

ELISA Kit for Measuring Human High-Sensitivity CRP

CircuLex High-Sensitivity CRP ELISA Kit

Cat# CY-8071

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Intended Use

The MBL Research Product **CircuLex High-Sensitivity CRP ELISA Kit** is used for the quantitative measurement of Human C-reactive protein (CRP) in serum.

Individual users should determine appropriate conditions when using other types of samples.

This assay kit is for research use only and not for use in diagnostic or therapeutic procedures.

Storage

- Upon receipt store all components at 4°C.
- Don't expose reagents to excessive light.

Introduction

C-Reactive protein (CRP) is a pentameric acute phase reactant that is synthesized by the liver. Its production is controlled primarily by interleukin-6. The serum CRP concentration may increase by up to 1000-fold with infection, ischemia, trauma, surgery, and other acute inflammatory events (1). Thus CRP can be used as an extremely sensitive systemic marker of inflammation. Especially in situations where microbiological diagnosis is difficult or too slow in the clinical context, CRP measurements can be used to infer the presence of bacterial infection (2).

Ligand-bound or aggregated CRP binds C1q and in so doing activates the classical complement pathway (3). Although primarily synthesized in hepatocytes, there is evidence for local expression of CRP in macrophages of the lung and the brain (4–6). In atherosclerotic plaques, it has been found associated with complement proteins and within foam cells (7, 8).

A growing number of studies suggest that CRP is an independent risk factor for atherosclerotic vascular disease. Plasma CRP concentrations in the highest quartile are associated, depending on the subject group, with 1.5- to 7-fold increases in relative risk of symptomatic atherosclerosis (1, 9) The baseline plasma concentration of C-reactive protein predicts the risk of future myocardial infarction and stroke (10) and is associated with a poor prognosis in unstable angina (11–13).

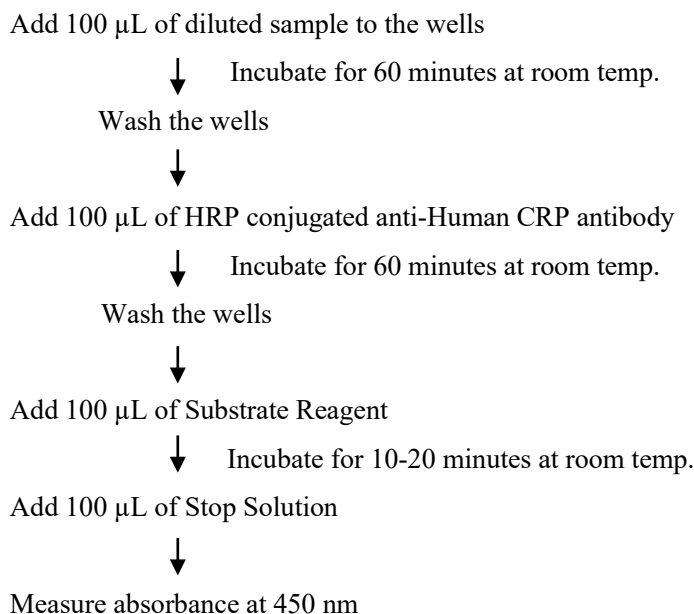
Plasma CRP levels are also strongly associated with obesity and obesity-related diseases, including insulin resistance, diabetes mellitus, and hyperlipidemia (14–17). Although a recent report indicated that the plasma CRP level decreased during weight reduction (17), the precise interaction of CRP with obesity has not been fully elucidated.

Principle of the Assay

The CircuLex High-Sensitivity CRP ELISA Kit employs the quantitative sandwich enzyme immunoassay technique. An antibody specific for Human CRP has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and the immobilized antibody binds any Human CRP present. After washing away any unbound substances, an HRP conjugated antibody specific for Human CRP is added to the wells. Following a wash to remove any unbound antibody HRP conjugate, the remaining conjugate is allowed to react with the substrate H₂O₂-tetramethylbenzidine. The reaction is stopped by addition of acidic solution and absorbance of the resulting yellow product is measured at 450 nm. The absorbance is proportional to the concentration of Human CRP. A standard curve is constructed by plotting absorbance values versus Human CRP concentrations of calibrators, and concentrations of unknown samples are determined using this standard curve.

