Flip Cap Microcentrifuge Tubes in the SpeedVac.
・Pre-heat the SpeedVac at 37℃.
・Prepare 2 of 1.5 ml tubes per library for Q.C. and label the sample name.

1.☐ Open the lid of the sample tube ⑫ and wrap with parafilm.

2.☐ Make 3 holes / well in parafilm with a syringe needle.

Important: To avoid contamination, wrap the opening of the tubes with parafilm and make 3 holes in it.

3.☐ Set the sample in the pre-heated SpeedVac at 37℃.

Note: Prepare balance prior to setting the sample in the SpeedVac.

4.☐ Set the timer at 1 : 15 (1 hr 15 min) and push the AUTO button to start the SpeedVac. [Refer to Fig.4]
5.☐ After the SpeedVac stopped, ensure that no solution is in the sample tube⑫. White precipitation will appear to the bottom of each well when the sample is completely dried.
6.☐ Dispense 10 µl/sample of H2O into each tube and dissolve well by tapping or pipetting. (P20)
7.☐ Dispense the sample for Q.C. Dispense 1 µl for Picogreen analysis and 2 µl for Bioanalyzer analysis.
8.☐ Store the samples at -20℃ until proceeding to the Step2<k>.

◆Step 2<j> final Q.C (2hrs)

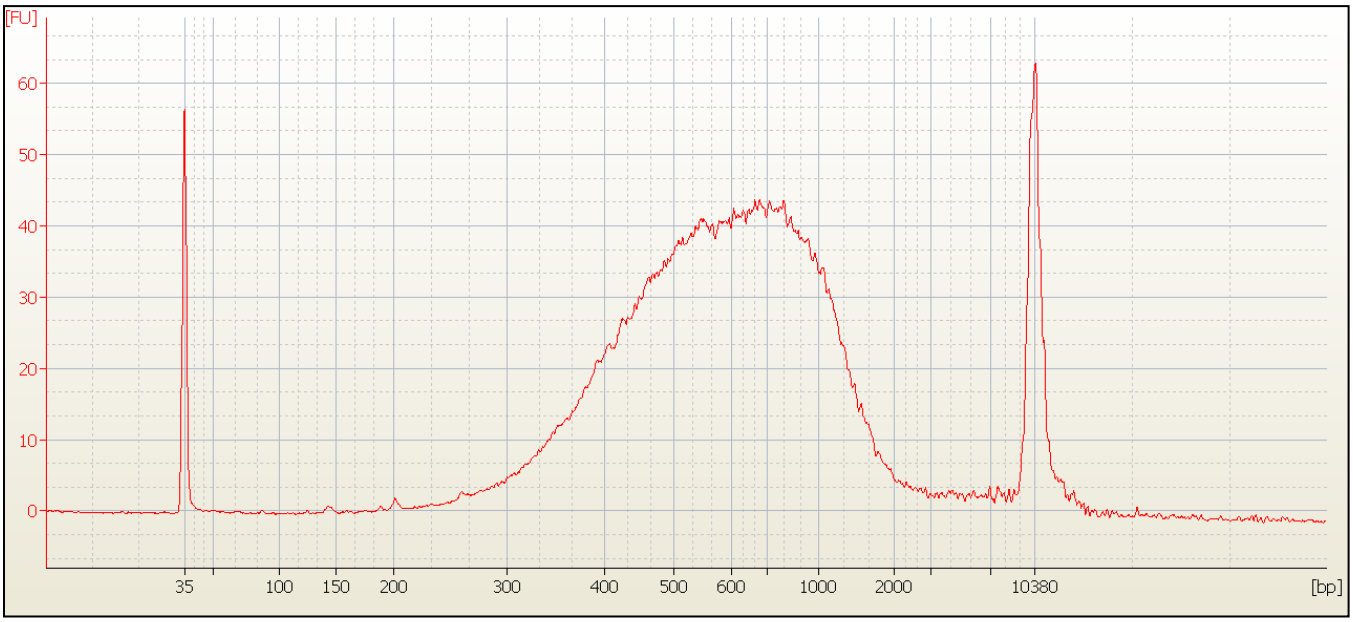
<QC3> Check concentration by Picogreen assay

Measure the amount of double strand cDNA by the Picogreen assay.
The protocol that comes with Picogreen or the protocol that is made by the laboratory can be used.

