For Research Use Only

October 3, 2011 Ver. 3

[Rabbit CRP ELISA Kit]

(Code No.:AKRCR-017)

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

Rabbit CRP ELISA Kit is a sandwich ELISA system for quantitative measurement of rabbit C-Reactive Protein. This is intended for research use only.

2. Storage and expiration

When the intact kit is stored at 2-8°C, the kit is stable until the expiration date shown on the label on the box. Reagents, once opened, should be used as soon as possible to avoid losing its optimal assay performance by storage environment.

3. Introduction

APP (acute phase proteins) are also called APR (acute phase reactants) the blood levels of which are increase in acute phase of inflammation. In general, they are produced in the liver in inflammation induced by microbe infection, myocardial infarction, thrombosis, acute stress, shock, injury, allergic diseases and malignant tumors. Representative APP are C-reactive protein (CRP), serum amyloid A protein (SAA), α1-antitrypsin, haptoglobin, ceruloplasmin, fiblinogen, complements such as C3, C4.

Small amount of CRP is present in normal plasma (10μg/ml in human), and markedly increases within 12 hours in various inflammatory diseases (pyogenic diseases, acute infection, collagen diseases, etc.) as well as neumonia, in acute myocardial infarction, surface wound, burn, big operation, malignant tumor, rheumatic fever, stress, etc., and rapidly returns to normal level upon recovery. Measurement of blood CRP is useful as an indicator to estimate inflammation and activity of tissue destructive lesion.

WHHL (Watanabe heritable hyperlipidemic) rabbit and KHC (Kusanagi-Hypercholesterolemic) rabbit have been established as model animals for heritable early onset coronary arteriosclerosis followed by myocardial infarction and familial hypercholesterolemia, and researches are actively carried out using these rabbits. Measurement of rabbit CRP in such researches would give useful findings.

4. Assay principle

In Shibayagi's Rabbit CRP ELISA Kit, standards or samples are incubated in polyclonal anti-CRP antibody-coated wells to capture CRP. After 1 hour incubation and washing, peroxidase-conjugated anti-CRP antibody is added and incubated further for 1 hour to bind captured CRP. After washing, peroxidase-complex remaining in wells is 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 nearly proportional to CRP concentration. The standard curve is prepared by plotting absorbance against standard CRP concentrations. CRP 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.
- Residual 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°C strictly. Avoid airstream velocity over 0.4 m/sec. (including wind from air conditioner)(*①), and humidity less than 30%. For more details, watch our web movie [Assay circumstance]

6. Reagents supplied

Components	State	Amount
(A) Antibody-coated 96 well-plate (Dried-plate)	Use after washing	96 wells/1 plate
(B) Rabbit Standard CRP (2 μg/ml) (derived from rabbit)	Concentrated. Use after dilution	200 μl/1 vial
(C) Buffer solution	Ready for use.	60 ml/1 bottle
(D) Peroxidase-conjugated anti-CRP antibody	Concentrated. Use after dilution.	200 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 cover	−	1 plate
Instruction Manual	−	1 copy

7. Equipments 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 200-500 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 (600-1200rpm)
☐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) Rabbit standard CRP (2 μg/ml)]

Make a serial dilution of original standard solution to prepare each standard solution. Example is shown below.

Volume of standard solution	Buffer solution	Concentration(ng/ml)
Original solution : 50 μl	450 μl	200
200 ng/ml solution : 200 μl	200 μl	100
100 ng/ml solution : 200 μl	200 μl	50
50 ng/ml solution : 200 μl	200 μl	25
25 ng/ml solution : 200 μl	200 μl	12.5
12.5 ng/ml solution : 200 μl	200 μl	6.25
6.25 ng/ml solution : 200 μl	200 μl	3.13
0 (Blank)	200 μl	0

[(D) Peroxidase-conjugated anti-CRP antibody]

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

[(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 96 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) Rabbit standard CRP (2 μg/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) Peroxidase-conjugated anti-CRP antibody]