The CircuLex High-Sensitivity CRP ELISA Kit is designed to measure the concentration of Human CRP from cell lysate of cultured human cell lines, cell culture conditioned medium and human serum/plasma.

Summary of Procedure



Materials Provided

All samples and standards should be assayed in duplicate. The following components are supplied and are sufficient for the one 96-well microplate kit.

Microplate: One microplate supplied ready to use, with 96 wells (12 strips of 8-wells) in a foil, zip-lock bag with a desiccant pack. Wells are coated with anti-human CRP antibody as a capture antibody.

10X Wash Buffer: One 100 mL bottle of 10X buffer containing Tween®-20

Dilution Buffer: One bottle containing 50 mL of 1X buffer; use for sample dilution. Ready to use.

Human CRP Standard*: One vial containing X* ng of lyophilized Human CRP

***The amount is changed depending on lot. See the real "User's Manual" included in the kit box.**

HRP conjugated Detection Antibody: One vial containing 12 mL of HRP (horseradish peroxidase) conjugated anti-Human CRP antibody. Ready to use.

Substrate Reagent: One bottle containing 20 mL of the chromogenic substrate, tetra-methylbenzidine (TMB). Ready to use.

Stop Solution: One bottle containing 20 mL of 1 N H₂SO₄. Ready to use.

* It contains human sera. Handle as if capable of transmitting infectious agents. The sera were tested and found to be negative for HIV, HBV and HCV. However, because no test method can offer complete assurance that HIV, HBV, HCV or other infectious agents are absent, this component should be handled at the Bio-safety Level 2 (BSL 2) as recommended for any potentially infectious human serum or blood specimen in the CDC / NIH manual "Biosafety in microbiological and Biomedical Laboratories".

Materials Required but not Provided

- **Pipettors:** 2-20 μL , 20-200 μL and 200-1,000 μL precision pipettors with disposable tips.
- **Precision repeating pipettor**
- **Orbital microplate shaker**
- **Microcentrifuge and tubes** for sample preparation.
- **Vortex mixer**
- **(Optional) Microplate washer:** Manual washing is possible but not preferable.
- **Plate reader** capable of measuring absorbance in 96-well plates at dual wavelengths of 450 nm/540 nm. Dual wavelengths of 450/550 or 450/595 nm can also be used. The plate can also be read at a single wavelength of 450 nm, which will give a somewhat higher reading.
- **(Optional) Software package facilitating data generation and analysis**
- **500 or 1,000 mL graduated cylinder.**
- **Reagent reservoirs**
- **Deionized water of the highest quality**
- **Disposable paper towels**

Precautions and Recommendations

- **Although we suggest to conduct experiments as outlined below, the optimal experimental conditions will vary depending on the parameters being investigated, and must be determined by the individual user.**
- Allow all the components to come to room temperature before use.
- All microplate strips that are not immediately required should be returned to the zip-lock pouch, which must be carefully resealed to avoid moisture absorption.
- Do not use kit components beyond the indicated kit expiration date.
- Use only the microtiter wells provided with the kit.
- Rinse all detergent residue from glassware.
- Use deionized water of the highest quality.
- Do not mix reagents from different kits.
- The buffers and reagents in this kit may contain preservatives or other chemicals. Care should be taken to avoid direct contact with these reagents.
- Do not mouth pipette or ingest any of the reagents.
- Do not smoke, eat, or drink when performing the assay or in areas where samples or reagents are handled.
- Dispose of tetra-methylbenzidine (TMB) containing solutions in compliance with local regulations.
- Avoid contact with the acidic Stop Solution and Substrate Solution, which contains hydrogen peroxide.
- Wear gloves and eye protection when handling immunodiagnostic materials and samples of human origin, and these reagents. In case of contact with the Stop Solution and the Substrate Solution, wash skin thoroughly with water and seek medical attention, when necessary.
- **“Human CRP Standard” contains human sera. Handle as if capable of transmitting infectious agents. The sera were tested and found to be negative for HIV, HBV and HCV. However, because no test method can offer complete assurance that HIV, HBV, HCV or other infectious agents are absent, this component should be handled at the Bio-safety Level 2 (BSL 2) as recommended for any potentially infectious human serum or blood specimen in the CDC / NIH manual "Biosafety in microbiological and Biomedical Laboratories".**
- **Biological samples may be contaminated with infectious agents. Do not ingest, expose to open wounds or breathe aerosols. Wear protective gloves and dispose of biological samples properly.**
- **Sulfuric Acid is a strong acid. Wear disposable gloves and eye protection when handling Stop Solution.**

Sample Collection and Storage

Serum: Use a serum separator tube and allow samples to clot for 60 ± 30 minutes. Centrifuge the samples at 4°C for 10 minutes at 1,000 x g. Remove serum and assay immediately or store samples on ice for up to 6 hours before assaying. Aliquots of serum may also be stored at below -70°C for extended periods of time. Avoid repeated freeze-thaw cycles.

