Instruction manual for GenNext RamDA-seq Single Cell Kit 202011

GenNext™ RamDA-seq™ Single Cell Kit

RMD-101 96 reactions
RMD-101T 24 reactions

Store at −20°C

Contents

CAUTION
All reagents in this kit are intended for research purposes. Do not use for diagnostic or clinical purposes. Please observe general laboratory safety precautions while using this kit.

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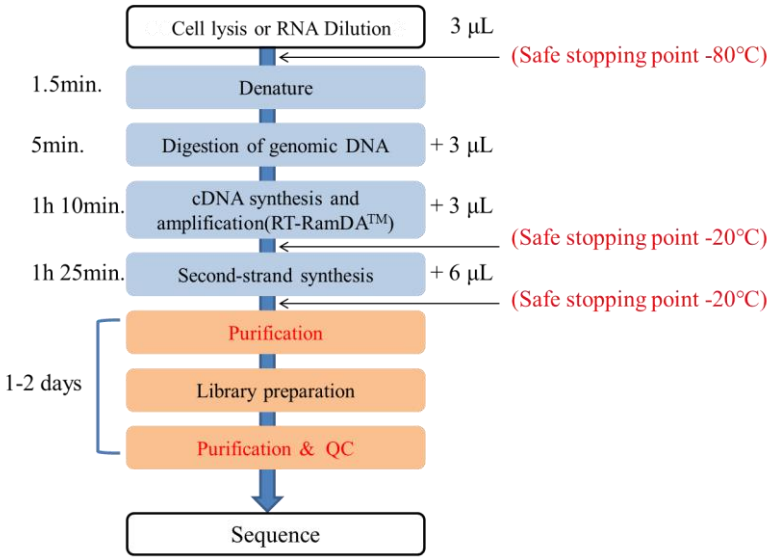
[1] Introduction

Description

The RamDA-seq™ Single Cell Kit is a kit for preparing cDNA for subsequent NGS analysis from single cells or trace amounts of RNA. With the use of this kit, it is possible to prepare cDNA from full-length total RNA.

This kit uses Reverse Transcription with Random Displacement Amplification (RT-RamDA™); see method reference (1). RT-RamDA™ is a novel cDNA amplification method that utilizes the strand displacement activity of reverse transcriptase, and this kit can detect not only poly(A) RNA but also non-poly(A) RNA with high sensitivity. For this reason, the RT-RamDA™ method is characterized by being able to detect more genes than conventional technology.

This kit does not include library preparation reagents or magnetic beads.



Process Workflow

Features

1. cDNA can be prepared from a single cell or small amount of input RNA
1–100 cells or 10 pg–1 ng total RNA

2. Full-length cDNA can be analyzed
cDNA that covers the entire length of the target RNA of 10 kb or more can be prepared.

3. Various RNAs can be detected, and the number of genes detectable is higher than that with conventional technology.
◇ Identification of isoforms and alternative splicing
◇ Detection of poly(A) RNA and non-poly(A) RNA (histone RNA and lncRNA)
◇ Detection of nuclear RNA (pre-mRNA and lncRNA)

[2] Components

The kits include the following reagents that can be used for 96 (RMD-101) and 24 (RMD-101T) reactions. All reagents should be stored at −20°C.

Important: Do not store any solutions once mixed. All reagent cocktails should be prepared shortly before use.

RamDA-seq™ Single Cell Kits (Cat. No. RMD-101 and RMD-101T)

	RMD-101	RMD-101T
①Lysis Buffer	480 µL	120 µL
②Lysis Enhancer	108 µL	27 µL
③RNase Inhibitor	22 µL	6 µL
④Nuclease-free water	960 µL	240 µL
⑤RT-RamDA™ Buffer	240 µL	60 µL
⑥RT-RamDA™ Enzyme Mix	54 µL	14 µL
⑦RT-RamDA™ Primer Mix	54 µL	14 µL
⑧gDNA Remover	54 µL	14 µL
⑨2nd strand Synthesis Buffer	330 µL	83 µL
⑩2nd strand Synthesis Enzyme	55 µL	14 µL
⑪2nd strand Synthesis Primer Mix	275 µL	69 µL

NSR Primer Set for human samples (Cat. No. NSR-101)

1st NSR Primer Mix for human	54 µL
2nd NSR Primer Mix for human	275 µL

NSR Primer Set for mouse samples (Cat. No. NSR-102)

