For Research Use Only

November 12, 2012 ver. 7

[GLP-1 (Active) ELISA Kit]

(Code No.:AKMGP-011)

Please, read this instruction carefully before use.

This kit is manufactured by Shibayagi Co., Ltd.
Use only the current version of Instruction Manual enclosed with the kit! For the detailed assay procedure, refer to Key points for ELISA by movie on our website: http://www.shibayagi.co.jp/index-E.htm

1. Intended use

GLP-1 (Active) ELISA Kit is a sandwich ELISA system for quantitative measurement of mouse/rat active GLP-1(7-36)amide. This is intended for research use only.

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

The amino acid sequence of GLP-1(glucagon-like peptide-1) is included in the glucagon precursor. Glucagon precursor, which is expressed in pancreas, intestine and hypothalamus, has been found to contain various bioactive substances relating to glucose metabolism, i.e. glucagon, glycentin, oxyntomodulin, glucagon-like peptide-1, and –2. Owing to the specificity of the processing enzyme, pancreas produces mostly glucagon, and intestine produces glycentin and oxyntomodulin. GLP-1 and GLP-2 are present in the C-terminal half region of the precursor.

GLP-1 is constituted from 37 amino acid residues, but its active forms, GLP-1(7-36)amide and GLP-1(7-37) are found in both pancreas and intestine.

GLP-1: hdeferhaegtftsdvssylegqaakefiawlvkgrg
GLP 1(7-37): haegtftsdvssylegqaakefiawlvkgrg
GLP 1(7-36) amide: haegtftsdvssylegqaakefiawlvkgr-NH_2

In the hypothalamus, GLP-1(7-36)amide occupies 55-94% of immunoreactive GLP-1, while in the ileum, its population is 27-73%, while only a minute amount is observed in the pancreas.
GLP-1 is mainly secreted in the form of GLP-1(7-36)amide (Ref. 1,2)

The amino acid sequence of GLP-1 is common among various mammalian species such as human, rat, mouse, cow, sheep, pig, and dog.

GLP-1 is considered to be one of the incretins together with GIP. It was reported that basal level of plasma amidated GLP-1 was 4.1 pmol/l, and that it increased to 15.4 pmol/l at 10 minutes after the administration of 1g/kg of glucose to the stomach.(Ref. 3)

GLP-1 enhances glucose-dependent insulin secretion, inhibits stomach movement and acid secretion, inhibits glucagon secretion, stimulates somatostatin secretion, lowers appetite, induces the intestinal epithelial growth, influences LH, TSH, CRH, oxytocin, vasopressin section in the pituitary gland, enhances glucose disposal in the peripheral

independent of insulin, and induces pancreatic islets growth including beta cell proliferation.

In plasma GLP-1(7-36)amide is quickly metabolized and loses N-terminal 2 amino acids by dipeptidyl peptidase IV (DPP IV, a serine enzyme which is inactivated by DFP, diisopropyl fluorophosphonate) to become GLP-1(9-36)amide, and the other active form, GLP-1(7-37), is transformed to GLP-1(9-37). When incubated with dog plasma *in vitro* at 37°C, the half life of GLP-1(7-36)amide was reported to be 61±9 minutes, and that of GLP-1(7-37) was 132±16 minutes (Ref. 4). For GLP-1 assay, it is necessary to use a DPP IV inhibitor in sampling.

Another incretin, GIP, strongly enhances GLP-1 secretion. GLP-1 secretion from the lower intestine (ileum) may be caused by cholinergic impulse and stimulation of peptidergic mediators and not by the direct stimulation by food (Ref. 5).

4. Assay principle

In Shibayagi's GLP-1(Active) ELISA Kit, standard or sample are incubated in monoclonal anti-GLP-1(7-36)amide antibody-coated wells to capture GLP-1(7-36)amide. After 2 hours' incubation and washing, biotin-labeled anti-GLP-1(7-36)amide antibody is added and incubated further for 2 hours to bind with captured GLP-1(7-36)amide. After washing, HRP (horse radish peroxidase)-labeled 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. The absorbance is proportional to GLP-1 concentration. The standard curve is prepared by plotting absorbance against standard GLP-1 concentrations. GLP-1(7-36)amide 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.
- 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 seal supplied, during incubation.
- ELISA can be easily affected by your laboratory environment. Room temperature should be at 20-25°C strictly. Avoid airstream velocity over 0.4 m/sec. ① (including wind from air conditioner), and humidity less than 30%. ①For airstream, refer to [Assay circumstance] on our web site.

