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LeukoComplete™

Cellular immunity assay kit based on gene detection

- LeukoComplete™ Plate and RT Kit
- LeukoComplete™ Antigen Coated Plate
- LeukoComplete™ Gene Detection Kit

Instruction for Use

Introduction

Thank you for purchasing this kit.
We recommend the methods described in this instruction manual for use.
This product is sold **for research purposes only** and cannot be used for any other purpose.

LeukoComplete™

A series of LeukoComplete™ products is specifically designed to evaluate cell-mediated immune response by quantifying mRNA. LeukoComplete™ Plate and RT Kit isolates immune cells from sample, extracts the mRNA, and synthesizes cDNA from the mRNA as the template. Multiple mRNA genes of interest can be quantified with the synthesized cDNA by LeukoComplete™ Gene Detection Kit.
As a practical application, we report the kit is useful for the implementation of Ex vivo Activation of Gene in Leukocyte (EAGL) method.[1] The high performance with clinical samples is realized with our 2 proprietary plates: Leukocyte Isolation Plate and mRNA Capture Plate in LeukoComplete™ Plate and RT Kit.[2]

Feature

<High sensitivity & Data quality>

- mRNA amplification and quantification by RT-qPCR
- Detection of immune response with <100 μL sample/well
- Antigen stimulation time as short as 4 hours
- Clinical performance comparable to ELISpot assay

<High throughput>

- The same day assay; at least 100 samples per day

<Variety of sample and target gene>

- Whole blood compatible
- cDNA sample for multiple qPCR assay
- Selection of genes of interest by using widely available primer-design tools

<Ready to use>

- No dedicated instrument
- All-in-one kit that is extensively validated for use of cell-mediated immune response assay

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1. Kit Contents

• LeukoComplete™ Plate and RT Kit

Component	Volume	Storage Temp.
PT Buffer	15 mL × 1	2 - 30℃
Leukocyte Isolation Plate (Filter Plate)	1	2 - 30℃
Deep Well Plate	1	2 - 30℃
Proteinase K	30 µL × 1	2 - 30℃
TCEP	300 µL × 1	2 - 30℃
Lysis Buffer	6 mL × 1	2 - 30℃
mRNA Capture Plate	1	2 - 30℃
Wash Buffer A	30 mL × 1	2 - 30℃
Wash Buffer B	50 mL × 1	2 - 30℃
Aluminum Seal	1	2 - 30℃
M-MLV Reverse Transcriptase	40 µL × 1	≦-20℃
RNase Inhibitor	10 µL × 1	≦-20℃
RT Buffer	1.5 mL × 2	≦-20℃

• LeukoComplete™ Antigen Coated Plate

Component	Volume	Storage Temp.
Antigen Coated Plate for SARS-CoV-2*	1 (24 test)	2 - 30℃

* Overlap peptide pool for spike protein from SARS-CoV-2 (15 a.a, 11 overlap).
Each test requires 4 wells (blank, 2 antigens, and positive control).

• LeukoComplete™ Gene Detection Kit

Component	Volume	Storage Temp.
Primer Mix*	100 µL × 1	≦-20℃
PCR Enzyme Mix	500 µL × 1	≦-20℃

* Independent Cat. # is assigned to each gene.

