



ab181416 – C-Reactive Protein (CRP) Human SimpleStep ELISA[®] Kit

Instructions for Use

For the quantitative measurement of C-Reactive Protein (CRP) in human cell culture supernatant, serum and plasma samples.

This product is for research use only and is not intended for diagnostic use.

This kit was updated on 27th April 2015. Please read the booklet carefully before proceeding with the assay.

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1. BACKGROUND

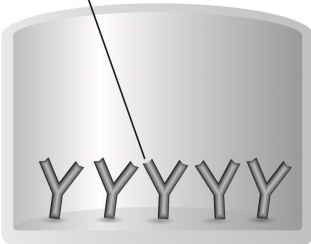
Abcam's C-Reactive Protein (CRP) *in vitro* SimpleStep ELISA® (Enzyme-Linked Immunosorbent Assay) kit is designed for the quantitative measurement of CRP protein in human cell culture supernatant, serum and plasma samples.

The SimpleStep ELISA® employs an affinity tag labeled capture antibody and a reporter conjugated detector antibody which immunocapture the sample analyte in solution. This entire complex (capture antibody/analyte/detector antibody) is in turn immobilized via immunoaffinity of an anti-tag antibody coating the well. To perform the assay, samples or standards are added to the wells, followed by the antibody mix. After incubation, the wells are washed to remove unbound material. TMB substrate is added and during incubation is catalyzed by HRP, generating blue coloration. This reaction is then stopped by addition of Stop Solution completing any color change from blue to yellow. Signal is generated proportionally to the amount of bound analyte and the intensity is measured at 450 nm. Optionally, instead of the endpoint reading, development of TMB can be recorded kinetically at 600 nm.

CRP displays several functions associated with host defense: it promotes agglutination, bacterial capsular swelling, phagocytosis and complement fixation through its calcium-dependent binding to phosphorylcholine. CRP can interact with DNA and histones and it may scavenge nuclear material released from damaged circulating cells. CRP is secreted; it forms a homopentamer pentaxin (or pentraxin) which have a discoid arrangement of 5 non-covalently bound subunits. CRP binds 2 calcium ions per subunit. The concentration of CRP in plasma increases greatly during acute phase response to tissue injury, infection or other inflammatory stimuli. It is induced by IL1/interleukin-1 and IL6/interleukin-6.

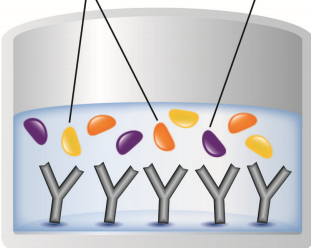
2. ASSAY SUMMARY

Immobilization Antibody



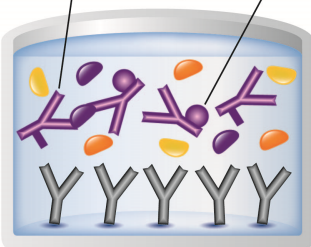
Remove appropriate number of antibody coated well strips. Equilibrate all reagents to room temperature. Prepare all reagents, samples, and standards as instructed.

Matrix Proteins Target Analyte



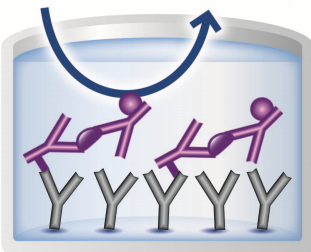
Add standard or sample to appropriate wells.

Capture Antibody Detector Antibody



Add Antibody Cocktail to all wells. Incubate at room temperature.

Substrate Color Development



Aspirate and wash each well. Add TMB Substrate to each well and incubate. Add Stop Solution at a defined endpoint. Alternatively, record color development kinetically after TMB substrate addition.

3. PRECAUTIONS

Please read these instructions carefully prior to beginning the assay.

All kit components have been formulated and quality control tested to function successfully as a kit. Modifications to the kit components or procedures may result in loss of performance.

4. STORAGE AND STABILITY

Store kit at 2-8°C immediately upon receipt.

Refer to list of materials supplied for storage conditions of individual components. Observe the storage conditions for individual prepared components in sections 9 & 10.

5. MATERIALS SUPPLIED

Item	Amount	Storage Condition (Before Preparation)
10X CRP Capture Antibody	600 µL	+2-8°C
10X CRP Detector Antibody	600 µL	+2-8°C
CRP Human Lyophilized Recombinant Protein	2 Vials	+2-8°C
Antibody Diluent CPI	6 mL	+2-8°C
10X Wash Buffer PT	20 mL	+2-8°C
TMB Substrate	12 mL	+2-8°C
Stop Solution	12 mL	+2-8°C
Sample Diluent NS	50 mL	+2-8°C
Pre-Coated 96 Well Microplate (12 x 8 well strips)	96 Wells	+2-8°C
Plate Seal	1	+2-8°C

6. MATERIALS REQUIRED, NOT SUPPLIED

These materials are not included in the kit, but will be required to successfully utilize this assay:

- Microplate reader capable of measuring absorbance at 450 or 600 nm.
- Method for determining protein concentration (BCA assay recommended).
- Deionized water.
- PBS (1.4 mM KH₂PO₄, 8 mM Na₂HPO₄, 140 mM NaCl, 2.7 mM KCl, pH 7.4).
- Multi- and single-channel pipettes.
- Tubes for standard dilution.
- Plate shaker for all incubation steps.
- Phenylmethylsulfonyl Fluoride (PMSF) (or other protease inhibitors).