6. Reagents supplied

Components	State	Amount
(A) Antibody-coated 96 well-plate	Use after washing	96 wells/1 plate
(B) Standard GLP-1(7-36)amide (500pg/ml) (Synthetic GLP-1(7-36)amide)	Concentrated. Use after dilution	200 µl/1 vial
(C) Buffer solution	Ready for use.	60 ml/1 bottle
(D) Biotin-labeled anti-GLP-1 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_2SO_4) Be careful!	Ready for use.	12 ml/1 bottle
(I) Concentrated washing buffer (10x)	Concentrated. Use after dilution.	100 ml/1 bottle
Plate seal	—	4 sheets
Instruction Manual	—	1 copy

7. Equipments or supplies required but not supplied ☐Use as a check box

☐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 50µl precisely, and another for 200-500µl.
☐Syringe-type repeating dispenser like Eppendorf multipette plus which can dispense 50µl.
☐Paper towel to remove washing buffer remaining in wells.
☐A vortex-type mixer.
☐A shaker for 96 well-plate (600-1,200rpm)
☐An automatic washer for 96 well-plate (if available), or a washing bottle with a jet nozzle
(refer to our web movie [Washing of microplate]).
☐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).
□DPP-IV Inhibitor to add when collecting blood sample to avoid the degradation of GLP-1.

8. Preparation of reagents

◆Bring all reagents of the kit to room temperature (20-25 °C) before use.

◆Prepare reagent solutions in appropriate volume for your assay. Do not store the diluted reagents.

【Concentrated reagents】

[(B) Standard GLP-1(7-36)amide (500pg/ml)]

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

Volume of standard solution	Buffer solution	Concentration (pg/ml)	Concentration (pmol/l) MW:3298
Original solution: 50 µl	450µl	50.0	15.16
50 pg/ml solution: 200µl	200µl	25.0	7.58
25 pg/ml solution: 200µl	200µl	12.5	3.79
12.5 pg/ml solution: 200µl	200µl	6.25	1.895
6.25 pg/ml solution: 200µl	200µl	3.13	0.948
3.13 pg/ml solution: 200µl	200µl	1.56	0.474
0 (Blank)	200µl	0	0

[(D) Biotin-labeled anti-GLP-1]

Prepare working solution by dilution of (D) with the buffer solution (C) to 1:100.
10 ml of the diluted solution is enough for 96 wells.

[(E) HRP-avidin conjugate]

Prepare working solution by dilution of (E) with the buffer solution (C) to 1:100.
10 ml of the diluted solution is enough for 96 wells.

[(I) 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】

[(A) Antibody-coated well-plate]

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.

[(B) Standard GLP-1(7-36)amide (500pg/ml)]

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

[(C) Buffer solution] and [(F) Chromogenic substrate solution]

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.

[(D) Biotin-labeled anti-GLP-1] & [(E) HRP-avidin conjugate]

Unused working solution (already diluted) should be disposed.

[(H) Reaction stopper (1 M H_2SO_4)]

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

[(I) Concentrated washing buffer (10x)]

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

9. Technical tips

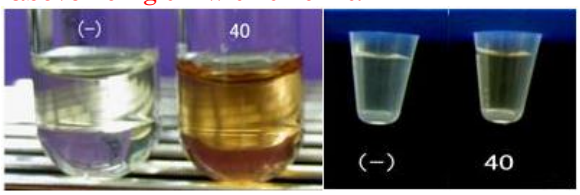
- In manual operation, proficiency in pipetting technique is recommended.
- The reagents are prepared to give accurate results only when used in combination within the same box. Therefore, do not combine the reagents from kits with different lot numbers. Even if the lot number is the same, it is best 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 over 0.4 m/sec. and the humidity less than 30%, seal the well plate with a plate seal and place the well plate in an incubator or a styrofoam box in each step of incubation. For more details, watch our web movie [Assay circumstance].

10. Preparation of samples

This kit is intended to measure GLP-1(7-36)amide in mouse/ rat serum, plasma and culture medium.
In sampling, to avoid enzymatic degradation of GLP-1(7-36)amide, **use DPP IV inhibitor**.
Samples should be immediately assayed or stored below –35 °C for several days. Frozen stored samples should be mixed thoroughly for best results.
Hemolytic and hyperlipemic serum samples are not suitable.
* To avoid influence of blood (high lipid or hemolysis, etc.), if your original samples have heavy chyle or hemolysis as the pictures below, do not use them for assay. Abnormal value might be obtained with hemolysis above 40mg/dL with this kit.



