For *in vitro* laboratory use only

March 1, 2010 Ver.1

# [Rat TSH ELISA Kit (R-type)]

(Code No.:AKRTS-010R)

Please, read this instruction carefully before use.

Contents



This kit is manufactured by Shibayagi Co., Ltd.

Use only the current version of Instruction Manual enclosed with the kit!

## 1. Intended use

Rat TSH ELISA kit (R-type) is a sandwich ELISA system for quantitative measurement of rat TSH (Thyroid-stimulating hormone), and is intended for research use only.

**Features**

(1) Highly sensitive assay with the standard range of 0.288~36ng/ml.
(2) This kit is for TSH in rat serum, culture medium and tissue extract.
(3) Assay sample volume is 10μl in the standard procedure.
(4) Assay format is 96 wells.
(5) Standard preparation is rat origin.
(6) Components of the kit are provided ready to use or in concentrated form.

## 2. Storage and expiration

When the complete kit is stored at 2-8°C, the kit is stable until the expiration date shown on the label on the box. Opened reagents should be used as soon as possible to avoid less than optimal assay performance caused by storage environment.

## 3. Introduction

TSH (Thyroid-stimulating hormone, Thyrotropin) is a glycoprotein hormone of 28kDa consisting of α- and β-subunits. The former is common to other pituitary glycoproteins, LH and FSH, while the latter has specific structure to TSH. TSH shows microheterogeneity due to the difference in sugar chain structure. TSH is produced and secreted by basophilic cells called thyrotrophs in the anterior pituitary throughout vertebrates. TSH enhances thyroid hormone synthesis and release by increasing inorganic iodide uptake and iodination of thyroglobulin in the thyroid gland, stimulates glucose utilization and degradation of lipid, and also causes exophthalmos. TSH secretion is promoted directly by TRH (thyrotropin-releasing hormone) and also indirectly by estrogen and insulin. Cold exposure stress increases blood TSH level. TSH secretion is lowered by somatostatin, thyroid hormones, GH, glucocorticoids, and β-endorphin, and also by stress and starvation. Blood TSH level shows diurnal variation.

## 4. Assay principle

In Shibayagi's Rat TSH ELISA Kit (R-type), biotin-conjugated anti-TSH and standard or sample are incubated in monoclonal anti-TSH antibody-coated wells. After 15~18 hours' incubation and washing, HRP (horse radish peroxidase)-conjugated avidin is added, and incubated for 30 minutes. After washing, HRP-complex remaining in

wells are reacted with a chromogenic substrate (TMB) for 30 minutes, and reaction is stopped by addition of acidic solution, and absorbance of yellow product is measured spectrophotometrically at 450 nm(sub-wavelength is 620nm). The absorbance is nearly proportional to TSH concentration. The standard curve is prepared by plotting absorbance against standard TSH concentrations. TSH concentrations in unknown samples are determined using this standard curve.

## 5. Precautions

- For professional use only, beginners are advised to use this kit under the guidance of experienced person.
- Wear gloves and laboratory coats when handling assay materials.
- Do not drink, eat or smoke in the areas where assays are carried out.
- In treating assay samples of animal origin, be careful for possible biohazards.
- This kit contains components of animal origin. These materials should be handled as potentially infectious.
- Be careful not to allow the reagent solutions of the kit to touch the skin, eyes and mucus membranes. Especially be careful for the reaction stopper because it is 1 M sulfuric acid. The reaction stopper and the substrate solution may cause skin/eyes irritation. In case of contact with these, wash skin/eyes thoroughly with water and seek medical attention, when necessary.
- The materials must not be pipetted by mouth.
- Unused samples and used tips should be rinsed in 1% formalin, 2% glutal aldehyde, or more than 0.1% sodium hypochlorite solution for more than 1 hour, or be treated by an autoclave before disposal.
- Dispose consumable materials and unused contents in accordance with applicable regional/national regulatory requirements.
- Use clean laboratory glassware.
- In order to avoid dryness of wells, contamination of foreign substances and evaporation of dispensed reagents, never forget to cover the well plate with a plate cover supplied, during incubation.
- ELISA can be easily affected by your laboratory environment. Room temperature should be at 20-25℃ strictly. Avoid airstream velocity over 0.4 m/sec. (including wind from air conditioner), and humidity less than 30%.