1st NSR Primer Mix for mouse	54 µL
2nd NSR Primer Mix for mouse	275 µL

Required materials not included

· Thermocycler

· Library preparation reagents
The RamDA-seq™ Single Cell Kits are specifically for use with the Illumina Nextera™ XT DNA Sample Kit.
Nextera™ XT DNA Sample Kit (24 Samples), Cat. no. FC-131-1024
Nextera™ XT DNA Sample Kit (96 Samples), Cat. no. FC-131-1096

· SPRI (Solid Phase Reversible Immobilization) paramagnetic beads
Agencourt™ AMPure™ XP Beads (Beckman Coulter, Cat. no. A63880 or A63881)

· TE Buffer pH 8.0 (10 mM Tris-HCl,,1mM EDTA)

· Magnetic rack/stand for magnetic bead separation

· 80% Ethanol (freshly prepared)

[3] Protocol

This kit can be used with cells or purified total RNA. Start with Section 1A when using FACS to obtain the cells, Section 1B when using non-FACS cells, and Section 1C when using purified total RNA. Then proceed with the denaturation in Section 2.

Sample		Cell lysis or RNA Dilution	Denaturation
Cells	FACS	Section 1A	Continue with Section 2 for all samples.
	Non-FACS	Section 1B	
Purified RNA		Section 1C	

1. Cell lysis or RNA dilution

A. *When acquiring cells with FACS*
(1) Prepare a cocktail of the cell lysis reagents in a single tube using the volumes listed in the table below. The volumes used will need to be scaled depending on the number of reactions you wish to perform.

	1 Reaction (µL)	20 Reactions (µL)*	100 Reactions (µL)*
①Lysis Buffer	2	44	220
②Lysis Enhancer	0.45	9.9	49.5
③RNase Inhibitor	0.05	1.1	5.5
④Nuclease-free water	0.5	11	55
Total	3	66	330

*An extra 10% in volume has been added here to account for pipetting error.

(2) Dispense 3 µL of cell lysis cocktail per well into a 96-well plate or 8-tube strip. Dispense on ice, and immediately after dispensing, seal with a qPCR seal or thermocompression seal.

(3) Store the plate on ice or at 4°C until sorting, and perform cell sorting according to the FACS user manual and the parameters recommended by the manufacturer.

(4) After sorting, seal the plate or tubes and centrifuge briefly to collect the solution at the bottom of the wells/tubes.

(5) Proceed immediately to the next step or store the samples at −80℃.

B. *Cell acquisition using methods other than FACS*

When obtaining the cells by manual picking, etc., the volume of the cell sample should be 0.5 µL or less.

(1) Prepare the reagents necessary for cell lysis in one tube per reaction using the volumes listed in the table below, and then add the cell samples.

If the volume of the cell sample is less than 0.5 µL, adjust the amount of water added when preparing the cell lysis reaction.

	1 Reaction (µL)
①Lysis Buffer	2
②Lysis Enhancer	0.45
③RNase Inhibitor	0.05
Sample	~0.5
④Nuclease-free water	X (adjust as needed based on sample volume)
Total	3

Example: When the cell sample volume is 0.5 µL

	1 Reaction (µL)
①Lysis Buffer	2
②Lysis Enhancer	0.45
③RNase Inhibitor	0.05
Sample	0.5
Total	3

(2) Dispense 3 µL of each cell lysis reaction per well into a 96-well plate or 8-tube strip. Dispense on ice, and immediately after dispensing, seal with a qPCR seal or thermocompression seal.

(3) Seal the plate or tubes and centrifuge briefly to collect the solution at the bottom of the wells/tubes.

(4) Proceed immediately to the next step or store the samples at −80℃.

C. *When using purified total RNA*

(1) Prepare the reagent cocktail using the volumes listed in the table below. The volumes used will need to be scaled depending on the number of reactions you wish to perform. For example:

Preparation of cell lysis cocktail

	100 Reactions (μL)*
①Lysis Buffer	220
②Lysis Enhancer	49.5
③RNase Inhibitor	5.5
④Nuclease-free water	55
Total	330

*An extra 10% in volume has been added here to account for pipetting error.