Normal | Hemolysis 40mg/dL | normal | Hemolysis 40mg/dL

Normal | Chyle (Highly lipid sample) | Normal | Chyle (Highly lipid sample)

Do not use Ether. If presence of interfering substance is suspected, examine by dilution test at more than 2 points. Turbid samples or those containing insoluble materials should be centrifuged before testing to remove any particular matter.
Blood sample should be withdrawn into an injection syringe containing proper volume of DPP IV inhibitor solution. Mix well and pipette 50 µl of diluted sample (e.g. sample 10µl + buffer 40µl) into a well for assay. Before starting assay, samples should be diluted between 2.5x and 5x in test tubes (PP or PE). In sample calculation never forget to multiply the assay value by this dilution rate.
For example of blood sampling in the assay of rat and mouse using DPP IV inhibitor, refer to section **14. Reference assay data**.
If you use aprotinin and heparin together, read section 15. Trouble shooting and Q&A prior to assay.

Storage and stability

GLP-1(7-36)amide in samples will be inactivated if store 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.

11. Assay procedure

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

(1) Wash the anti-GLP-1 coated plate (A) by filling the wells with washing buffer and discard 3 times(*②), then strike the plate upside-down onto several layers of paper towels to remove buffer residual buffer in the wells.
(2) Pipette 50µl of diluted sample to the designated sample wells.
(3) Pipette 50µl of standard solution to the wells designated for standards.
(4) Shake the plate gently on a plate shaker (*③).
(5) Stick a plate seal (*④) on the plate and incubate for 2 hours at 20-25°C.
(6) Discard the reaction mixture and rinse wells as step (1).
(7) Pipette 50µl of biotin-labeled anti-GLP-1 solution to all wells, and shake as step (4).
(8) Stick a plate seal (*④) on the plate and incubate the plate for 2 hours at 20-25°C.
(9) Discard the reaction mixture and rinse wells as step (1).
(10) Pipette 50µl of HRP-conjugated avidin solution to all wells, and shake as step (4).
(11) Stick a plate seal (*④) on the plate and incubate the plate for 30 minutes at 20-25°C.
(12) Discard the reaction mixture and rinse wells as step (1).
(13) Pipette 50µl of chromogenic substrate solution to wells, and shake as step (4).
(14) Stick a plate seal (*④) on the plate and incubate the plate for 30 minutes at 20-25°C.
(15) Add 50 µl 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

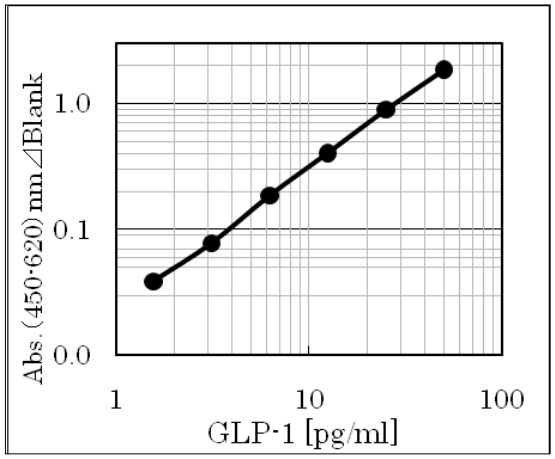
30 minutes.
*Refer to the page 8 for notes of *②, *③ and *④.

12. Calculations

(1) Prepare a standard curve using semi-logarithmic or two-way logarithmic section paper by plotting absorbance* (Y-axis) against GLP-1 concentration (pg/ml) on X-axis.
*Absorbance at 450nm minus absorbance at 620nm.

(2) Using the standard curve, read the GLP-1 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.
Physiological or pathological situation of animals should be judged comprehensively taking other examination results into consideration.



GLP-1 assay standard curve (an example)
Absorbance may change due to assay environment.

13. Performance characteristics

● Assay range
The assay range of the kit is 1.56 pg/ml ~ 50 pg/ml. (For 5x dilution of sample, 7.8 ~ 250 pg/ml)
If some samples show absorbance more than that of 50 pg/ml standard, please repeat the assay after proper dilution of samples.

● Specificity
The antibodies used in this kit are specific to GLP-1(7-36)amide. Cross-reactivity with GLP-1(7-37) is less than 0.1%, and no cross reaction was observed with mouse/rat GLP-1(1-37), mouse/rat GLP-1(9-36)amide, mouse/rat GLP-2, mouse/rat glucagon(1-29), mouse/rat insulin, mouse/rat secretin, mouse/rat GIP, mouse/rat VIP, mouse GRF, bovine glucagon(1-29), bovine VIP, porcine glucagon(1-29), or porcine VIP, when tested at 1 ng/ml.