## 6. Technical tips

- For manual operation, proficiency in pipetting technique is recommended.
- The reagents are prepared to give accurate results only when used in combination within the same box. Therefore, do not combine the reagents from kits with different lot numbers. Even if the lot number is the same, it is best not to mix the reagents with those that have been preserved for some period.
- Be careful to avoid any contamination of assay samples and reagents. We recommend the use of disposal pipette tips, and 1 tip for 1 well.
- Optimally, the reagent solutions of the kit should be used immediately after reconstitution.
- Time the reaction from the pipetting of the reagent to the first well.
- Prepare a standard curve for each assay.
- Dilution of the assay sample must be carried out using the buffer solution provided in the kit.
- The chromogenic substrate (TMB) solution should be almost colorless before use. It turns blue during reaction, and gives yellowish color after addition of reaction stopper. Greenish color means incomplete mixing.
- To avoid denaturation of the coated antibody, do not let the plate go dry.
- As the antibody-coated plate is module type of 8wells x 12 strips, each strip can be separated by cutting the cover sheet with a knife and used independently.
- When ELISA has to be done under the airstream velocity of over 0.4 m/sec. and the humidity of less than 30%, completely close each well in addition to cover the well plate with a plate cover in each step of incubation.
  Ex.) Cover the well plate with parafilm, and put the plate cover on it. Or place the well plate with the plate cover in an incubator, or in a styrofoam box. Take the best way depending on situation of each laboratory.

## 7. Reagents supplied

| Components | State | Amount |
|---|---|---|
| (A) Anti-TSH-coated plate | Use after washing | 96 wells/1 plate |
| (B) Standard rat TSH (360ng/ml) | Concentrated. Use after dilution | 200 μl/1 vial |
| (C) Buffer solution | Ready for use. | 60 ml/1 bottle |
| (D) Biotin-conjugated anti-TSH | Concentrated. Use after dilution. | 50 μl/1 vial |
| (E) HRP-conjugated avidin | Concentrated. Use after dilution. | 100 μl/1 vial |
| (F) Chromogenic substrate reagent (TMB) | Ready for use. | 12 ml/1 bottle |
| (H) Reaction stopper (1M $H_2SO_4$) | Ready for use. | 12 ml/1 bottle |
| (I) Concentrated washing buffer (10x) | Concentrated. Use after dilution. | 100 ml/1 bottle |
| Plate cover | — | 1 plate |
| Instruction Manual | — | 1 copy |

Biotin-conjugated anti-TSH (D), and HRP-conjugated avidin (E): Vials contain more than volumes shown in the list so that you can take out 50 μl from vials.

## 8. Equipments or supplies required but not supplied

- Purified water (distilled water)
- Test tubes for preparation of standard solution series.
- Glassware for dilution of washing buffer (a graduated cylinder, a bottle)
- Pipettes (disposable tip type). One should be able to deliver 10 μl precisely, and another for 100-200 μl.
- Syringe-type repeating dispenser like Eppendorf multipette plus which can dispense 50-100 μl.
- Paper towel to remove washing buffer remaining in wells.
- A vortex-type mixer.
- A shaker for 96 well-plate (~800rpm)
- An automatic washer for 96 well-plate (if available), or a washing bottle with a jet nozzle.
- A 96 well-plate reader (450nm ±10nm, 620nm: 600-650nm)
- Software for data analysis, if available. Shibayagi is proposing the use of assay results calculation template for EXCEL. Please check our website (http://www.shibayagi.co.jp/en/tech_003.html).
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## 9. Preparation of reagents

- ✧ Bring all reagents of the kit to room temperature before use.
- ✧ Prepare reagent solutions in appropriate volume for your assay. Do not store the diluted reagents.
- ✧ Do not use the reagent after expiration date.

### ● Reagents ready for use after return to room temperature

[Anti-TSH-coated plate]

Storage and stability

If seal is not removed, put the strip back in a plastic bag with zip-seal originally used for well-plate container and store at 2-8 °C. The strip will be stable until expiration date.

[Buffer solution] and [Chromogenic substrate reagent]

Storage and stability

If not opened, store at 2-8 °C. It maintains stability until expiration date. Once opened, we recommend using them as soon as possible to avoid influence by environmental condition.

[Reaction stopper (1 M $H_2SO_4$)] *Careful handling, please.

