For Research Use Only December 3, 2012 Ver. 9

[Human Apo B-48 ELISA Kit]

(Code No.: AKHB48)

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

Human Apo B-48 ELISA Kit is a sandwich ELISA system for quantitative measurement of human Apolipoprotein B-48 (Apo B-48). 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 loss in optimal assay performance caused by storage environment.

3. Introduction

Plasma lipoproteins are complex substances composed of certain ratios of lipids and proteins, and are important factors for serum lipids transportation. On the surface of the lipoprotein structure, apolipoproteins are present and playing roles in stabilization of lipoprotein structure, in activation of enzymes relating to lipoprotein metabolism, and in binding with lipoprotein receptors on the cell surface. Apolipoprotein-B48 (ApoB-48) has 48% amino acid sequence of apolipoprotein B-100 which is present in lipoproteins, VLDL, LDL and HDL of liver origin. ApoB-48 is an apolipoprotein specific to a lipoprotein, chylomicron (CM) which is formed in intestine and carries exogenous lipids derived from foods to the liver and peripheral tissues. Measurement of ApoB-48 is useful in observation and pursuit of postprandial dynamics of lipoprotein, lipid-soluble nutrients and drugs. Co-estimation of LDL-, HDL-cholesterol and B48 will give important information about exogenous and endogenous cholesterol (see reference), and also estimation of CM-remnant that is thought to be a risk factor of cardiovascular atherosclerosis.

Reference: Otokozawa S, et al. Atherosclerosis (2009) 205: 197-201

4. Assay principle

In Shibayagi's Human Apo B-48 ELISA Kit, standards or samples are incubated in monoclonal antibody-coated wells to capture Apo B-48. After 1 hour's incubation and washing, biotin-conjugated anti-Apo B-48 antibody is added and incubated further for 1 hour to bind with captured Apo B-48. After washing, HRP- (horse radish peroxidase) conjugated avidin is added, and incubated for 30 minutes. After washing, bound HRP-conjugated avidin is reacted with a chromogenic substrate reagent (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 nearly proportional to Apo B-48 concentration. The standard curve is prepared by plotting absorbance against standard Apo B-48 concentrations. Apo B-48 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.
- Do not drink, eat or smoke in the areas where assays are carried out.
- 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 reagent containing 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) Anti-apo B-48-coated plate	Ready for use.	96 wells/1 plate
(B) Standard human apo B-48 (derived from human)	Frozen-dried.	128 ng/1 vial
(C) Buffer solution	Ready for use.	60 ml/1 bottle
(D) Biotin-conjugated anti-apo B-48 antibody	Concentrated. Use after dilution.	100 μl/1 vial
(E) Peroxidase-conjugated streptavidin	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) 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 50 μl precisely, and another for 200 μl and 400 μl.
☐Syringe-type repeating dispenser like Eppendorf multipette plus which can dispense 50 μl.
☐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 wash 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).

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 human apo B-48 (Frozen-dried)]

Add 400 μl of distilled water of room temperature to Standard human apo B-48 (Frozen-dired) and let it stand still for 10 minutes (Original solution). Avoid bubbling of the solution, and shake it sufficiently at 1800-2000 rpm for 10 seconds x 3 times. Dissolve the standard in vial completely, and let it be clear solution.

Caution for vortexing: Put a vial on Vortex and start mixing. Do not put vial on Vortex while moving. That caused bubbling.

Reconstituted solution (original solution) is highly viscous. Before preparing standard solutions, get accustomed to treat highly viscous solution (for pipetting, we recommend co-washing procedure). In order to prepare the standard solution precisely, it is very important to pipette correctly the first 200 μl.

Sufficient shaking with Vortex (1800-2000 rpm for 10 seconds x 3 times) in every step of dilution is very important. Be careful not to make bubbles while mixing. Bubbling causes bad reproducibility.

Below is an example of preparing each standard solution.

