

Cat. No.: RSHAKRLH-010R

This kit is manufactured by Shibayagi Co., Ltd.
Use only the current version of Instruction Manual enclosed with the kit!

1. INTENDED USE

Rat LH ELISA Kit (TMB) is a sandwich ELISA system for quantitative measurement of rat LH.

Features

1. This is intended for research use only.
2. Rapid assay (total reaction time: 3 hours 50 minute).
3. This kit is for LH in rat serum, plasma, culture medium and tissue extract.
4. Assay format is 96 wells.
5. Standard LH is derived from the rat anterior pituitary gland.
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

LH is produced and stored in basophilic cells called gonadotrophs in the anterior pituitary gland. LH is found in all vertebrates from fishes to mammals. The content of LH in rat anterior pituitary gland is 6~7 μg (as NIH-LH S1)/gland in males, and 3~4 μg /gland in females. In females, the content of LH changes during the sexual cycle. Some reports indicated the presence of LH-like substance in the testis.

LH has a heterodimer structure consisted of α -subunit and β -subunit with a total molecular weight of approximately 29,000. α -Subunit is a glycoprotein which is common to other pituitary hormones follicle-stimulating hormone (FSH) and thyroid-stimulating hormone (TSH), while β -subunit is specific to LH, and is also a glycoprotein.

α -Subunit has two oligosaccharide chains and β -subunit has one chain bound to asparagine moieties. There are many disulfide bonds within both subunits, keeping their rigid three-dimensional structure.

LH shows microheterogeneity deriving from oligosaccharide chains, and from the difference of the number of sialic acids locating at the end of the chains, seven isomers or components having different isoelectric points are present in rat pituitary gland (1). These components reflect various stages of processing to the final intake of sialic acid at the end of each chain. These components are also different in the affinity with the receptor, so that are different in the potency to cause the androgen production in the interstitial cells (Leidig cells) in vitro. Components with basic pI's show high potency, and those with low pI's owing to the addition of sialic acid moieties show lower potencies with more sialic acid moieties (2).

It was also found that the addition of sialic acid moieties caused elongation of biological half lives of the LH components(3). The elimination of oligosaccharide chain from HCG, a closely similar to LH from its structure and action, resulted in the loss of LH-like biological activity though the binding ability to the receptor remains. So deglycosylated HCG acts as a competitive inhibitor of LH (4).

The receptor of LH is a transmembrane receptor of G-protein coupled type, which relates to PKA system and penetrates the cell membrane 7 times.

In females, LH acts on matured granulosa cells in ovarian follicle, where it causes, in cooperation with FSH, follicular maturation, estrogen production, and ovulation, and after ovulation, acts on corpora lutea and promotes progesterone production and secretion.

In males, LH acts on the interstitial cells (Leidig cells) in the testis, causing production and secretion of androgens, and secondarily promotes spermatogenesis via androgen.

Deficiency of LH leads to the lower secretion of sex steroids, atrophy of interstitial

cells, and failure of ovulation and luteinization, while excessive LH causes hyperplasia of testicular interstitial cells followed by atrophy, increased secretion of estrogen or androgen, super-ovulation, and accelerated sexual maturation.

Human diseases showing low blood LH levels: hypo-gonadotropic eunuchoidism, primary central amenorrhea, Sheehan syndrome, Chiari-Frommel syndrome, anorexia nervosa, granulosa-cell tumor, adrenogenital syndrome, luteal dysfunction, craniopharyngioma, etc.

Diseases showing high blood LH levels: azospermia, Klinefelter syndrome, Turner syndrome, premature adolescence, premature menopause, polycystic ovary syndrome (Stein - Leventhal syndrome), ovarian growth and developmental disorder, castration, menopause, etc. For details of LH biosynthesis and secretion under various physiological states check our website (English version in http://www.shibayagi.co.jp/en/tech_010_01.html).

NIH-LH S1 (ovine origin) is the first biological standard preparation of animal LH, and 1 mg was defined as 1 NIH Unit. Providing succeeding lots like S-2, S-3, and so on has continued this standard preparation. Each lot has nearly the same potency.