Storage and stability

Close the stopper tightly and store at 2-8 ° C. It maintains stability until expiration date.

### ● Concentrated reagents

[Concentrated washing buffer (10x)]

Dilute 1 volume of the concentrated washing buffer (10x) to 10 volume with purified (distilled) water to prepare working solution. Example: 100 ml of concentrated washing buffer (10x) and 900ml of purified (distilled) water.

Storage and stability

The rest of undiluted buffer: if stored tightly closed at 2-8 °C, it is stable until expiration date.

Dispose any unused diluted buffer.

**[Master Standard rat TSH (360ng/ml)]**

Make a serial dilution of master standard solution to prepare each standard solution.

An example of preparation.

| Volume of standard solution | Buffer | Concentration (ng/ml) | Concentration (μIU/ml) |
|---|---|---|---|
| **Master solution 50μl** | 450μl | 36 | 1260 |
| 36ng/ml solution 200μl | 300μl | 14.4 | 504 |
| 14.4ng/ml solution 200μl | 200μl | 7.2 | 252 |
| 7.2ng/ml solution 200μl | 300μl | 2.88 | 101 |
| 2.88ng/ml solution 200μl | 300μl | 1.15 | 40 |
| 1.15ng/ml solution 100μl | 300μl | 0.288 | 10 |
| 0 (Blank) | 300μl | 0 | 0 |

*Conversion to IU is based on 35IU/mg.

Storage and stability

Standard solutions prepared above should be used as soon as possible, and should not be stored.

The master standard solution, if stored tightly closed at 2-8 °C, it is stable until expiration date.

[Biotin-conjugated anti-TSH]

You can take out 50μl of (D). Prepare working solution by dilution of (D) with the buffer solution (C) to 1:200.

Storage and stability

Unused working solution (already diluted) should be disposed. If you used only a part of (D), the rest of (D) is stable until expiration date, if stored tightly closed at 2-8 °C.

[HRP-conjugated avidin]

You can take out 50µl of (E). Prepare working solution by dilution of (E) with the buffer solution (C) to 1:200.

Storage and stability

Unused working solution (already diluted) should be disposed. If you used only a part of (E), the rest of (E) is stable until expiration date, if stored tightly closed at 2-8 °C.

## 10 Preparation of assay samples

This kit is principally intended to measure TSH in rat serum and plasma. Tissue extracts and incubation or culture media can be also assayed for TSH if confirmed by assay validation tests.

○For preparation of plasma, we recommend to use EDTA-2Na at final concentration of 1mg/ml.

○Deep ether anesthesia at blood sampling may lower blood TSH level, we recommend barbiturates as anesthesia, though they also influence hypothalamus to minimize TRH release (Endocrinology 99: 875-880, 1975).

○High hemolysis (more than 120mg/dl hemoglobin) may influence assay results.

○If sample is turbid or contains insoluble materials, centrifuge and use clear supernatant fluid.

○Organic solvents may influence assay results.

○Assay samples soon after preparation.

○Sample dilution should be carried out with the buffer solution of the kit using small test tubes before assay. Mix well, and pipette 50 µl of diluted sample into a well. In the standard assay procedure, the dilution rate is 5x. The minimum dilution rate is 2.5x. Undiluted (neat) serum and plasma are not suitable because their pH may influence assay results.

○Frozen stored samples should be thawed just before assay and mixed well to make them homogenous.

○If the presence of any interfering substances is suspected, confirm dilution linearity using more 2 different dilutions or more.

Stability and storage of samples

In immediate assay, samples can be kept in a refrigerator, and brought to room temperature just before assay. If they have to kept for a long period, tightly close the container and store lower than –35°C. Avoid repeated freezing and thawing.

●Testing for the compatibility of your samples with Shibayagi's kit using a positive sample.

Due to various factors of your sampling conditions (anesthetics, preservatives, anticoagulants, sample pH shift caused by loss of $CO_2$ during storage, evaporation and condensation during storage, etc), sometimes the kit does not work well with your samples. If the standard curve is in a good shape, while your samples give low absorbance, please check the compatibility of your samples (serum, plasma, or culture medium) by a simple recovery test as follows.