RNA dilution to obtain 1 ng input

	(μL)
E.g., 10 ng/μL total RNA	1
Nuclease-free water	29
Total	30

Use 3 μL of the diluted 333pg/μL total RNA solution per reaction

RNA dilution to obtain 10 pg input

	(μL)
E.g., 333 pg/μL total RNA	1
Nuclease-free water	99
Total	100

Use 3 μL of the diluted 3.3 pg/μL total RNA solution per reaction.

(2) Dispense 3 μL of the reagent cocktail per well into a 96-well plate or 8-strip tube

(3) After dispensing, seal and centrifuge briefly to collect the solutions at the bottom of the wells/tubes.

(4) Proceed immediately to the next step or store the samples at −80℃.

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2. Denaturation

(1) Centrifuge the sample plate or tubes at 4℃ and incubate as described in the table below.

Note: When using frozen samples, thaw the samples first at 4℃ before heat denaturation and then proceed to heat denaturation after brief centrifugation.

Step	Temperature	Time
Denature	70℃	1.5 min.
Hold	4℃	∞

3. Digestion of genomic DNA

(1) Prepare a cocktail of the reagents necessary for genomic DNA removal using the volumes listed in the table below. The volumes used will need to be scaled depending on the number of reactions you wish to perform.

Note: When using control RNA such as ERCC RNA, adjust the volume of nuclease-free water added.

	1 Reaction (μL)	20 Reactions (μL)*	100 Reactions (μL)*
⑤RT-RamDA™ Buffer	0.3	6.6	33
⑧gDNA Remover	0.45	9.9	49.5
④Nuclease-free water	2.25	49.5	247.5
Total	3	66	330

*An extra 10% in volume has been added here to account for pipetting error.

(2) Add 3 μL of the genomic DNA removal cocktail per well to the 96-well plate or 8-tube strip that contains the heat-denatured samples from the previous step. Centrifuge briefly and incubate at the following temperatures.

Step	Temperature	Time
Genomic DNA digestion	30℃	5 min.
Hold	4℃	∞

(3) Proceed immediately to the next step after this reaction.

4. cDNA synthesis and amplification reaction (RT-RamDA™ reaction)

(1) Prepare a cocktail of the necessary reagents for the RT-RamDA™ reaction in a single tube using the volumes listed in the table below. The volumes used will need to be scaled depending on the number of reactions you wish to perform.

Note: When using the NSR Primer, use the 1st NSR Primer Mix for human samples or the 1st NSR Primer Mix for mouse samples instead of the RT-RamDA™ Primer Mix.

	1 Reaction (μL)	20 Reactions (μL)*	100 Reactions (μL)*
⑤RT-RamDA™ Buffer	1.5	33	165
⑥RT-RamDA™ Enzyme Mix	0.45	9.9	49.5
⑦RT-RamDA™ Primer Mix	0.45	9.9	49.5
④Nuclease-free water	0.6	13.2	66
Total	3	66	330

*An extra 10% in volume has been added here to account for pipetting error.

(2) Add 3 μL of the RT-RamDA™ cocktail solution per well to the 96-well plate or 8-tube strips in which the genome removal reactions were performed. After brief centrifugation, incubate the plate/tubes at the following temperatures.

Step	Temperature	Time
Priming 1	25°C	10 min.
Priming 2	30°C	10 min.
Reverse transcription and amplification	37°C	30 min.
Reverse transcription 2	50°C	5 min.
Inactivation	98°C	5 min.
Hold	4°C	∞

(3) Proceed immediately to the next step after the RT-RamDA™ reaction or store at −20°C to −30°C.

5. 2nd strand synthesis reaction

(1) Prepare the reagents necessary for the 2nd strand synthesis reaction in a tube using the volumes listed in the table below. The volumes used will need to be scaled depending on the number of reactions you wish to perform.

Note: When using the NSR Primer, use the 2nd NSR Primer Mix for human samples and the 2nd NSR Primer Mix for mouse samples instead of the 2nd strand Synthesis Primer Mix.

	1 Reaction (μL)	20 Reactions (μL)*	100 Reactions (μL)*
⑨2nd strand Synthesis Buffer	3	63	315
⑩2nd strand Synthesis Enzyme	0.5	10.5	52.5
⑪2nd strand Synthesis Primer Mix	2.5	52.5	262.5
Total	6	126	630

*An extra 5% in volume has been added here to account for pipetting error.

(2) Add 6 μL of the 2nd strand synthesis cocktail per well of the 96-well plate or 8-tube strips that contains the RT-RamDA™ reactions and incubate at the following temperatures after brief centrifugation.