Other biological samples: MBL has not tested.

(e.g. Remove any particulates by centrifugation and assay immediately or aliquot and store samples at below -70°C. Avoid repeated freeze-thaw cycles. Individual users should determine appropriate conditions when using other types of samples.)

Detailed Protocol

The MBL Research Product **CircuLex High-Sensitivity CRP ELISA Kit** is provided with removable strips of wells so the assay can be carried out on separate occasions using only the number of strips required for the particular determination. Since experimental conditions may vary, an aliquot of the Human CRP Standard within the kit, should be included in each assay as a calibrator. Disposable pipette tips and reagent troughs should be used for all liquid transfers to avoid cross-contamination of reagents or samples.

Preparation of Working Solutions

All reagents need to be brought to room temperature prior to the assay. Assay reagents are supplied ready-to-use, with the exception of **10X Wash Buffer** and **Human CRP Standard**.

1. Prepare a working solution of Wash Buffer by adding 100 mL of the **10X Wash Buffer** to 900 mL of deionized (distilled) water. Mix well. Store at 4°C for two weeks or -20°C for long-term storage.
2. Reconstitute **Human CRP Standard** with **X* mL** of **Dilution Buffer**. The concentration of the human CRP in vial should be **15.2 ng/mL**, which is referred to as a **Master Standard** of human CRP. ***The amount is changed depending on lot. See the real "User's Manual" included in the kit box.**

Prepare Standard Solutions as follows:

Use the **Master Standard** to produce a dilution series (below). Mix each tube thoroughly before the next transfer. The 3,800 pg/mL standard (Std.1) serves as the high standard. The **Dilution Buffer** serves as the zero standard (Blank).

	Volume of Standard	Dilution Buffer	Concentration
Std.1	150 µL of Master Standard	450 µL	3,800 pg/mL
Std.2	300 µL of Std. 1 (3,800 pg/mL)	300 µL	1,900 pg/mL
Std.3	300 µL of Std. 2 (1,900 pg/mL)	300 µL	950 pg/mL
Std.4	300 µL of Std. 3 (950 pg/mL)	300 µL	475 pg/mL
Std.5	300 µL of Std. 4 (475 pg/mL)	300 µL	237.5 pg/mL
Std.6	300 µL of Std. 5 (237.5pg/mL)	300 µL	118.8 pg/mL
Std.7	300 µL of Std. 6 (118.8 pg/mL)	300 µL	59.4 pg/mL
Blank	-	300 µL	0 pg/mL

Note: Do not use a Repeating pipette. Change tips for every dilution. Wet tip with Dilution Buffer before dispensing. Unused portions of Standards should be aliquoted and stored at below -70°C immediately. Avoid multiple freeze and thaw cycles.

Sample Preparation

Dilute samples with **Dilution Buffer**.

- Serum samples may require a 2,500-fold dilution.
 - e.g. First: Make 50-fold dilution (2 µL of sample + 98 µL of Dilution Buffer)
 - Second: Make 2,500-fold dilution (5 µL of 50-fold diluted sample + 245 µL Dilution Buffer)
 - Don't store diluted samples.