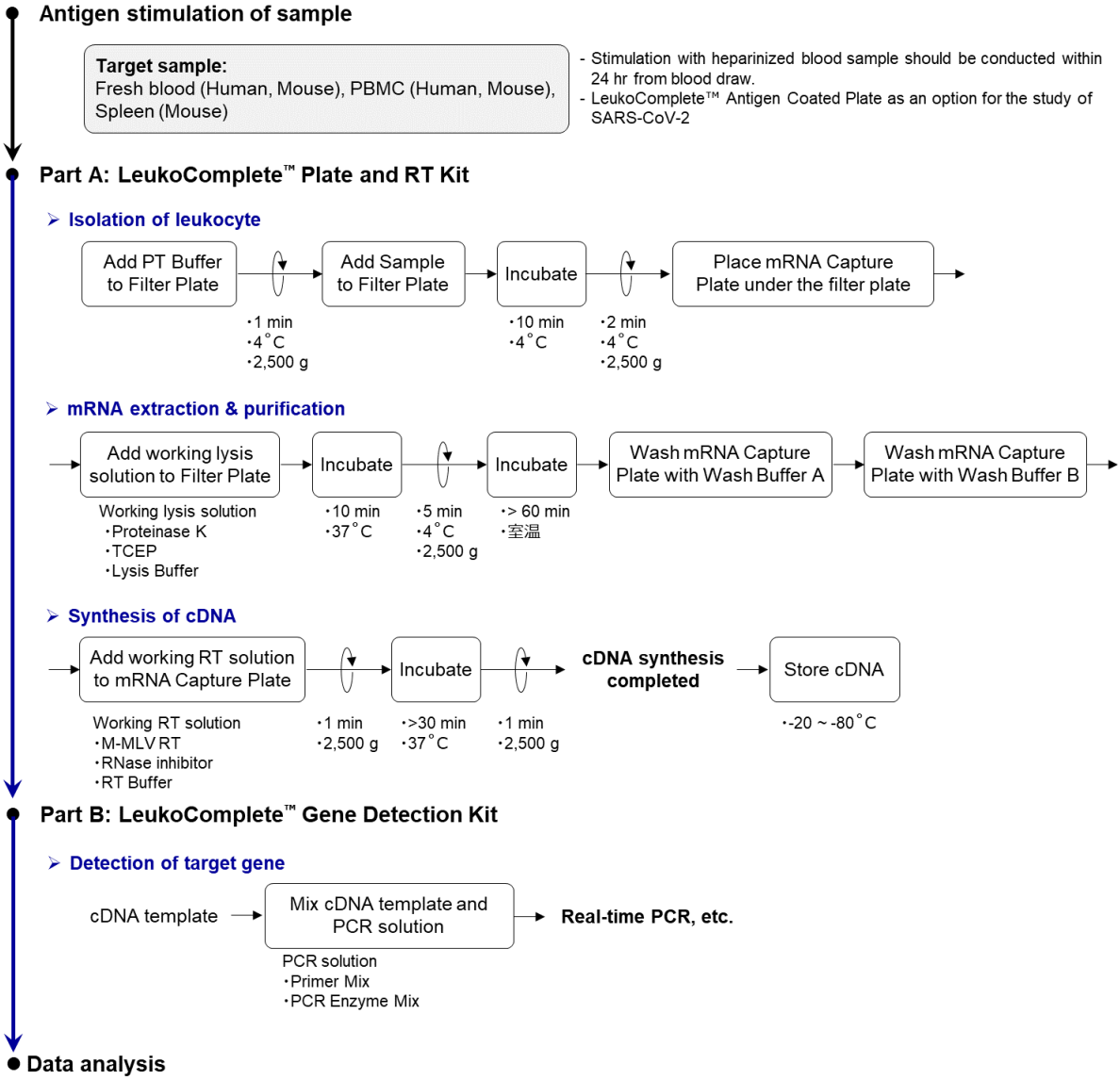
2. Equipment and Supplies

- **Equipment**
  - Centrifuge with microplate carriers and rotor
  - Incubator
  - Real-time PCR instrument
    (Applied Biosystems 7500 Fast Real-Time PCR System (Thermo Fisher Scientific Inc.), etc.)

- **Supplies**
  - Ice pan
  - 8 channel pipettor
  - Tip with hydrophobic filter
  - Reservoir
  - 96 well cell culture plate
  - 96 well reaction plate for real-time PCR
  - 8 channel aspirator (recommended)

3. Protocol Overview

Described below is a workflow with LeukoComplete™ products comprising 2 parts: cDNA preparation with LeukoComplete™ Plate and RT Kit (Part A) and RT-qPCR with LeukoComplete™ Gene Detection Kit (Part B).



Please refer to the following for the approximate time required for each process.

Part A: 180 min

Part B: 60 min/run (gene)

4. Sample preparation and Antigen stimulation methods

➢ **Target Sample**

- Whole blood* : Human, Mouse
- Peripheral blood mononuclear cell (PBMC) : Human, Mouse
- Spleen Crushing Solution : Mouse

* To evaluate immune response, use heparinized blood collection tube and the fresh blood for your assay within 24 hours from blood draw.

➢ **Antigen Stimulation Methods**

When evaluating immune response, please consider the stimulation conditions (time, concentration, target gene) in advance.