Place 90 µl of your sample (e.g. a sample from control group in your experiment) in a small test tube, then add 10 µl of the highest standard solution (50pg/ml). Assay this mixture together with the original sample, and compare the assay values. The assay value of the mixture will be around [0.9 x original sample + 0.1 x highest standard concentration]. If the assay value is increased as expected, the assay system is working well with your sample.

Especially when you use Shibayagi's kit for the first time, we recommend you to run this simple recovery test.

●Quality control samples

We recommend preparing quality control samples of your own laboratory by storing many aliquots of serum, plasma or culture medium with known amount of the substance to be measured after initial testing. Keep them in small and tightly capped sample tubes below -35 °C. If the sample tube is too big, water will be lost during storage. If possible, prepare high and low controls.

Measure these control samples along with your samples in every run to confirm the reproducibility and successful performance of the assay system.

## 11.1 Assay procedure

○ **Peel off the cover sheet from the plate after the plate is brought back to room temperature.**

**First day**

(1) Bring back all the necessary reagents (plate, buffer solution, standard solutions, biotin-labeled anti TSH, washing buffer) to room temperature.

(2) Wash the anti-TSH coated plate (A) by filling the wells with washing buffer and discard 4 times*, then strike the plate upside-down onto several layers of paper towels to remove buffer residual buffer in the wells.

(3) Pipette 50µl of biotin-conjugated anti-TSH (diluted 200x) to all wells, and shake the plate gently on a plate shaker (800rpm for 10 seconds x 3 times).

(4) Pipette 50µl of standard solution to the wells designated for standards, and Pipette 50µl of sample to the designated sample wells.

(5) Shake the plate gently on a plate shaker (800rpm for 10 seconds x 3 times).

(6) Put the plate cover on the plate and incubate for 15~18 hours at 2~8°C.

**Second day**

(7) Bring the buffer solution to room temperature, and dilute HRP-conjugated avidin solution to 200x.

(8) Discard the reaction mixture in the wells, and rinse wells by filling the wells with washing buffer and discard 4 times, then strike the plate upside-down onto several layers of paper towels to remove buffer residual buffer in the wells.

(9) Pipette 100µl of HRP-conjugated avidin solution to all wells, and shake the plate gently on a plate shaker (800rpm for 10 seconds x 3 times).
(10) Put a plate cover on the plate and incubate for 30 minutes at room temperature (20~25°C).
(11) Discard the reaction mixture. Rinse wells by filling the washing buffer and discard 4 times, then strike the plate upside-down onto folded several sheets of paper towel to remove buffer drops remaining in wells.
(12) Pipette 100µl of chromogenic substrate to wells, and shake the plate gently on a plate shaker (800rpm for 10 seconds x 3 times).
(13) Put a plate cover on the plate and incubate the plate for 20 minutes at room temperature.
(14) Add 100 µl of the reaction stopper to all wells, and shake the plate gently on a plate shaker (800rpm for 10 seconds x 3 times). After shaking, measure the absorbance of each well at 450 nm (reference wavelength, 620*nm) using a microplate reader within 30 minutes. (600~650nm is allowed for reference wavelength:)

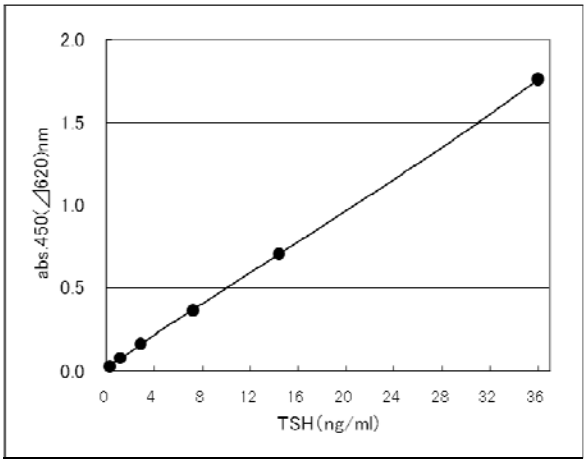
*Detailed description about manual washing is shown in "Shibayagi's Manual Operation" and "How to operate Shibayagi's ELISA Kits (Power point)" on our Home Page. Volume of washing buffer is about 300 µl/well. Setting of pressure for automatic washer is 5~25ml/min depending on nozzle diameter. Be careful not to dry plate after discarding liquid.