Standard preparations have been attached to RIA kits for various species of animals. For rat LH, NIDDK rat LH RP-1 was provided in the early period of NIDDK RIA kit. But this standard preparation was so crude, however, in many reports the results were expressed as the weight of RP-1. We have to be careful in reading such reports.

Later, successive reference preparations RP-2 and RP-3 were provided. The potency of RP-3 is the same to I-9, and 0.9 x NIH-LH-S1 which corresponds to 888IU/mg of human 2nd IRP HMG according to the instruction paper for NIDDK rat LH RIA kit, 19-Jan-95, by Dr. A. F. Parlow.

4. ASSAY PRINCIPLE

In Shibayagi's Rat LH ELISA Kit, standards or samples are incubated in monoclonal anti-LH α antibody-coated wells to capture LH. After 2 hours' incubation and washing, biotin-labeled anti-LH β antibody is added and incubated further for 1 hours to bind with captured LH. After washing, HRP (horse radish peroxidase)-labeled avidin is added, and incubated for 30 minutes. After washing, HRP-complex remained in wells are reacted with a chromogenic substrate (TMB) for 20 minutes, and reaction is stopped by addition of acidic solution, and absorbance of yellow product is measured spectrophotometrically at 450 nm. The absorbance is proportional to LH concentration. The standard curve is prepared by plotting absorbance against standard LH concentrations. LH 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.
- Avoid contact with the acidic Reaction stopper solution and Chromogenic substrate solution, which contains hydrogen peroxide and tetramethylbenzidine (TMB). Wear gloves and eye and clothing protection when handling these reagents.
- 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.

6. TECHNICAL TIPS

In manual operation, proficiency in pipetting technique is requested.

- Be careful to avoid any contamination to assay samples and reagents. We recommend the use of disposal pipette tips. The reagents are prepared to give accurate results by their combination within the kit. So, do not combine the reagents in the kit of other lot number. Even the lot number is the same, do not mix the reagents with those that have been preserved for some period
- Be careful to avoid any contamination to assay samples and reagents. We recommend the use of disposal pipette tips.
- The reagent solutions of the kit should be used principally immediately after reconstitution. Otherwise, store them in a dark place at 2-8 °C, and use them within 3 days.

- Time the reaction from the pipetting of reagent to the first well.
- Prepare the standard curve in 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 8 wells x 12 strips, each strip can be separated by cutting the cover sheet with a knife and used independently.

7. REAGENTS SUPPLIED

Components	State	Amount
A. Antibody-coated 96 well-plate	Use after washing	96 wells/1 plate
B. Rat standard LH (100 ng/ml)	Concentrated. Use after dilution	200 µl/1 vial
C. Buffer solution	Ready for use.	60 ml/1 bottle
D. Biotin-labeled anti-LH antibody	Concentrated. Use after dilution.	100 µl/1 vial
E. HRP-avidin conjugate	Concentrated. Use after dilution.	100 µl/1 vial
F. Chromogenic substrate (TMB) solution	Ready for use.	12 ml/1 bottle
H. Reaction stopper (1M H ₂ SO ₄)	Ready for use.	12 ml/1 bottle
I. Concentrated washing buffer (10x)	Concentrated. Use after dilution.	100 ml/1 bottle
Instruction Manual	—	1 copy

Biotin-labeled anti-LH (D), and HRP-avidin conjugate (E): Vials contain more than volumes shown in the list.

So, you can take out 100 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 multipipette 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 (~800 rpm)
- An automatic washer for 96 well-plate (if available), or a wash bottle with a jet nozzle.
- A 96 well-plate reader (450 nm \pm 10 nm, 620 nm: 600-650 nm)
- 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).

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

Antibody-coated well-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 solution

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 H₂SO₄)

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 deionized water to prepare working solution. Example: 100 ml of concentrated washing buffer (10x) and 900ml of deionized 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.