Assay Procedure

1. Prepare the assay protocol, assigning the appropriate wells for setting up Standard solutions, diluted patient specimens and any internal laboratory controls in duplicate.
Remove the appropriate number of microtiter wells from the foil pouch and place them into the well holder. Return any unused wells to the foil pouch, refold, seal with tape and store at 4°C.
2. Dilute samples with **Dilution Buffer**. (See “Sample Preparation” above.)
3. Pipette **100 µL** of **Standard Solutions (Std1-Std7, Blank)** and **diluted samples** in duplicates, into the appropriate wells.
4. Incubate the plate **at room temperature (ca.25°C) for 60 minutes, shaking at ca. 300 rpm on an orbital microplate shaker.**
5. Wash 4-times by filling each well with Wash Buffer (350 µL) using a squirt bottle, multi-channel pipette, manifold dispenser or microplate washer.
6. Add **100 µL** of **HRP conjugated Detection Antibody** into each well.
7. Incubate the plate **at room temperature (ca.25°C) for 60 minutes, shaking at ca. 300 rpm on an orbital microplate shaker.**
8. Wash 4-times by filling each well with Wash Buffer (350 µL) using a squirt bottle, multi-channel pipette, manifold dispenser or microplate washer.
9. Add **100 µL** of **Substrate Reagent**. Avoid exposing the microtiter plate to direct sunlight. Covering the plate with e.g. aluminum foil is recommended. Return Substrate Reagent to 4°C immediately after the necessary volume is removed.
10. Incubate the plate **at room temperature (ca. 25°C) for 10-20 minutes, shaking at ca. 300 rpm on an orbital microplate shaker.** The incubation time may be extended up to 30 minutes if the reaction temperature is below than 20°C.
11. Add **100 µL** of **Stop Solution** to each well in the same order as the previously added Substrate Reagent.
12. Measure absorbance in each well using a spectrophotometric microplate reader at dual wavelengths of 450/540 nm. Dual wavelengths of 450/550 or 450/595 nm can also be used. Read the microplate at 450 nm if only a single wavelength can be used. Wells must be read within 30 minutes of adding the Stop Solution.

Note-1: Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.

Note-2: Reliable standard curves are obtained when either O.D. values do not exceed 0.2 units for the blank (zero concentration), or 2.5 units for the highest standard concentration.

Note-3: If the microplate reader is not capable of reading absorbance greater than the absorbance of the highest standard, perform a second reading at 405 nm. A new standard curve, constructed using the values measured at 405 nm, is used to determine Human CRP concentration of off-scale samples. The readings at 405 nm should not replace the on-scale readings at 450 nm.

Calculations

Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density. Plot the optical density for the standards versus the concentration of the standards and draw the best curve. To determine the Human CRP concentration of each sample, first find the absorbance value on the y-axis and extend a horizontal line to the standard curve. At the point of intersection, extend a vertical line to the x-axis and read the corresponding Human CRP concentration. If the samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

1. The results of unknown samples can be calculated with any computer program having a 5-parameter logistic function. It is important to make an appropriate mathematical adjustment to accommodate for the dilution factor.
2. Most microtiter plate readers perform automatic calculations of analyte concentration. The calibration curve is constructed by plotting the absorbance (Y) of calibrators versus log of the known concentration (X) of calibrators, using the four-parameter function. Alternatively, the logit log function can be used to linearize the calibration curve (i.e. logit of absorbance (Y) is plotted versus log of the known concentration (X) of calibrators).

Measurement Range

The measurement range is 59.4 pg/mL to 3,800 pg/mL. Any sample reading higher than the highest standard should be diluted with Dilution Buffer in higher dilution and re-assayed. Dilution factors need to be taken into consideration in calculating the human CPR concentration.

Troubleshooting

1. All samples and controls should be assayed in duplicate, using the protocol described in the **Detailed Protocol**. Incubation times or temperatures significantly different from those specified may give erroneous results.
2. Poor duplicates, accompanied by elevated values for wells containing no sample, indicate insufficient washing. If all instructions in the **Detailed Protocol** were followed accurately, such results indicate a need for washer maintenance.
3. Overall low signal may indicate that desiccation of the plate has occurred between the final wash and addition of Substrate Reagent. Do not allow the plate to dry out. Add Substrate Reagent immediately after wash.

Reagent Stability

All of the reagents included in the MBL Research Product **CircuLex High-Sensitivity CRP ELISA Kit** have been tested for stability. Reagents should not be used beyond the stated expiration date. Upon receipt, kit reagents should be stored at 4°C, except the reconstituted Human CRP Standard must be stored at below -70°C. Coated assay plates should be stored in the original foil bag sealed by the zip lock and containing a desiccant pack.