● Precision of assay
Within assay variation (2 samples, 5 replicates assay,) Mean CV is less than 5 %.

● Reproducibility
Between assay variation (2 samples, 4 days, assayed in 4 replicates) Mean CV is less than 5 %

● Recovery test
Standard GLP-1 was added in 3 concentrations to 2 serum samples and were assayed in duplicates. The recoveries were 95.8 ~103%

● Dilution test
Two serum samples were serially diluted by 3 steps.
The dilution curves showed excellent linearity. R^2= 0.998-0.999.

14. Reference assay data

Mouse/rat sample assay data

Strain	Age	Sex	Number	Assay data (pg/ml)		Remarks
				mean	SD	
C57BL/6	6w	♂	8	21.1	2.56	Sera, fasting: 16hrs
db/db	5w	♂	10	30.6	4.01	Sera, fed *ad libitum*
ob/ob	8w	♂	4	10.9	2.13	Sera, fed *ad libitum*
CD (rat)	8w	♂	12	20.1	4.55	Plasma, fasting :24hrs

Sampling condition
Serum: Eppendorf tubes placed in ice were added blood samples withdrawn by syringes, and after clotting, centrifuged for 30 minutes at 1800xg at 4°C. DPP IV inhibitor (Cat. DPP4, Millipore) was added to separated serum samples at a concentration of 20 µl/ml, and samples were stored at -80°C until assay (Freezing and thawing was only once).

Plasma: Eppendorf tubes containing EDTA-2Na* and aprotinin** in amounts to make final concentrations after addition of blood sample of 1 mg/ml and 500 KIU/ml, respectively, were placed in ice. Blood samples were added to those tubes, stirred and centrifuged for 10 minutes at 1000xg at 4°C to obtain plasma. DPP IV inhibitor (Cat. DPP4, Millipore) was added to the plasma at a concentration of 20 µl/ml. Samples were stored at -80°C until assay (Freezing and thawing was only once).

* EDTA-2Na, Wako Pure Chemicals, Code# 3002E-A101x

** Aprotinin, Wako Pure Chemicals, Code#595-01285

Anesthesia: Isoflurane, Sampling by heart puncture using intact syringes'

These data should be considered as guidance only, as the assay values may change owing to conditions of keeping, blood sampling, and sample storage. Each laboratory should establish its own normal and pathological reference ranges for GLP-1 levels independently.

*Comparison of direct assay and assay after absorption and elution using Oasis® HLB 96-well µ elution plate with 18 plasma samples of mouse and rat gave the equation: y= 0.9007x + 0.1061 with a correlation coefficient of 0.868. (x: direct assay, y: assay after treatment). This result shows that GLP-1 in mouse and rat samples can be assayed without extraction.

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.

- The OD of blank is higher than that of the lowest standard concentration (1.56pg/ml)

  Possible explanations:

  Improper or inadequate washing. (Change washing repetition from 3 times to 4-6 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.

- Q-3: When thawing samples, cloud-like substance appeared. Does this influence the assay?

  A-3: It might lower the assay result or be out of detection limit. Especially when adding aprotinin and heparin together, fibrin might appear in samples. Raise heparin concentration or use EDTA-2Na.

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

1) Holst, J. J., and Orskov, C., Incretin hormones—an update, Scand J Lan Invest Suppl, 234, 75-85, 2001

2) Nauck, M. A., Is glucagon-like peptide 1 an incretin hormone?, Diabetologia, 42, 1148-9, 1999

3) van Delft, J., Uttenthal, L.O., Hermida, O.G., et al., Identification of amidated forms of GLP-1 in rat tissues using a highly sensitive radioimmunoassay., Regul Pept, 70, 191-198, 1997.

4) Pridal, L., Deacon, C.F., Kirk, O., et al., Glucagon-like peptide-1(7-37) has a larger volume of distribution than glucagon-like peptide-1(7-36)amide in dogs andis degradated more quickly in vitro by dog plasma., Eur J Drug Metab Pharmacokinet, 21, 51-59, 1996

5) Herrmann-Rinke, C., Vöge, A., Hess, M.,Göke, B., Regulation of glucagon-like peptide-1 secretion from rat ileum by neurotransmitters and peptides., J Endocrinol, 147, 25-31, 1995

Summary of assay procedure ☐ : Use as a check box

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

For more details, watch our web movie **[ELISA by MOVIE]** on our website.