1. Antigen solution dissolved to any concentration, blank and positive control (see Table 1) are dispensed into 96-well cell culture plate.
   （Antigen Coated Plate for SARS-CoV-2 (see Figure 1) can be used instead.）

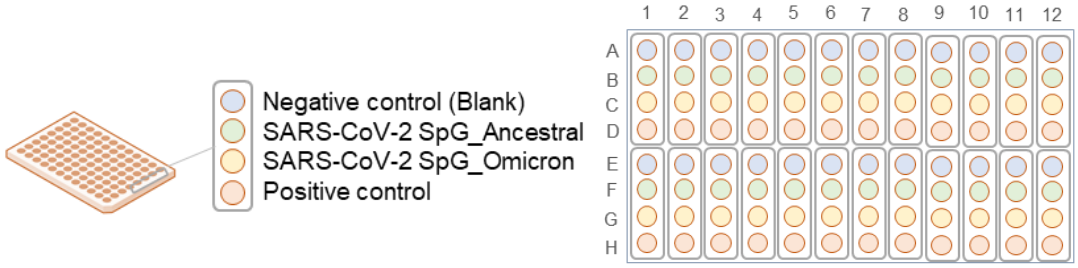


Figure 1. Antigen Coated Plate for SARS-CoV-2

2. Dispense an arbitrary amount* of fresh blood (unfrozen heparin blood within 24 hours of collection or other sample brought to room temperature into the plate prepared in step 1, and mix by pipetting.
   * Recommended amounts.
   - Fresh blood : 50 - 120 µL / well
   - PBMC / Spleen crushing solution : 0.25 - 1.0×10^6 cells /well

   When using Antigen-Coated Plate for SARS-CoV-2, the peptide concentration in the reaction solution can be adjusted to 1 µg/mL by adding 100 µL/well of sample.
3. Incubate at 37℃ for 4 hours.
4. Perform Part. A or freeze samples at -80℃ after antigen stimulation.

Table 1. Selection of Positive Controls and Target Genes

Species / Genes \ Positive control antigen		PMA/IM	PHA	LPS	ConA	CEF	Cutoff of Ct
Human	*IFNG*	+++	++	+++	++	+	32 >
	IL2	+++	++	+/-	+/-	N.D.	34 >
	TNFSF2	++	+	++	+	+/-	32 >
	IL4	+++	++	N.D.	N.D.	N.D.	34 >
	IL5	++	+/-	N.D.	N.D.	N.D.	34 >
	IL6	++	++	+++	++	N.D.	34 >
	IL10	++	+	++	+/-	N.D.	34 >
	IL13	+++	++	+/-	+/-	N.D.	34 >
	CSF2	+++	++	++	++	N.D.	32 >
	CXCL10	N.D.	++	++	++	++	32 >
Mouse	*IFNG*	+++	+/-	+/-	++	–	–
	IL10	+++	++	N.D.	++	–	–
	IL17	++	N.D.	+/-	N.D.	–	–
Final conc. of each positive control		PMA: 100 ng/mL IM: 1000 ng/mL	10-100 µg/mL	1-100 µg/mL	100 µg/mL	1-10 µg/mL	Instrument: ABI Fast7500 Threshold line: 0.1

Human: 100 µL of fresh blood was mixed with each antigen and incubated for 4hr under 37°C.
Mouse: 10^6 cells of spleen was mixed with each antigen and incubated for 4hr under 37°C.

Abbreviation	
PMA	Phorbol 12-myristate 13-acetate
IM	Ionomycin
PHA	Phytohemagglutinin
LPS	Lipopolysaccharide
ConA	Concanavalin A
CEF	CEF control peptide pool

Rank	Mean relative mRNA expression (log_2FC, n=12)
+++	> 8
++	5-8
+	3-5
+/-	1.5-3
N.D.	1.5 >
–	Not evaluated

5. Part A: LeukoComplete™ Plate and RT Kit

Please refer to the components in the LeukoComplete™ Plate and RT Kit listed under Kit Contents.

➢ **Leukocyte Isolation**

1. Place the Leukocyte Isolation Plate (Filter plate) on the top of the Deep Well Plate (see Figure 2).
2. Add 150 µL/well of cold PT Buffer to the Filter Plate.
3. Centrifuge at 2,500 g for 1 min at 4℃.
4. Add the sample* to the Filter plate**.
   * If samples are stored frozen, incubate at room temperature for 30 min to dissolve.
   ** Sample volume should be less than 200 µL.
5. Incubate at 2 - 8℃ for 10 min.
6. Centrifuge at 2,500 g for 2 min at 4℃.
7. Remove the Deep well Plate and place the mRNA Capture Plate under the Filter Plate (see Figure 2).