Rat standard LH (100ng/ml)

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

Volume of standard solution	Buffer solution	Concentration (ng/ml)
Original solution : 50 µl	450 µl	10.0
10 ng/ml solution : 200 µl	200 µl	5.0
5 ng/ml solution : 200 µl	200 µl	2.5
2.5 ng/ml solution : 200 µl	200 µl	1.25
1.25 ng/ml solution : 200 µl	200 µl	0.625
0.625 ng/ml solution : 200 µl	200 µl	0.313
0 (Blank)	200 µl	0

Storage and stability

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

Biotin-labeled anti-LH

Prepare working solution by dilution of (D) with the buffer solution (C) to 1:100.

Storage and stability

Unused working solution (already diluted) should be disposed.

HRP-avidin conjugate

Prepare working solution by dilution of (E) with the buffer solution (C) to 1:100.

Storage and stability

Unused working solution (already diluted) should be disposed.

10. PREPARATION OF SAMPLES

This kit is intended to measure rat serum, plasma, culture medium and tissue/cell extracts.

Serum samples are most recommended. If plasma samples are used, we recommend EDTA-2Na (at a final concentration of 1mg/ml). Heparin is not suitable. Anesthesia while sampling may influence the assay system. We do not recommend ether anesthesia.

Samples should be immediately assayed or stored below –20 °C for several days. Defrosted samples should be mixed thoroughly for best results. Hemolytic and hyperlipemic serum samples are not suitable. If presence of interfering substance is

suspected, examine by dilution test at more than 2 points. Dilution of a sample should be made in a test tube using buffer solution prior to adding them to wells. Turbid samples or those containing insoluble materials should be centrifuged before testing to remove any particulate matter.

Before starting assay, samples should be diluted to 5x with the buffer solution(C) in small test tubes. The limit of dilution is 2.5x.

Storage and stability

LH in samples will be inactivated if stored at 2-8 °C. If you have to store assay samples for a longer period, snap-freeze samples and keep them below -35 °C. Avoid repeated freeze-thaw cycles.

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

Due to various factors of your sampling conditions (anesthesia, preservatives, anticoagulants, raised sample pH caused by loss of CO₂ during storage, preservative used, evaporation and condensation during storage in a freezer, 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 (10 ng/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 analyte 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. ASSAY PROCEDURE

Remove the cover sheet of the 96 well-plate after bringing up to room temperature.

1. Wash the anti-LH coated plate (A) by filling the well with washing buffer and discard 4 times, then strike the plate upside-down onto several layers of paper towels to remove residual buffer in the wells.
2. Pipette 50 μ l of sample to the designated sample wells.
3. Pipette 50 μ l of standard solution to the wells designated for standards.
4. Shake the plate gently on a plate shaker (800 rpm for 10 seconds x 3 times).
5. Incubate for 2 hours at room temperature (20-25 °C).
6. Discard the reaction mixture. Rinse wells by filling the wells with the washing buffer and discard 4 times, then strike the plate upside-down onto several layers of paper towels to remove residual buffer in the wells.
7. Pipette 50 μ l of biotin-labeled anti-LH solution to all wells, and shake as step (4).
8. Incubate the plate for 1 hour at room temperature.
9. Discard the reaction mixture. Rinse wells by filling the wells with the washing buffer and discard 4 times, then strike the plate upside-down onto several layers of paper towels to remove residual buffer in the wells.
10. Pipette 50 μ l of HRP-conjugated avidin solution to all wells, and shake as step (4).
11. Incubate the plate for 30 minutes at room temperature.
12. Discard the reaction mixture. Rinse wells by filling the wells with the washing buffer and discard 4 times, then strike the plate upside-down onto several layers of paper towels to remove residual buffer in the wells.
13. Pipette 50 μ l of chromogenic substrate solution to wells, and shake as step (4).
14. Incubate the plate for 20 minutes at room temperature.
15. Add 50 μ l of the reaction stopper to all wells and shake as step (4).
16. Measure the absorbance of each well at 450 nm (reference wavelength, 620*nm) using a plate reader within 30 minutes.

Note: For manual washing procedure see “Kit operation (Power point)” in our website.

* 600-650 nm can be used as reference wavelength.