An example of work sheet

| | Strip 1&2 | Strip 3&4 | Strip 5&6 | Strip 7&8 | Strip 9&10 | Strip 11&12 |
|---|---|---|---|---|---|---|
| A | 36 ng/ml | Sample 1 | Sample9 | Sample17 | Sample25 | Sample33 |
| B | 14.4 ng/ml | Sample2 | Sample10 | Sample 18 | Sample26 | Sample34 |
| C | 7.2ng/ml | Sample3 | Sample11 | Sample19 | Sample27 | Sample35 |
| D | 2.88 ng/ml | Sample4 | Sample12 | Sample20 | Sample28 | Sample36 |
| E | 1.15ng/ml | Sample5 | Sample13 | Sample21 | Sample29 | Sample37 |
| F | 0.288ng/ml | Sample6 | Sample14 | Sample 22 | Sample30 | Sample38 |
| G | 0 | Sample7 | Sample15 | Sample23 | Sample31 | Sample39 |
| H | Positive control | Sample8 | Sample16 | Sample24 | Sample32 | Sample40 |

## 12. Calculations

(1) Prepare a standard curve in every assay by plotting absorbance* (Y-axis) against TSH concentration (ng/ml) on X-axis.
*Absorbance at 450nm minus absorbance at 620nm.
(2) Using the standard curve, read TSH concentration of samples at their absorbance, and multiply the assay value by dilution rate.
Though the assay range is wide enough, in case the absorbance of some samples is higher than that of the highest standard, please repeat the assay after proper dilution of samples with the buffer solution.
(3) We recommend the use of 3rd order regression curve for log-log plot, or 4 parameters method for log-normal plot in computer calculation. We are providing a convenient assay calculation template for EXCEL on our home page.
(4) Physiological or pathological situation of animals should be judged comprehensively taking other examination data into consideration.




**TSH assay standard curve (an example above)**
Absorbance may change due to assay environment.

## 13. Performance characteristics

● Assay range

The assay range of the kit is 0.288~36 ng/ml. (for 5x sample dilution, 1.44~180 ng/ml)
If some samples show absorbance more than that of 36 pg/ml standard, please repeat the assay after proper dilution of samples.

● Specificity

Monoclonal antibodies specific to TSH are used in this kit.
Reactivity for closely related substances to TSH is shown below.

| Substance | Cross reactivity | Substance | Cross reactivity |
|---|---|---|---|
| Rat TSH | 100% | Rat FSH | No cross reaction |
| Rat LH | No cross reaction | Rat GH | No cross reaction |

Cross reactivity was examined at 10ng/ml.

TSH is a heterodimer consisting of α- and β-subunits. Some RIA assays based on competitive binding recognize only one epitope, and have the possibility of measuring free b-subunit. Shibayagi's TSH ELISA kit uses two antibodies, and each of them recognizes α-subunit and β-subunit, respectively. So the assay system is specific to native TSH. So, the TSH assay values after thyroidectomy or goitorogen administration may be different from the data reported with RIA measurement. Ref. Mori M, Oshima K, et al. Acta Endocrinol, 105: 49-56, 1984

- Precision of assay

Within assay variation (3 samples, 5 replicates assay,) Mean CV is less than 5 %.

- Reproducibility

Between assay variation (3 samples, 4 days, assayed in 4 replicates ) Mean CV is less than 5 %

- Recovery test

Standard TSH was added in 3 concentrations to 2 serum samples and were assayed in duplicates.
The recoveries were 98.8 ~103%

- Dilution test

Two serum samples were serially diluted and assayed in triplicates.
The dilution curves showed excellent linearity with $R^2$ of 0.9977 and 0.9998.

## 14. Reference assay data

Rat TSH assay data: Mean 3.43 ng/ml, SD 2.34ng/ml
Serum samples obtained between 14:00 to 16:00 clock hour from 8 adult CD(SD) male rats.
These are reference data. Blood TSH levels show diurnal change, and may change due to breeding, sampling, and sample storage conditions.

## 15. Trouble shooting

- Low absorbance in all wells

Possible explanations:

1) The standard or samples might not be added.
2) Reagents necessary for coloration such as Biotin-labeled antibody, HRP-conjugated avidin, or TMB might not be added.
3) Wrong reagents related to coloration might have been added. Wrong dilution of biotin-labeled antibody or HRP-avidin conjugate.
4) Contamination of enzyme inhibitor(s).
5) Influence of the temperature under which the kits had been stored.
6) Excessive hard washing of the well plate.
7) Addition of TMB solution soon after taken out from a refrigerator might cause poor coloration owing to low temperature.