➢ **Cell Lysis and mRNA Isolation**

8. Prepare the working lysis solution* by mixing Proteinase K, TCEP, and Lysis Buffer** (see Table 2).
   * Prepare more working lysis solution than the number of samples required.
   ** If Lysis Buffer have crystallization, warm up at 37℃ for a couple of hours to dissolve the crystallization.
9. Add 60 µL/well of working lysis solution to the Filter Plate and cover with lid.
10. Incubate at 37℃ for 10 min.
11. Centrifuge at 2,500 g for 5 min at 4℃.
12. Remove the Filter Plate and cover the mRNA Capture Plate with lid.
13. Incubate the mRNA Capture Plate with lid at room temperature for at least 1 hour.
14. Place the mRNA Capture Plate, Wash Buffer A* and Wash Buffer B on ice pan filled with ice**.
   * If Wash Buffer A have crystallization, warm up at 37℃ for a couple of hours to dissolve the crystallization.
   ** Keep the plate and Wash Buffer cold during the washing process from14 to 17.
15. Aspirate the lysates in the mRNA Capture Plate.
16. Add 100 µL/well of Wash Buffer A (cold) and aspirate. Repeat two more times (total of three times).
17. Add 150 µL/well of Wash Buffer B (cold), incubate for 3 min with a lid then aspirate. Repeat two more times (total of three times).
18. Remove any remaining liquid in the wells by tapping the plate face down several times on lint-free cloth.

➢ **Synthesis of cDNA**

19. Prepare the required amount* of working RT solution by mixing M-MLV Reverse Transcriptase (M-MLV RT), RNase Inhibitor, and RT Buffer (see Table 3).
    * Prepare more working RT solution than the number of samples required.
20. Add 30 µL/well of working RT solution to the mRNA Capture Plate and seal with Aluminum Seal.
21. Centrifuge at 2,500 g for 1 min at room temperature.
22. Incubate at 37℃ for 30 min.
23. Centrifuge at 2,500 g for 1 min at room temperature.
24. Perform Part B or store the mRNA Capture Plate containing cDNA template at -20 - -80℃.

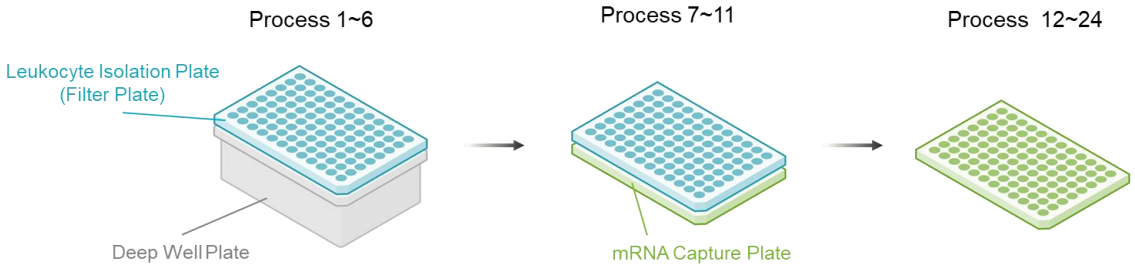


Figure 2. Placement of each plate

Table 2. Preparation of working lysis solution

Component	per well	*e.g.* 1 plate (100 well)
Proteinase K	0.3 µL	30 µL
TCEP	3.0 µL	300 µL
Lysis Buffer	56.7 µL	5670 µL
Working lysis solution	60 µL	6000 µL

Table 3. Preparation of working RT solution

Component	per well	*e.g.* 1 plate (100 well)
M-MLV Reverse Transcriptase	0.4 µL	40 µL
RNase Inhibitor	0.1 µL	10 µL
RT Buffer	29.5 µL	2950 µL
Working RT solution	30 µL	3000 µL

6. Part B: LeukoComplete™ Gene Detection Kit

Please refer to the components in the LeukoComplete™ Gene Detection Kit listed under Kit Contents.