☐Bring the well-plate and all reagents to 20-25°C for 2 hours.

☐Concentrated washing buffer must be diluted to 10 times by purified water that returned to 20-25°C .

☐Standard GLP-1 solution dilution example:

Concentration (pg/ml)		50	25.0	12.5	6.25	3.13	1.56	0
Std. GLP-1 solution	(µl)→ Orig.sol.	50	200*	200*	200*	200*	200*	0
Buffer solution	(µl)	450	200	200	200	200	200	200

*One rank higher standard.

		Precautions & related info
☐	Antibody-coated 96 well-plate	
☐	↓Washing 3 times (*②)	*⑥
☐	Diluted Samples+ /Standards 50 µl	*⑦ [Handling of pipetting]
☐	↓Shaking(*③), Incubation for 2 hours at 20-25°C. (Standing(*④))	*⑧ [Assay circumstance]
☐	Biotin conjugated anti GLP-1(D) Dilute to 100 times by using buffer solution(C) and use.	Dilute reagents during the first reaction.
☐	↓Washing 3 times (*②)	*⑥
☐	Biotin-labeled anti-GLP-1 antibody 50 µl	*⑦ [Handling of pipetting]
☐	↓Shaking(*③), Incubation for 2 hours at 20-25°C. (Standing(*④))	*⑧ [Assay circumstance]
☐	HRP conjugated streptavidin(E) Dilute to 100 times by using buffer solution(C) and use.	Dilute reagents during the second reaction.
☐	↓Washing 3 times (*②)	*⑥
☐	HRP-conjugated avidin 50 µl	*⑦ [Handling of pipetting]
☐	↓Shaking(*③), Incubation for 30 minutes at 20-25°C. (Standing(*④))	*⑧ [Assay circumstance]
☐	↓Washing 3 times (*②)	*⑥
☐	Chromogenic substrate (TMB) 50 µl	*⑦ [Handling of pipetting]
☐	↓Shaking(*③), Incubation for 30 minutes at 20-25°C (Standing(*④))	*⑧ [Assay circumstance]
☐	Reaction stopper (1M H_2SO_4) 50 µl	
☐	↓Shaking(*③)	
☐	Measurement of absorbance (450nm, Ref 620nm(*⑤)	

*②After dispensing wash buffer to wells, lightly shake the plate on your palm for 10 sec and remove the buffer. Guideline of washing volume: 300µl/well for an automatic washer and for a pipette if the washing buffer is added by pipette. In case of washing by using 8 channel pipette, sometimes the back ground tends to be high. If so, change washing frequency from 3 times to 4-6 times at the constant stroke after the reaction with HRP conjugated streptavidin. Standard of plate-washing pressure: 5-25ml/min. (Adjust it depending on the nozzle's diameter.) Refer to our web movie **[Washing of microplate]**.

*③Guideline of shaking: 600-1,200rpm for 10 seconds x 3 times.

*④Seal the plate during the reaction after shaking. Peel off the protective paper from the seal and stick the seal on the plate. Do not reuse the plate seal used once.

*⑤600-650 nm can be used as reference wavelength.

*⑥After removal of wash buffer, immediately dispense the next reagent.

*⑦Refer to our web movie **[Handling of pipetting]**.

*⑧Refer to our web movie **[Assay circumstance]**.

Worksheet example

	Strip 1&2	Strip 3&4	Strip 5&6	Strip 7&8	Strip 9&10	Strip 11&12
A	50 pg/ml	Sample 1	Sample 9	Sample 17	Sample 25	Sample 33
B	25 pg/ml	Sample 2	Sample 10	Sample 18	Sample 26	Sample 34
C	12.5 pg/ml	Sample 3	Sample 11	Sample 19	Sample 27	Sample 35
D	6.25 pg/ml	Sample 4	Sample 12	Sample 20	Sample 28	Sample 36
E	3.13 pg/ml	Sample 5	Sample 13	Sample 21	Sample 29	Sample 37
F	1.56 pg/ml	Sample 6	Sample 14	Sample 22	Sample 30	Sample 38
G	Blank	Sample 7	Sample 15	Sample 23	Sample 31	Sample 39
H	Pos. Control.	Sample 8	Sample 16	Sample 24	Sample 32	Sample 40

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.)

This kit is manufactured by **Shibayagi Co., Ltd.**
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