The amount of double strand cDNA may be different depending on the type of the RNA sample and the amount of single starand cDNA that is used for the mixture. As reference, a few to a few dozens ng of double strand cDNA per a library is applicable.

<QC4> Check the final product using the Highsensitivity DNA kit

Check the size distribution of the double strand cDNA and the presence of their unspecific peak using the Bioanalyzer High Sensitivity DNA kit.
The protocol that comes with High Sensitivity DNA kit or the protocol that is made by the laboratory can be used.
This step is not essential for sequencing analysis and can be skipped.
We recommend that to keep the record of the peak size in case unspecific peak (for example, due to the linkers that are used to prepare the library) appears, because it may affect the sequencing results.



Reference: A result from analysis with High Sensitivity DNA kit

◆Step 2<k> sample preparation for cluster generation(1hr)

Purpose: Prepare sample for cluster generation.

It is confirmed that the protocol provided from illumine, Inc. does not provide sufficient number of read. We strongly recommend changing the dilution rate for the sample and the amount of sample to be used for cluster generation referring to the following steps.
We recommend requesting the condition change disclosing the following condition when sequencing is outsourced.

Important: Cluster generation can be started just after the sample preparation.

Table 79 : Required reagents, consumables and equipment for sample preparation for cluster generation

Reagents not included in the kit	Consumable	Equipment
☐ H_2O (R1)	☐ 10 µl filter tip	☐ P2 pipette
☐ 2 M NaOH	☐ 20 µl filter tip	☐ P20 pipette
☐ 1 M Tris-HCl(pH7.0)	☐ 200µl filter tip	☐ P200 pipette
☐ Hybridization buffer HT1 (Included in illumina cluster generation kit)	☐ 1.5 ml tube	☐ Ice bucket ☐ Tabletop centrifugal machine

Preparation

・Dissolve the sample prepared in the Step2<i> 8 on ice.
・Stand 1 M Tris-HCl (pH7.0) and Hybridization buffer HT1 for more than 30 min on ice.
・Prepare a 1.5 ml tube and label with the sample name. (This is sample tube ⑬)

<sample preparation>

1.☐ Based on the concentration that is measured in the QC3, dispense 3ng of the sample into the sample tube ⑬. (P2/P20)
Note: 3ng is preferable to acquire sufficient number of read. However, if the acquired amount of sample from the library preparation is less than 3ng, use the whole sample (7µl) for the sequencing.
2.☐ Add required amount of H_2O to make the total volume 19 µl to the sample tube ⑬ according to Table 81. Mix well by tapping. (P20)

Table 80 : Dilution of library solution

Reagent	1 sample	Equipment
☐ CAGE Library	☐ x µl (3ng)	P2/P20
☐ H_2O	☐ (19-x) µl	P20
Total volume	19 µl	

3.☐ Spindown in the tabletop centrifuge to collect the solution to the bottom of the tube.
4.☐ Add 1 µl of 2 M NaOH to the sample tube ⑬. Mix well by tapping. (P2)
5.☐ Spindown in the tabletop centrifuge to collect the solution to the bottom of the tube.
6.☐ Incubate for 5 min at room temperature.
7.☐ Transfer the sample tube ⑬ to ice. Add 20 µl/sample of 1 M Tris-HCl(pH7.0). Set the scale of the pipette at 30 and mix well by pipetting 10 times. (P200)
8.☐ Add 110 µl/sample of Hybridization buffer HT1 to the sample tube ⑬ on ice. Set the scale of the pipette at 100 and mix well by pipetting 10 times. (P200)
9.☐ Spindown in the tabletop centrifuge to collect the solution to the bottom of the tube.

10.☐ Load 120µl (out of the total amount 150 µl) of the sample from the sample tube ⑬ into the cluster generation(c-Bot).