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. Otherwise, store them in a dark place at 2-8 °C.
- 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. For more details, watch our web movie [Assay circumstance]

10. Preparation of samples

This kit is intended to measure CRP in rabbit serum or plasma. Use serum or plasma samples collected by proper method. Dilute samples properly using the kit's buffer so as to be in the assay range (3.13-200 ng/ml). Normal rabbit serum can be diluted 100x in standard procedure. Sample dilution should be carried out with the buffer solution of the kit using small test tubes before assay, and dispense to the wells. Samples should be immediately assayed or stored below –35 °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. Turbid samples or those containing insoluble materials should be centrifuged before testing to remove any particulate matter.

Storage and stability

If you have to store assay samples for a longer period, snap-freeze samples and keep them below –35°C. Avoid repeated freezing and thawing 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 CO_2 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 diluted 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 (200ng/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

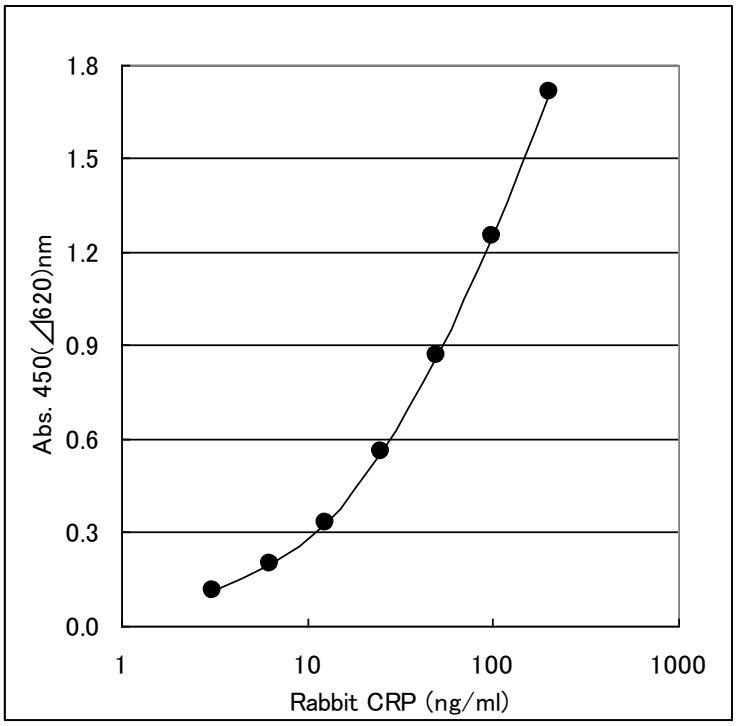
Remove the cover sheet of the 96 well-plate after bringing up to room temperature.

(1) Wash the antibody coated plate (A) by filling the well with washing buffer and discard 3 times, (*②) then strike the plate upside-down onto several layers of paper towels to remove residual buffer in the wells.
(2) Pipette 50 µl of diluted samples to the designated sample wells.
(3) Pipette 50 µl of standard solution to the wells designated for standards.
(4) Shake the plate on a plate shaker (*③).
(5) Put a plate cover 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 50 µl of peroxidase-conjugated anti-CRP antibody solution (D) to all wells, and shake as step (4).
(8) Put a plate cover 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 50 µl of chromogenic substrate solution (F) to wells, and shake as step (4).
(11) Put a plate cover on the plate and incubate the plate for 30 minutes at 20-25°C.
(12) Add 50 µl of the reaction stopper (H) to all wells and shake as step (4).
(13) 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 for notes of *② and *③.

12. Calculations

(1) Prepare a standard curve using semi-logarithmic or two-way logarithmic section paper by plotting absorbance* (Y-axis) against CRP concentration (ng/ml) on X-axis. Physiological or pathological situation of animals should be judged comprehensively taking other examination results into consideration.
(2) Using the standard curve, read the CRP concentration of a sample at its absorbance*, and multiply the assay value by dilution factor. 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.