In case of washing by using 8 channel pipette, sometimes the back ground tends to be high. If so, change the washing frequency from 4 times to 6-8 times. Especially, the washing after the reaction with HRP-avidin conjugate is important.

Standard of plate-washing pressure: 5-25 ml/min. (Adjust it depending on the nozzle's diameter.)

Worksheet example

	Strip 1&2	Strip 3&4	Strip 5&6	Strip 7&8	Strip 9&10	Strip 11&12
A	10 ng/ml	Sample 1	Sample 9	Sample 17	Sample 25	Sample 33
B	5 ng/ml	Sample 2	Sample 10	Sample 18	Sample 26	Sample 34
C	2.5 ng/ml	Sample 3	Sample 11	Sample 19	Sample 27	Sample 35
D	1.25 ng/ml	Sample 4	Sample 12	Sample 20	Sample 28	Sample 36
E	0.625 ng/ml	Sample 5	Sample 13	Sample 21	Sample 29	Sample 37
F	0.313 ng/ml	Sample 6	Sample 14	Sample 22	Sample 30	Sample 38
G	0	Sample 7	Sample 15	Sample 23	Sample 31	Sample 39
H	Pos. Control.	Sample 8	Sample 16	Sample 24	Sample 32	Sample 40

12. CALCULATIONS

1. Prepare a standard curve using semi-logarithmic or two-way logarithmic section paper by plotting absorbance* (Y-axis) against LH concentration (ng/ml) on X-axis. Physiological or pathological situation of animals should be judged comprehensively taking other examination results into consideration.

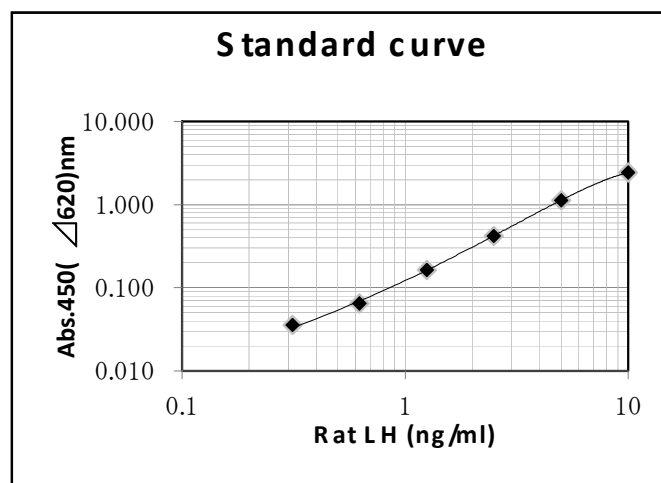
Rat LH assay standard curve (an example)

Absorbance may change due to assay situation.

*Absorbance at 450nm minus absorbance at 620nm.

2. Using the standard curve, read the LH concentration of a sample at its absorbance*, and multiply the assay value by dilution factor if the sample has been diluted. 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.

* We recommend the use of 3rd order regression curve for log-log plot, or 4 parameters method for log-normal plot in computer calculation.



13. PERFORMANCE CHARACTERISTICS

- Assay range

The assay range of the kit is 0.313 ~ 10 ng/ml.

If some samples show absorbance more than that of 10ng/ml standard, please repeat the assay after proper dilution of samples.

- Specificity

The kit uses a monoclonal antibodies specific to LH.

- .Precision of assay

Within assay variation (2 samples, 8 replicates assay,)

Mean CV is less than 5 %.

- Reproducibility

Between assay variation (3 samples, 4 days, 4 replicates assay)

Mean CV is less than 5 %

- Recovery test

Standard LH was added in 3 concentrations to 2 serum samples and was assayed in duplicates.

The recoveries were 97.7 ~103 %

- Dilution test

Two serum samples were serially diluted and assayed in triplicates.