Assay Characteristics

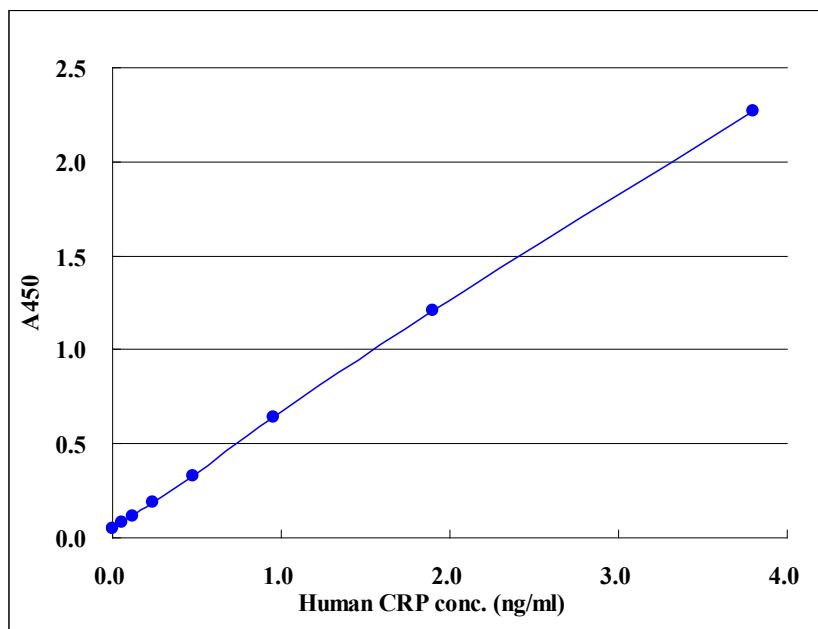
1. Sensitivity

The limit of detection (defined as such a concentration of Human CRP giving absorbance higher than mean absorbance of blank* plus three standard deviations of the absorbance of blank: A blank + 3SD blank) is better than 28.6 pg/mL of sample.

* Dilution Buffer was pipetted into blank wells.

Eighty assays were evaluated and the minimum detectable dose (MDD) of Human CRP ranged from 21.5- 34.3 pg/mL. The mean MDD was 28.6 pg/mL. The MDD was determined by adding three standard deviations to the mean optical density value of twenty zero standard replicates and calculating the corresponding concentration.

Typical standard curve



2. Specificity

The antibodies in the CircuLex High-Sensitivity CRP ELISA Kit are highly specific of Human CRP, with no detectable cross-reactivity to other serum protein and to other acute phase reactant including SAA.

3. Precision

Intra-assay Precision (Precision within an assay)

Three samples of known concentration were tested twelve times on one plate to assess intra-assay precision.

- Intra-assay (Within-Run, n=12) CV = 3.8, 1.8, 2.6 %

CRP conc.in human Serum (ug/ml)			
	A	B	C
1	1.18	4.47	7.83
2	1.22	4.29	8.57
3	1.21	4.27	8.24
4	1.28	4.26	8.07
5	1.24	4.28	8.41
6	1.32	4.38	8.54
7	1.24	4.37	8.40
8	1.20	4.44	8.30
9	1.28	4.23	8.18
10	1.31	4.30	8.23
11	1.32	4.37	8.27
12	1.30	4.37	8.56
MAX.	1.32	4.47	8.57
MIN.	1.18	4.23	7.83
MEAN	1.26	4.34	8.30
S.D.	0.05	0.08	0.22
C.V.	3.8%	1.8%	2.6%

Inter-assay Precision (Precision between assays)

Three samples of known concentration were tested in five separate assays to assess inter-assay precision.

- Inter-assay (Run-to-Run, n=5) CV = 5.2, 5.2, 2.6 %

CRP conc. (ug/ml)			
Day	Serum 1	Serum 2	Serum 3
1	1.16	3.94	7.29
2	1.14	3.88	7.33
3	1.01	3.58	7.44
4	1.11	3.76	7.13
5	1.11	3.48	6.97
MAX.	1.16	3.94	7.44
MIN.	1.01	3.48	6.97
MEAN	1.10	3.73	7.23
S.D.	0.058	0.195	0.186
C.V.	5.2%	5.2%	2.6%

4. Spiking Recover

Serum samples were spiked with different amounts of Human CRP and assayed.

The recovery of Human CRP spiked to levels throughout the range of the assay was evaluated.