Step	Temperature	Time
Second-strand synthesis	16℃	60 min.
Denaturation	70℃	10 min.
Hold	4℃	∞

(3) Proceed immediately to the next step after the 2nd strand synthesis reaction or store the reactions at −20°C to −30°C.

6. Library preparation

Important: The library preparation protocol for use with this kit now recommends using 1/4 of capacity typically recommended by Illumina for use with their Nextera™ XT DNA Library Prep Kit. Please note that this change is included in the following protocol and is only applicable to this kit.
Please refer to the Illumina Nextera™ XT DNA Library Prep Kit manual for an in-depth description of the library preparation procedure.

A. Purification of double-stranded cDNA

(1) Make 1/4 diluted AMPure XPbeads before the purification step.When diluting, centrifuge AMPure XP beads and use the supernatant as diluent.

Example) How to make 1/4 diluted AMPure XP beads for 8 samples
a. Place 1 × AMPure XP Beads 220 µL on a magnetic stand, and when the magnetic beads are completely absorbed, use the supernatant as a diluent.
b. Mix 180 µL of the supernatant prepared in step a with 60 µL of 1 × AMPure XP beads and use as 1/4 diluted AMPure XP beads.

(2) Add 27 µL of 1/4 diluted AMPure XP beads to 15 µL of double-stranded cDNA solution. After that, centrifuge, at 2,000 rpm for 2 minutes at 25 ° C, and let stand for 5 minutes.

(3) The reaction tubes or 96-well plate is placed on the magnetic stand to allow the solutions to become clear. Remove the supernatant by careful pipetting.

(4) Add 150 µL of 80% ethanol per well/tube, mix briefly by pipetting the bead suspension up and down five times, and incubate the solutions for 30 sec at room temperature.

(5) Place the tubes or plate back onto the magnetic stand and wait until the solutions become clear. Remove the ethanol by careful pipetting.

(6) Add 150 µL of 80% ethanol per well/tube, mix briefly by pipetting five times, and incubate the solutions for 30 sec at room temperature.

(7) Place the tubes or plate back onto the magnetic stand and wait until the solutions become clear. Remove the ethanol by careful pipetting.

(8) To remove any residual ethanol, leave the tubes or plate open on the magnetic stand at room temperature to dry for up to 5 min. or until the magnetic beads begin to visibly crack (if any ethanol remains, the subsequent reactions may not be successful). Adjust the drying time according to the ambient temperature and humidity in your laboratory.

(9) Prepare the reagents necessary for elution in a separate tube while waiting for the beads to dry, using the volumes listed in the table below. The volumes used will need to be scaled depending on the number of reactions you wish to perform.

	1 Reaction (μL)	100 Reactions (μL)*
Tagment DNA Buffer (Nextera™ XT DNA Library Prep Kit)	2.5	270
④Nuclease-free water	1.25	135
Total	3.75	405

*An extra 8% in volume has been added here to account for pipetting error.

(10) Add 3.75 μL of the elution cocktail to each reaction in the 96-well plate or 8-tube strips and briefly centrifuge.

(11) Vortex until the beads are completely dispersed, incubate at 25°C for 5 min., and briefly centrifuge to collect the suspension at the bottom of the wells/tubes.

(12) Place the tube or plate on the magnetic stand for 5 minutes or until the solutions have cleared.

(13) Transfer 3.75 μL of the clear supernatant to new tubes or a plate and proceed to the next step.

B. Tagmentation (Nextera™ XT DNA library preparation)

(1) Add 1.25 μL of Amplicon Tagment Buffer (Nextera™ kit) to each of the 3.75 μL solutions containing purified double-stranded cDNA. Centrifuge briefly and incubate at the following temperatures.

Step	Temperature	Time
Tagmentation	55℃	5 min.
Hold	10℃	∞

C. Neutralization (Nextera™ XT DNA library preparation)

(1) Add 1.25 μL per well of Neutralization Buffer to the tagged double-stranded cDNA prepared in the previous step. Centrifuge briefly, incubate at 25°C and 2,000 rpm for 1 min., and then let the reactions stand for 5 min.

D. Library enrichment (Nextera™ XT DNA library preparation)

(1) Add 3.75 µL of NPM PCR Master Mix to each reaction containing the neutralized double-stranded cDNA and briefly centrifuge. Then, add 1.25 µL each of the index primers S5XX and S7XX to each well and briefly centrifuge.