Rabbit CRP assay standard curve (an example)
Absorbance may change due to assay situation.

13. Performance characteristics

- Assay range

The assay range of the kit is 3.13 ~ 200 ng/ml.

- Specificity

The antibody used in this kit is specific to CRP.

*Cross reaction at 5,000 ng/ml.

Samples	Cross reaction
Rat CRP	-
Mouse CRP	-
Human CRP	Less than 1.7%

- Precision of assay

Within assay variation (3 samples, 8 replicates assay,) Mean CV was 3.6 %.

- Reproducibility

Between assay variation (3 samples, 3 days, duplicates assay) Mean CV was less than 5 %

- Recovery test

CRP was added in 4 concentrations to 2 serum samples and was assayed.
The recoveries were 100 ~ 108%

- Dilution test

Serum samples were serially diluted by 3 steps.
The dilution curves showed excellent linearity. (R^2= 0.9999)

14. 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 peroxidase-conjugated CRP antibody or TMB might not be added.
3) Wrong reagents related to coloration might have been added. Wrong dilution of peroxidase-conjugated CRP antibody.
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.

- Blank OD was higher than that of the lowest standard concentration (3.13 ng/ml).

Possible explanations: Improper or inadequate washing. (Change washing frequency from . 3 times to 4-6 times at the constant stroke after the reaction with HRP-conjugated anti-CRP antibody.)

- 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 96 well-plate is empty when I opened the box.

A-2: As this kit is dried type, not preservation stabilizer is added.

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).

15. References

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 to 20-25°C for 2 hours.

☐Concentrated washing buffer must be diluted to 10 times by purified water.

☐Standard solution dilution example:

Concentration (ng/ml)	200	100	50	25	12.5	6.25	3.13	0
Std. solution (µl) orig.sol.	50	200*	200*	200*	200*	200*	200*	0
Buffer solution (µl)	450	200	200	200	200	200	200	200

*One rank higher standard.

☐Make the positive control.

			Precautions & related info
☐	Antibody-coated 96 well-plate (Dried-plate)		
☐	↓ Washing 3 time(*②)		*⑥
☐	Diluted Samples / Standards	50 µl	*⑦ [Handling of pipetting]
☐	↓ Shaking(*③), Incubation for 1 hour at 20-25°C. (Standing(*④))		*⑧ [Assay circumstance]
☐	Dilute peroxidase-conjugated anti-CRP antibody (D) to 100x with buffer (C) returned to 20-25°C.		
☐	↓ Washing 3 times(*②)		*⑥
☐	Peroxidase-conjugated anti-CRP antibody	50 µl	*⑦ [Handling of pipetting]
☐	↓ Shaking(*③), Incubation for 1 hour at 20-25°C. (Standing(*④))		*⑧ [Assay circumstance]
☐	↓ Washing 3 times(*②)		*⑥
☐	Chromogenic substrate (TMB)	50 µl	After dispense, the color turns to blue depending on the concentration.
☐	↓ Shaking(*③), Incubation for 30 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.

*②Guideline 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.

*④Put a plate cover on the plate during the reaction after shaking.

*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	**200 ng/ml**	Pos. Control	Sample 8	Sample 16	Sample 24	Sample 32
B	**100 ng/ml**	Sample 1	Sample 9	Sample 17	Sample 25	Sample 33
C	**50 ng/ml**	Sample 2	Sample 10	Sample 18	Sample 26	Sample 34
D	**25 ng/ml**	Sample 3	Sample 11	Sample 19	Sample 27	Sample 35
E	**12.5 ng/ml**	Sample 4	Sample 12	Sample 20	Sample 28	Sample 36
F	**6.25 ng/ml**	Sample 5	Sample 13	Sample 21	Sample 29	Sample 37
G	**3.13 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] 6 months from production (Expiration date is indicated on the container.)

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