➢ **Detection of target genes**

1. Prepare the required amount* of PCR solution by mixing Primer Mix** and PCR Enzyme Mix (see Table 4).
   * Prepare more PCR solution than the number of samples required.
   ** Primer Mix is different for each control gene and target gene.
   Please purchase the Gene Detection Kit for the gene of interest.
2. Apply 6 μL/well* of PCR solution (each gene corresponding well) to the 96-well PCR plate.
   * Add the appropriate sample volume for the real-time PCR system to be used.
   The total volume for the PCR reaction in this protocol is 10 μL per well.
3. Add 4 μL/well of the cDNA solution from Part A to each corresponding well.
4. Seal the PCR plate with optical film and centrifuge at 2,500 g for 1 min.
5. Set up real-time PCR instrument and run (see Table 5 for PCR parameters).

Table 4. Preparation of PCR solution

Component	per well	*e.g.* 1 plate (100 well)
Primer Mix	1.0 μL	100 μL
PCR Enzyme Mix	5.0 μL	500 μL
PCR solution	6.0 μL	600 μL

Table 5. Real-time PCR parameters

Fast Mode		
Stage 1	95℃, 20 sec	1 cycle
Stage 2	95℃, 3 sec 65℃, 30 sec	40 cycles
Melt curve stage		

Standard Mode		
Stage 1	95℃, 10 min	1 cycle
Stage 2	95℃, 30 sec 65℃, 1 min	40 cycles
Melt curve stage		

Equipment: Applied Biosystems 7500 Fast Real-Time PCR System (Thermo Fisher Scientific Inc.)

7. Results analysis

The comparative Ct method* allows relative comparison of target gene expression (see Figure 3).

Please refer to the following to calculate the change in expression of the target gene.

1. Calculate the ΔCt value for each sample.
   ΔCt = Ct [target gene] - Ct [control gene]
2. Calculate the change in expression of the target gene (-ΔΔCt) from ΔCt of the target sample and blank sample.
   -ΔΔCt = ΔCt [target gene of target sample] - ΔCt [target gene of blank sample]
3. The change in expression of the target gene is expressed as Log_2FC (Fold Change).
   -ΔΔCt = Log_2FC

*Comparative Ct method is a method of showing the expression of target gene relative to endogenous control gene.[3]

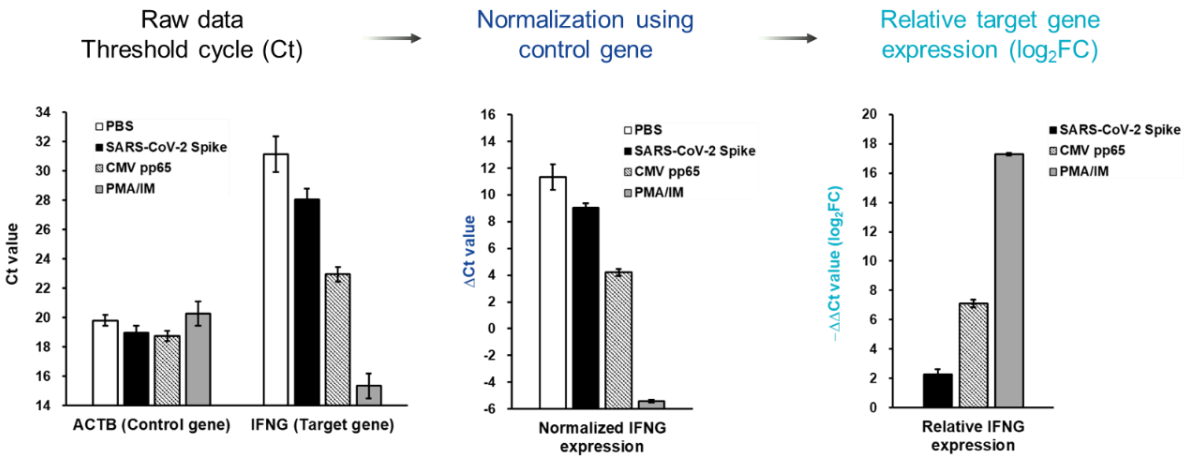


Figure 3. Analysis of comparative Ct method

8. Troubleshooting and Q&A

➢ **Troubleshooting**

- The target gene cannot be detected.

  (1) It is possible that the amount of cDNA synthesis has decreased or (2) the expression of the target gene is below limit of detection.

  In case of (1), we recommend reviewing some of the parameters in the protocol.