(2) After centrifugation, perform PCR with the following cycling conditions.

Step	Temperature	Time	No. of cycles
Anneal	72℃	3 min.	1
Denature	95℃	30 sec.	1
PCR	95℃ 55℃ 72℃	10 sec. 30 sec. 30 sec.	14*
Extension	72℃	5 min.	1
Hold	4℃	∞	1

*Change the number of PCR cycles according to the table below.

Estimated amount of total RNA per cell/total RNA amount	Typical number of PCR Cycles
>10 pg	14
5–10 pg	14–15

*If the amount of input RNA is less than 5 pg, increase the number of PCR cycles.
Note: If the library yield is too high, the number of PCR cycles can be reduced.

E. Library purification (Nextera™ XT DNA library preparation)

(1) Add 15 µL of 1× AMPure XP beads to 12.5 µL of each PCR reaction. Centrifuge briefly, incubate for 2 min. at 25℃ and 2,000 rpm, and then let the reactions stand for 5 min.

(2) Place the tubes or plate on a magnetic stand and allow the solutions to clear. Remove the supernatants by careful pipetting.

(3) While still on the magnetic stand, add 150 µL of 80% ethanol to each well/tube, mix briefly by pipetting five times, and incubate at room temperature for 30 sec.

(4) Remove the ethanol by careful pipetting.

(5) With the tubes or plate on the magnetic stand, add 150 µL of 80% ethanol per sample, mix briefly by pipetting five times, and incubate at room temperature for 30 sec.

(6) Remove the ethanol completely by careful pipetting.

(7) Leave the tubes or plate on the magnetic stand and allow the beads to dry at room temperature for 2 min. or until the magnetic beads begin to crack (if any ethanol remains, the subsequent reactions may not be successful). Adjust the drying time according to the ambient temperature and humidity of your laboratory.

(8) Add 24 µL of TE Buffer per well/tube and briefly centrifuge.

(9) Vortex until the beads are completely dispersed, incubate at 25°C for 2 min. and then briefly centrifuge.

(10) Place the tubes or plate on the magnetic stand and allow to stand for 2 min. until the solutions clear.

(11) Transfer 24 µL of the clear supernatant to a new plate or tubes and proceed to the QC step or store at −20°C.

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7. Library QC

Library quantification

Library quantification can be performed using 7. Library QC

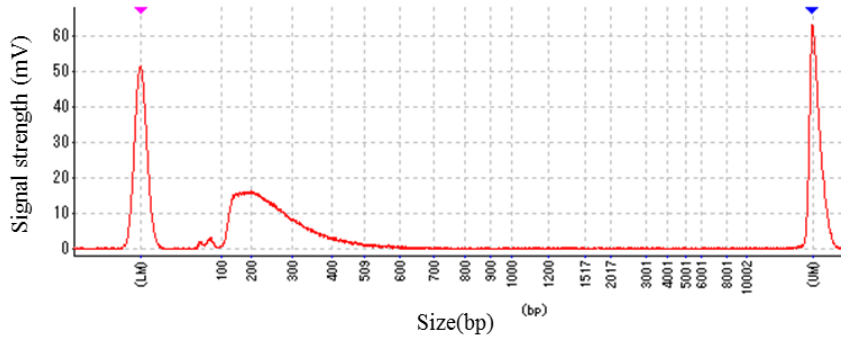
Use the Toyobo GenNext™ NGS Library Quantification Kit (Cat. No. NLQ-101) or equivalent. This kit allows the specific and accurate quantification of libraries bearing P5 and P7 adapters and can be applied to flow cell amplification.

Library quality control

Assess the quality of the library using a capillary electrophoresis device such as an Agilent Bioanalyzer or equivalent.

Library distribution example

When using the GenNext™ RamDA-seq™ Single Cell Kit together with the Nextera™ XT DNA Library Preparation Kit (14 cycles), and when preparing a library from mouse ES cells for, on average, a single cell per reaction, the library distribution was confirmed to be in the range of 100–600 bp as shown in the figure below. An eluted library concentration of 10–20 nM (with an elution volume of 24 µL in TE buffer) was obtained.



8. Sequence QC

It is recommended to check the coverage uniformity before analyzing the sequence data. With this kit, it is possible to obtain uniform coverage as shown below.