Volume of standard solution	Buffer solution	Concentration(ng/ml)
Original solution 200 μl	200 μl	160
160ng/ml solution 200 μl	200 μl	80
80 ng/ml solution 200 μl	200 μl	40
40 ng/ml solution 200 μl	200 μl	20
20 ng/ml solution 200 μl	200 μl	10
10 ng/ml solution 200 μl	200 μl	5.0
5.0 ng/ml solution 200 μl	200 μl	2.5
Blank	200 μl	0

[(D) Biotin-conjugated anti-apo B-48 antibody]

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) Proxidase-conjugated streptavidin]

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 dionized water.

【Storage and stability】

[(A) Anti-apo B-48-coated 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 human apo B-48 (Frozen-dried)]

The reconstituted standard solution (original solution) should be used within 24 hours if stored in a refrigerator. Dispose remaining prepared solution. For longer storage, freeze it immediately after reconstitution, and store it under -35 ° C.

[(C) Buffer solution] & [(F) Chromogenic substrate reagent]

Use only volume you need for your assay. Remaining reagents should be stored at 2-8 °C fastening the cap tightly. It maintains stability until expiration date. Once opened, we recommend using as soon as possible to avoid influence by environmental condition.

[(D) Biotin-conjugated anti-apo B-48 antibody] & [(E) Proxidase-conjugated streptavidin]

Unused working solution (already diluted) should be disposed. The rest of the undiluted solution: if stored tightly closed at 2-8 °C, it is stable until expiration date.

[(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.

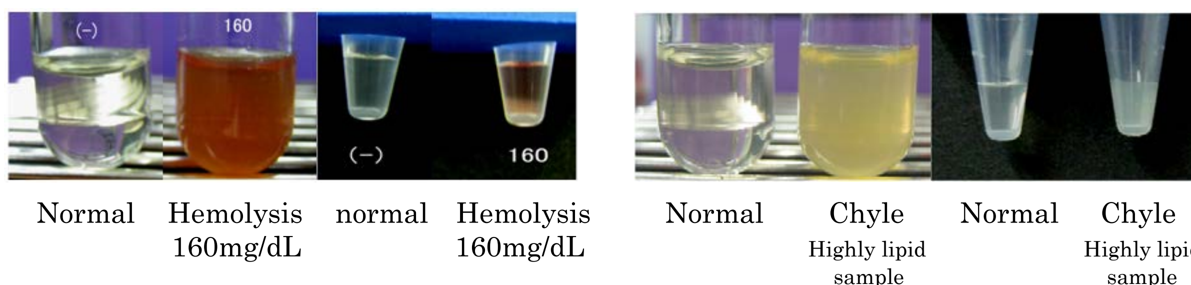
9. Technical tips

- 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. Otherwise, store them in a dark place at 2-8 °C.
- 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 sucstrate reagent (TMB) 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 apo B-48 in human serum or plasma (citric acid for plasma is not suitable). The least sample volume for the assay is 5μl. We recommend to use EDTA-2Na, EDTA-2K or heparin as anticoagulant. Don't use citric acid. Samples should be immediately assayed or stored below –35 °C until assay. Before starting assay, shake thawed samples sufficiently. Do not repeat freeze-and-thaw cycles. Turbid samples or those containing insoluble materials should be centrifuged before testing to remove any particulate matter. 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 PP or PE test tube using buffer solution prior to adding them to wells (Be careful with pipetting since samples diluted with buffer have highly viscosity). Mix well and pipette 50 μl of diluted sample into a well for assay.
Recommended dilution is 100x. For high value samples, more than 200x dilution may be needed.
When diluting samples, vortex them well (1800-2000rpm-10sec x 3) with care not to make bubbling.
Hemolytic and hyperlipemic 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 160mg/dL with this kit.



Normal | Hemolysis 160mg/dL | normal | Hemolysis 160mg/dL | Normal | Chyle (Highly lipid sample) | Normal | Chyle (Highly lipid sample)

Storage and stability

Apo B-48 in samples will be inactivated if stored at 2-8°C. If it is necessary to store samples in refrigerator (2-8°C), add aprotinin at final concentration of 100-500KIU/ml. (KIU: kallikrein inhibitor unit). If you have to store assay samples for a longer period, snap-freeze samples and keep them below –35°C. Defrosted samples should be mixed thoroughly for best results. Avoid repeated

freeze-thaw cycles.