The dilution curves showed excellent linearity. ($R^2= 0.999$)

14. REFERENCE ASSAY DATA

Rat serum assay data

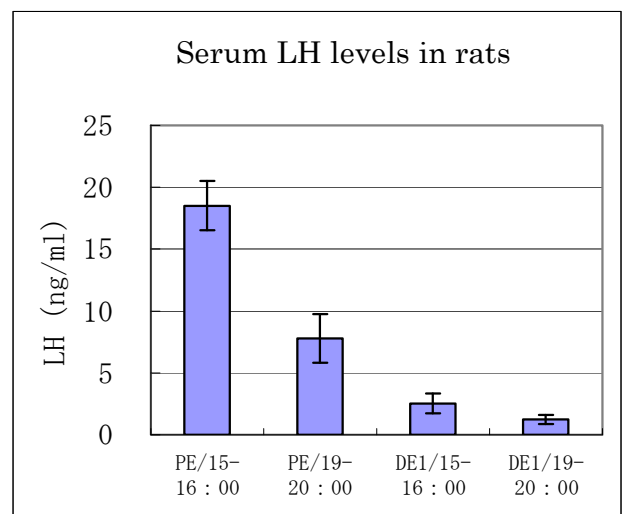
Strain: Wistar Imamichi

Age: 8 weeks

Samples: Serum samples were collected on proestrus (PE) and diestrus I (DE1) days.

These data should be considered as guidance only.

Each laboratory should establish its own normal and pathological reference ranges for LH levels independently.



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 taking out from a refrigerator might cause poor coloration owing to low temperature.

- Intense coloration in all wells including blank

Possible explanations:

1. Improper or inadequate washing. (Change washing frequency from 4times to 6-8 times after the reaction with HRP-avidin conjugate.)
2. Overdeveloping. Incubation time with chromogenic substrate solution should be decreased before addition of reaction stopper.
3. Too high incubation temperature. Adjust the temperature to 20-25 °C.

- 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).

16. REFERENCES

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Conformation of the β -subunit of deglycosylated human chorionic gonadotropin in the interaction at receptor sites.
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Summary of assay procedure

***First, read this instruction manual carefully and start your assay after confirmation of details.**

Bring the well-plate and all reagents to room temperature.

Concentrated washing buffer must be diluted to 10 times by purified water that returned to room temperature.

Standard LH solution dilution example:

Concentration (pg/ml)	10	5.0	2.5	1.25	0.625	0.313	0
Std. LH solution (μ l)→	50	200	200	200	200	200	0
Buffer solution (μ l)	450	200	200	200	200	200	200

Biotin conjugated anti LH(D) : Dilute to 100 times by using buffer solution(C) and use.

*Dilute Biotin conjugated anti LH during the incubation of 2 hours (refer to 11. Assay procedure-(5).)

HRP conjugated streptavidin(E) : Dilute to 100 times by using buffer solution(C) and use.

*Dilute HRP conjugated streptavidin during the incubation of 1 hour (refer to 11. Assay procedure-(8).)

Antibody-coated 96 well-plate

↓Washing 4 times*

Diluted Samples⁺ /Standards **50 μl**

↓Shaking**, Incubation for 2 hours at room temp.

↓Washing 4 times*

Biotin-labeled anti-LH antibody **50 μl**

↓Shaking**, Incubation for 1 hour at room temp.

↓Washing 4 times*

HRP-conjugated avidin **50 μl**

↓Shaking**, Incubation for 50 minutes at room temp.

↓Washing 4 times*

Chromogenic substrate (TMB) **50 μl**

↓Shaking**, Incubation for 20 minutes at room temp.

Reaction stopper (1 M H₂SO₄) **50 μl**

↓Shaking**

Measurement of absorbance (450 nm, Ref 620 nm)***

Use the value (abs.450nm — abs.620 nm)

: Use as a check box

Room temp:20~25 °C*

Guideline of washing volume: 300 μl/well for an automatic washer and for a pipette if the washing buffer is added by pipette. If the back ground is high, change the washing frequency from 4 times to 6-8 times. Especially, the washing after the reaction with HRP-avidin conjugate is important.

+ Samples diluted to 5x using assay buffer solution (minimum dilution: 2.5x)

** Guideline of shaking: 800rpm for 10 seconds x 3 times

*** 600-650 nm can be used as reference wavelength.

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 °C (Do not freeze).
Term of validity 6 months from production (Expiration date is indicated on the
container.)

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