  ・The sample dissolution time was too long, resulting in mRNA degradation. (5.1)

  ・The mRNA was degraded due to insufficient cooling during the washing process. (5.14~5.17)

  ・Any liquid remained in the wells and the efficiency of cDNA synthesis decreased. (5.18)

  In case of (2), the following methods to improve detection sensitivity may be effective.

  ・Extend mRNA capture time to 16 hours. (5.13)

  ・Increase the amount of cDNA template when performing real-time PCR. (6.3)

  ・Perform real-time PCR in Standard mode instead of Fast mode. (6.5)

- The change in expression of the target gene cannot be confirmed.

  If the positive control is not detected, review the above. If the positive control is detected, it is recommended to review the stimulation conditions (time, concentration, target gene). When using PBMC or spleen crushing solution that has been frozen and stored, it is also effective to perform recovery culture or to add serum components to the stimulation medium components.

- Insufficient amount of cDNA template.

  Reduce the amount of cDNA template when performing real-time PCR. (6.3)

- Peptide antigens do not dissolve.

  After dissolving in an organic solvent such as DMSO, dissolve in a buffer such as PBS.

  However organic solvents such as DMSO may be cytotoxic, so please prepare a final concentration of less than 0.1%.

- Centrifugation at 2,500 g cannot be performed.

  Centrifugation can be performed at 2,000 g twice.

➢ **Q&A**

Q1. Can I use animal samples other than Human and Mouse?

A1. cDNA can be obtained from Rat, Rabbit, and Monkey PBMC.

Q2. Is it possible to detect other genes not included in this kit?

A2. This kit purifies mature mRNA with a Poly A tail in leukocytes. In theory, any genes in leukocytes can be detected by designing appropriate primers.

Q3. Do the results correlate with existing cellular immunity assays?

A3. We confirmed correlation of our method with ELISpot, ELISA, and flow cytometry with a correlation coefficient of about 0.5. You can find more details in the references [1] and on the kit website. However, it is necessary to consider that cellular immunity assay may fluctuate in measurement values due to

stimulation conditions, etc.

Q4. Is there an expiration date?

A4. We do not set an expiration date, but please use the product as soon as possible after opening.

9. Precautions

Handling precautions

- The use of heparin blood is recommended in cellular immunity assay.
- PBS, DMSO, or a mixture of the two is recommended as the solvent for the peptide solution. The concentration of DMSO in the sample should be less than 1%.
- Frozen reagents should be mixed well after dissolving and lightly spin down before use.
- Do not allow mRNA Capture Plate to keep for prolonged periods in between steps. Once the protocol has been initiated, proceed continuously to completion.
- Since detection by real-time PCR is extremely sensitive, extreme care should be taken to prevent contamination of the experimental environment and instruments.
- To avoid contamination of sample solutions and reagents, use tips with hydrophobic filters when dispensing sample solutions and reagents.
- Avoid contamination by microorganisms (even small amounts of microorganisms or nucleases contained in human sweat or saliva can degrade RNA and cause false results).
- When handling samples and this kit, wear a mask, gloves, lab coat, and protective eyewear, and take care to avoid contact of the reagents with skin, eyes, and mucous membranes. Wash hands thoroughly after handling.
- When handling the real-time PCR, follow the instruction manual of the respective device.
- When disposing of the container after use, please dispose of it separately as medical waste or industrial waste in accordance with the waste regulations.

Other Notes

- The specifications, design, contents, etc. of this kit are subject to change without notice.
- This kit is for research purposes only and should not be used for commercial, industrial applications, for clinical diagnosis, treatment, and services.
- Do not analyze or reverse engineer this kit.
- All rights (ownership, patents, and other intellectual property right, etc.) related to this kit belong to us.
- We are not responsible for any damages incurred by you or any other third party resulting from this kit.
- The suitability of this kit for any application, including legal compliance, is to be determined by the user.
- If you breach any of your obligations set forth herein, we will demand compensation from

you any damages incurred as a result of or in connection with such breach.

- You represent and warrant that you do not have and will not have any socially reprehensible relationships with antisocial forces, and that you will not act in violation of the laws and regulations of each country and region, including antibribery laws.

10. References

[1]. Saito, T., et al., Biochem Biophys Res Commun, 2023. 694: p. 149398.

[2]. Mitsuhashi, M., et al., Clin Chem, 2006. 52(4): p. 634-42.

[3]. Schmittgen, T.D. and K.J. Livak, Nat Protoc, 2008. 3(6): p. 1101-8.