11. Assay procedure

Remove the cover sheet of the antibody-coated plate after bringing up to room temperature.

(1) Wash the anti-apo B-48-coated plate (A) by filling the wells with washing buffer and discard 4 times(*②), then strike the plate upside-down onto folded several sheets of paper towel to remove residual buffer in the wells.
(2) Pipette 50 μl of properly diluted samples 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 1 hour at 20-25°C.
(6) Discard the reaction mixture and rinse wells as step (1).
(7) Pipette 50μl of Biotin-conjugated anti-apo B-48 antibody to all wells, and shake as step (4).
(8) Stick a plate seal (*④) on the plate and incubate the plate for 1 hour at 20-25°C.
(9) Discard the reaction mixture and rinse wells as step (1).
(10) Pipette 50μl of Peroxidase-conjugated streptavidin 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 reagent to wells, and shake as step (4).
(14) Stick a plate seal (*④) on the plate and incubate the plate for 20 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.

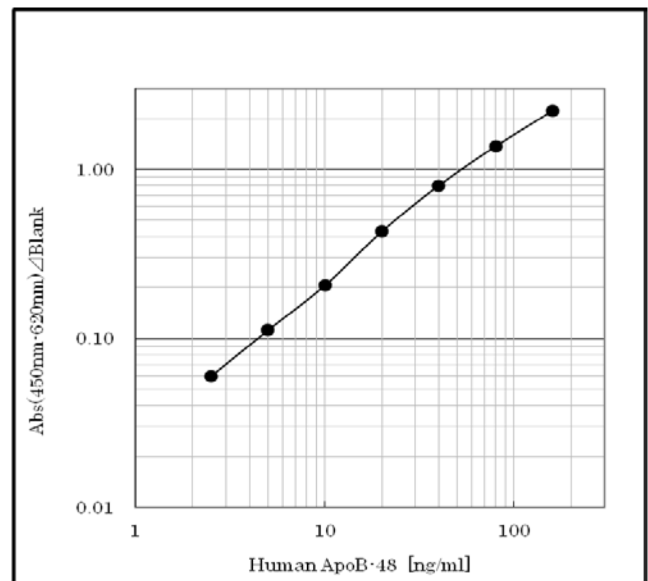
*Refer to the page 7-8 for notes of *②, *③ and *④.

12. Calculations

(1) Prepare a standard curve by plotting standard concentration on X-axis and absorbance on Y-axis.
(Refer to our web site for more detailed explanation about standard curve. Shibayagi is offering a convenient Excel template. http://www.shibayagi.co.jp/en/tech_003.html)

(2) Using the standard curve, read the apo B-48 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.

(3) Higher reaction temperature than 25°C tends to produce high absorbance on the whole. Though it depends on assay instruments, if coloring proceeds to area where there's low reliability of absorbance and slope of standard curve decreases, the area should not be used for calculation. Repeat assay by setting reaction temperature between 20-25 °C.



Human Apo B-48 assay standard curve (an example)
Absorbance may change due to assay environment.

* 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 human should be judged comprehensively taking other examination results into consideration.

13. Performance characteristics

- Assay range

  The assay range of the kit is 2.5 ng/ml ~ 160 ng/ml.
- Specificity

  The antibodies used in this kit are specific to apo B-48.
  Human Apo B-100 had no crossreactivity with this kit.
- Precision of assay

  Within assay variation (3 samples, 5 replicates assay,), Mean CV is 3.5%.
- Reproducibility

  Between assay variation (3 samples, 3 days, 3 replicates assay), CV is 2.8 ~ 8.6%
- Recovery test

  Standard apo B-48 was added in 4 concentrations to 2 serum samples and were assayed.
  The recoveries were 94.9 ~101%
- Dilution test

  Serum sample was serially diluted by 4 steps.
  The dilution curves showed linearity with R^2 = 0.996 ~ 0.999.