Sample Average % Recovery Range

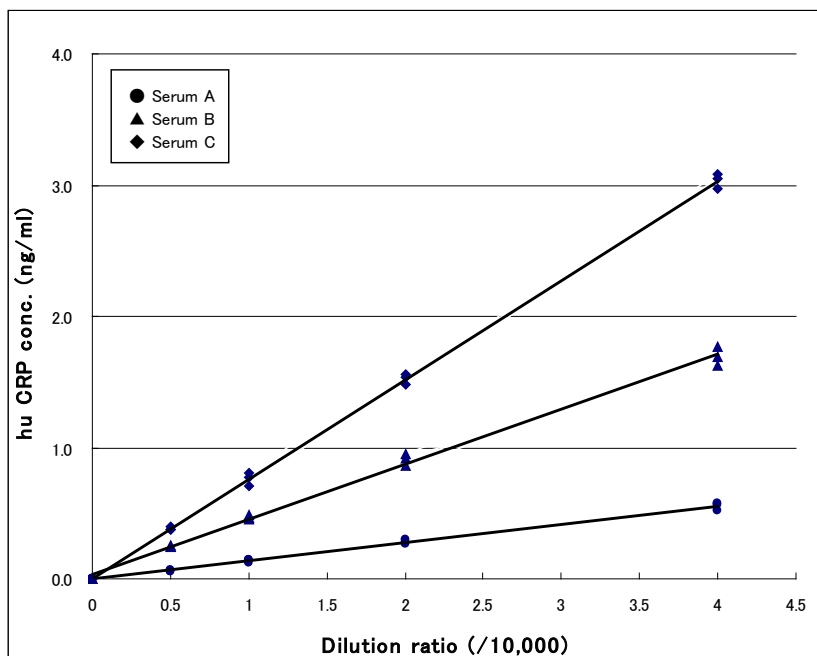
Cell culture media (n=4)

- Recovery = 104, 96, 112 %

5. Linearity

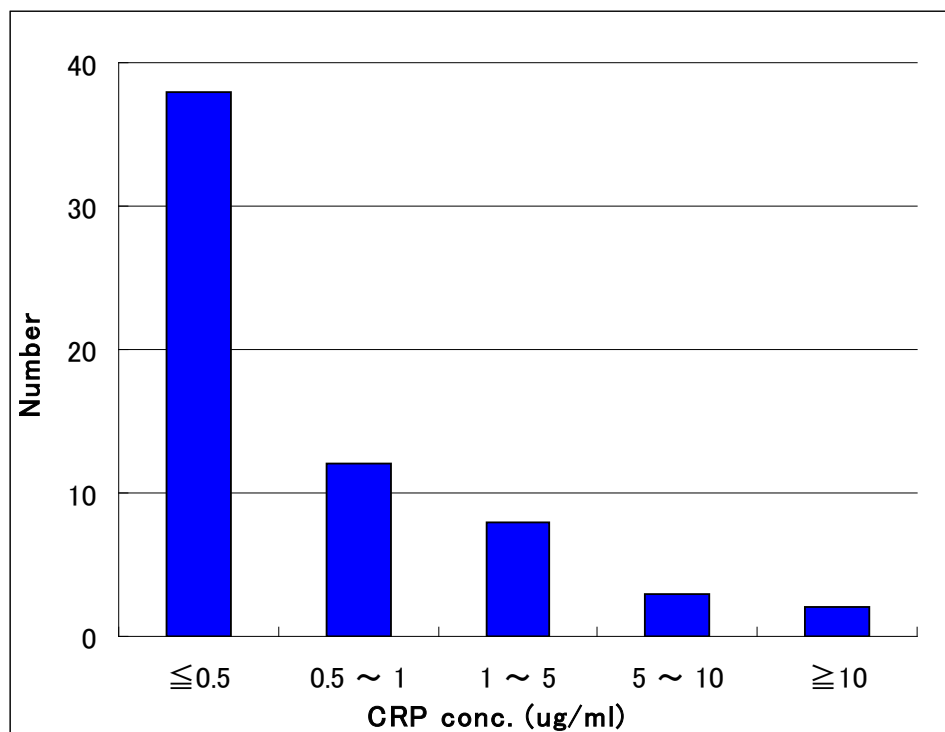
To assess the linearity of the assay, samples containing and/or spiked with high concentrations of Human CRP were serially diluted with the Dilution Buffer to produce samples with values within the dynamic range of the assay.

The linearity of the assay



Example of Test Results

Fig.1 Human CRP level in sera from 63 healthy volunteers



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