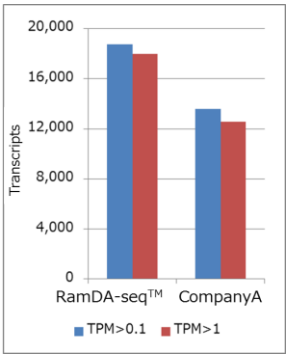
[4] Application data

1. Comparison of the number of detected transcripts and the alignment ratio within each region

<Method> Using next generation sequencing data, cDNA and double-stranded DNA were prepared using either the GenNext™ RamDA-seq™ Single Cell Kit (Cat. No. RMD-101) or a Company A kit, with a sample input of 10 pg of total RNA extracted from mouse ES cells. The GenNext™ RamDA-seq™ Single Cell Kit uses the NSR Primer Set for mouse samples (NSR-102). PCR for cDNA amplification, which is not required with the GenNext™ RamDA-seq™ Single Cell Kit, was performed for 18 cycles with the Company A kit. The libraries were then prepared with the Nextera™ XT DNA Library Preparation Kit and sequenced with the Illumina MiSeq instrument.

<Result>

RNA sourse		mouse ES RNA 10pg			
Percentage of reads(%)		RamDA-seq™		CompanyA	
Mapped reads		90.9	86.1	89.5	90.7
rRNA+mtRNA		24.0	23.0	9.0	9.9
CDS		27.8	26.3	41.9	41.5
UTR		16.5	15.8	21.9	22.3
Introns		16.2	14.8	9.1	9.0
Intergenic regions		6.4	6.2	7.6	8.0
Trans cripts	TPM>0.1	18,984	18,465	13,500	13,721
	TPM>1	18,338	17,629	12,575	12,616

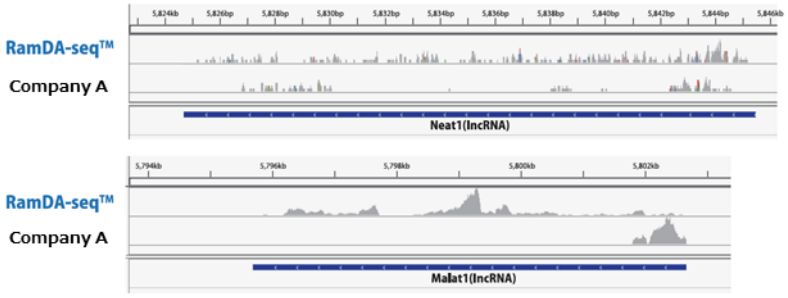


Thus, the GenNext™ RamDA-seq™ Single Cell Kit was shown to detect approximately 5,000 more genes than the Company A kit.

2. Comparison of the full-length coverage of long RNA molecules

<Method> Analysis was performed on the sequence data obtained with the method described for Example 1.

<Result>



The GenNext™ RamDA-seq™ Single Cell Kit can detect lncRNAs such as *Neat1* and *Malat1*, which were difficult to capture with the Company A kit over their entire length.

[5] Troubleshooting

Symptom	Cause	Solution
Low cDNA yield	RNA is degraded	· Check that the RNA is not degraded. · Check that RNase has not contaminated the tips or tubes used. · Perform all cell lysis and RNA dilution steps on ice.
Low library yield	Ethanol remaining after purification	· If ethanol remains among the magnetic beads after washing with ethanol, subsequent enzyme reactions may be hindered. Make sure that the magnetic beads are dry. · If the humidity in the laboratory is high, it may be difficult to dry the beads. It is recommended that the humidity in the work area be 55% or less.

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[6] Related products

Product name	No. of reactions	Catalog No.
RT-RamDA™ cDNA Synthesis Kit cDNA prep kit for qPCR	96	RMD-201
	24	RMD-201T
RamDA Cell Lysis Kit	1,152	RMD-301
NSR Primer Set for human simples	96	NSR-101
NSR Primer Set for mouse simples	96	NSR-102
GenNext™ NGS Library Quantification Kit For the quantification of NGS libraries prepared with Illumina™ instruments	500	NLQ-101

Reference (1)

Tetsutaro Hayashi*, Haruka Ozaki*, Yohei Sasagawa, Mana Umeda, Hiroki Danno and Itoshi Nikaido. Single-cell full-length total RNA sequencing uncovers dynamics of recursive splicing and enhancer RNAs. Nature Communications. 2018.