14. Reference assay data

Human apo B-48 assay data

Mean assay value: 4.60 μg/ml, SD: 1.54 μg/ml, n = 18, fasting

These data (Japanese) should be considered as guidance only. Each laboratory should establish its own normal and pathological reference ranges for apo B-48 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-conjugated anti-apo B-48 antibody, Peroxidase-conjugated streptavidin, or Chromogenic substrate reagent might not be added.
  3) Wrong reagents related to coloration might have been added. Wrong dilution of biotin-conjugated anti-apo B-48 antibody or peroxidase-conjugated streptavidin.
  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 chromogenic substrate reagent soon after taking out from a refrigerator might cause poor coloration owing to low temperature.
- Blank OD was higher that that of the lowest standard concentration (2.5 ng/ml).

  Possible explanations:

  Improper or inadequate washing. (Change washing frequency from 4 times to 5-8 times at the constant stroke after the reaction with peroxidase-conjugated streptavidin.)
- 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

Determination of apolipoprotein B-48 in serum by a sandwich ELISA.
Kinoshita,M., Kojima,M., Matsushima,T., and Teramoto,T. Clinica Chimica Acta, 351:115-120, 2005

Please, refer to [User's Publication] on our website.

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 back 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 solution dilution example

Concentration (ng/ml)	160	80	40	20	10	5.0	2.5	0
Std. solution (μl)→	Ori.Sol.200	200*	200*	200*	200*	200*	200*	0
Buffer solution (μl)	200	200	200	200	200	200	200	200

*One rank higher standard.

☐Prepare the positive sample.

	Procedure	Volume	Precautions & related info
☐	Anti-apo B-48-coated plate		
☐	↓ Washing 4 times(*②)		*⑥
☐	Diluted samples, or Standards	50 μl	*⑦ [Handling of pipetting]
☐	↓ Shaking(*③), Incubation for 1 hour at 20-25°C. (Standing(*④))		*⑧ [Assay circumstance]
☐	Dilute Biotin-conjugated anti-apo B-48 antibody to 100x with buffer returned to 20-25°C. (This should be prepared during incubation.)		
☐	↓ Washing 4 times(*②)		*⑥
☐	Bioton-conjugated anti-apo B-48 antibody	50 μl	*⑦ [Handling of pipetting]
☐	↓ Shaking(*③), Incubation for 1 hour at 20-25°C. (Standing(*④))		*⑧ [Assay circumstance]
☐	Dilute Peroxidase-conjugated streptavidin to 100x with buffer returned to 20-25°C. (This should be prepared during incubation.)		
☐	↓ Washing 4 times*		*⑥
☐	Peroxidase-conjugated streptavidin	50 μl	*⑦ [Handling of pipetting]
☐	↓ Shaking(*③), Incubation for 30 minutes at 20-25°C. (Standing(*④))		*⑧ [Assay circumstance]
☐	↓ Washing 4 times(*②)		*⑥
☐	Chromogenic substrate reagent (TMB)	50 μl	After dispense, the color turns to blue depending on the concentration.
☐	↓ Shaking(*③), Incubation for 20 minutes at 20-25°C. (Standing(*④))		*⑧ [Assay circumstance]
☐	Reaction stopper (1M H_2SO_4)	50 μl	After dispense, the color turns to yellow depending on the concentration.
☐	↓ Shaking(*③)		Immediately shake.
☐	Measurement of absorbance (450nm, Ref 620nm(*⑤))		Ref. wave cancels the dirt in the back of plate.

*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 4 times to 5-8 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	160 ng/ml	Pos.Control.	Sample 8	Sample 16	Sample 24	Sample 32
B	80 ng/ml	Sample 1	Sample 9	Sample 17	Sample 25	Sample 33
C	40 ng/ml	Sample 2	Sample 10	Sample 18	Sample 26	Sample 34
D	20 ng/ml	Sample 3	Sample 11	Sample 19	Sample 27	Sample 35
E	10 ng/ml	Sample 4	Sample 12	Sample 20	Sample 28	Sample 36
F	5.0 ng/ml	Sample 5	Sample 13	Sample 21	Sample 29	Sample 37
G	2.5 ng/ml	Sample 6	Sample 14	Sample 22	Sample 30	Sample 38
H	0	Sample 7	Sample 15	Sample 23	Sample 31	Sample 39

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] 12 months from production (Expiration date is indicated on the container.)

Shibayagi

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