- The OD of blank is higher than that of the lowest standard concentration (0.288ng/ml)

Possible explanations:

Improper or inadequate washing. (Change washing repetition from 4 times to 5-8 times after the reaction with HRP-avidin.)

- High coefficient of variation (CV)

Possible explanation:

1) Improper or inadequate washing.
2) Improper mixing of standard or samples.
3) Pipetting at irregular intervals.

- Q-1: Can I divide the plate to use it for the other testing?

A-1: Yes, cut off the clear seal on the plate with cutter along strip. Put the residual plate, which is still the seal on, in a refrigerator soon

- Q-2: I found there contains liquid in 96 well-plate when I opened the box. What is it?

A-2: When we manufacture 96 well-plate, we insert preservation stabilizer in wells.

For detailed FAQS and explanations, refer to **"Trouble shooting and Important Points in Shibayagi's ELISA kits**" on our website (http://www.shibayagi.co.jp/en/tech_004.html).

## Summary of assay procedure [Standard procedure]

***Please, confirm the detail procedure in the section 11. This is the summary for checking the process.**

☐Bring back all the necessary reagents (plate, buffer solution, standard solutions, biotin-conjugated anti-TSH, washing buffer) to room temperature.

☐Dilution of concentrated washing buffer: Dilute to 10x with purified water of room temperature.

☐Dilution of master standard(an example): Dilute with buffer of room temperature.

| Conc. (ng/ml) | | 36 | | 14.4 | | 7.2 | | 2.88 | | 1.15 | | 0.288 | 0 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Standard (µl) | Master: | **50** | → | 200* | → | 200* | → | 200* | → | 200* | → | **100*** | 0 |
| Buffer(µl) | | 450 | | 300 | | **200** | | 300 | | 300 | | 300 | 300 |

Note: *One rank higher standard

☐Preparation of positive sample

☐Dilution of biotin-labeled anti-TSH, 200x

| | | |
|---|---|---|
| **Day 1** | ☐ | **Anti-TSH-coated plate** |
| | ☐ | ↓Washing 4 times* |
| | ☐ | **Biotin-conjugated anti-TSH 50µl** |
| | ☐ | ↓Shaking** |
| | ☐ | **Diluted sample or standard solution 50µl** |
| | ☐ | ↓Shaking**, Reaction at 2～8℃, 15～18hours（Standing***） |
| **Day 2** | ☐ | Dilution of HRP-conjugated avidin with buffer of room temperature to 200x |
| | ☐ | ↓Washing 4 times* |
| | ☐ | **HRP-conjugated avidin 100µl** |
| | ☐ | ↓Shaking**, reaction at room temp. 30min (standing***) |
| | ☐ | ↓Washing 4 times* |
| | ☐ | **Chromogenic substrate reagent (TMB) 100µl** |
| | ☐ | ↓Shaking**, reaction at room temp. 20min (standing***) |
| | ☐ | **Reaction stopper（1 M $H_2SO_4$）**※Careful **100µl** |
| | ☐ | ↓Shaking** |
| | ☐ | **Measurement of absorbance（450nm, sub 620nm）** |

Room temp：20～25℃

*Washing buffer volume：300 µl/well
Plate washer pressure：5～25ml/min（depending on nozzle diameter）
Be careful not to try wells after removal of liquid.

**Guideline of shaking：800rpm-10sec.×3 times
Sub-wavelength(reference wavelength):600～650nm

***Put a plate cover on the plate while the reaction after shaking.

**Assay worksheet**

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | | | | | | | | | | | | |
| B | | | | | | | | | | | | |
| C | | | | | | | | | | | | |
| D | | | | | | | | | | | | |
| E | | | | | | | | | | | | |
| F | | | | | | | | | | | | |
| G | | | | | | | | | | | | |
| H | | | | | | | | | | | | |

[Storage condition] Store the kit at 2～8℃ in dark place (Do not freeze).
[Term of validity] 6 month from production (Expiration date is indicate upon the container)

This kit is manufactured by shibayagi Co., Ltd.
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