7. LIMITATIONS

- Assay kit intended for research use only. Not for use in diagnostic procedures.
- Do not mix or substitute reagents or materials from other kit lots or vendors. Kits are QC tested as a set of components and performance cannot be guaranteed if utilized separately or substituted.

8. TECHNICAL HINTS

- Samples generating values higher than the highest standard should be further diluted in the appropriate sample dilution buffers.
- Avoid foaming or bubbles when mixing or reconstituting components.
- Avoid cross contamination of samples or reagents by changing tips between sample, standard and reagent additions.

- Ensure plates are properly sealed or covered during incubation steps.
- Complete removal of all solutions and buffers during wash steps is necessary to minimize background.
- As a guide, typical ranges of sample concentration for commonly used sample types are shown below in Sample Preparation (section 11).
- All samples should be mixed thoroughly and gently.
- Avoid multiple freeze/thaw of samples.
- Incubate ELISA plates on a plate shaker during all incubation steps.
- When generating positive control samples, it is advisable to change pipette tips after each step.
- The provided Antibody Diluents and Sample Diluents contain protease inhibitor aprotinin. Additional protease inhibitors can be added if required.
- **To avoid high background always add samples or standards to the well before the addition of the antibody cocktail.**
- **This kit is sold based on number of tests. A ‘test’ simply refers to a single assay well. The number of wells that contain sample, control or standard will vary by product. Review the protocol completely to confirm this kit meets your requirements. Please contact our Technical Support staff with any questions.**

9. REAGENT PREPARATION

- Equilibrate all reagents to room temperature (18-25°C) prior to use. The kit contains enough reagents for 96 wells. **The sample volumes below are sufficient for 48 wells (6 x 8-well strips); adjust volumes as needed for the number of strips in your experiment.**
- Prepare only as much reagent as is needed on the day of the experiment. Capture and Detector Antibodies have only been tested for stability in the provided 10X formulations.

9.1 **1X Wash Buffer PT**

Prepare 1X Wash Buffer PT by diluting 10X Wash Buffer PT with deionized water. To make 50 mL 1X Wash Buffer PT combine 5 mL 10X Wash Buffer PT with 45 mL deionized water. Mix thoroughly and gently.

9.2 **Antibody Cocktail**

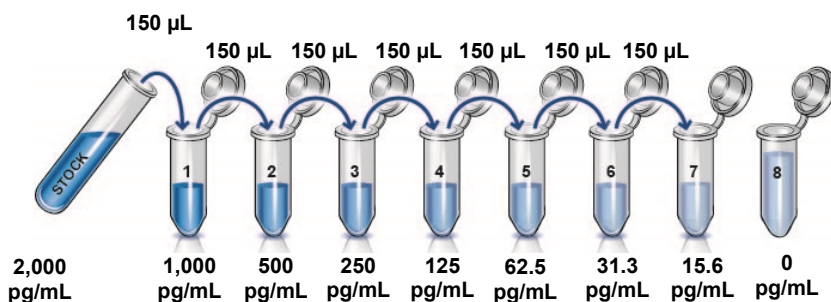
Prepare Antibody Cocktail by diluting the capture and detector antibodies in Antibody Diluent CPI. To make 3 mL of the Antibody Cocktail combine 300 μ L 10X Capture Antibody and 300 μ L 10X Detector Antibody with 2.4 mL Antibody Diluent CPI. Mix thoroughly and gently.

10. STANDARD PREPARATION

Prepare serially diluted standards immediately prior to use. Always prepare a fresh set of positive controls for every use.

The following table describes the preparation of a standard curve for duplicate measurements (recommended).

- 10.1 Reconstitute the CRP human protein standard sample by adding 200 μL water by pipette. Mix thoroughly and gently. Hold at room temperature for 5 minutes and mix gently. This is the 2,000 pg/mL **Stock Standard** Solution.
- 10.2 Label eight tubes, Standards 1– 8 and add 150 μL Sample Diluent NS into each tube.
- 10.3 Use the Stock Standard to prepare the following dilution series. Standard #8 contains no protein and is the Blank control:



11. SAMPLE PREPARATION

TYPICAL SAMPLE DYNAMIC RANGE	
Sample Type	Range
Human Serum	8,000 - 512,000X diluted
Human Plasma - Citrate	8,000 - 256,000X diluted
Human Plasma - EDTA	4,000 - 128,000X diluted
Human Plasma - Heparin	4,000 - 128,000X diluted

11.1 Plasma

Collect plasma using citrate, EDTA or heparin. Centrifuge samples at 2,000 x g for 10 minutes. Dilute samples into Sample Diluent NS and assay. Store un-diluted plasma samples at -20°C or below for up to 3 months. Avoid repeated freeze-thaw cycles.

11.2 Serum

Samples should be collected into a serum separator tube. After clot formation, centrifuge samples at 2,000 x g for 10 minutes and collect serum. Dilute samples into Sample Diluent NS and assay. Store un-diluted serum at -20°C or below. Avoid repeated freeze-thaw cycles.

11.3 Cell Culture Supernatants

Centrifuge cell culture media at 2,000 x g for 10 minutes to remove debris. Collect supernatants and assay. If needed, dilute samples into Sample Diluent NS. Store samples at -20°C or below. Avoid repeated freeze-thaw cycles.

Note: Due to the high dilutions required for certain Human serum and plasma samples, we recommend initially diluting your samples in 1X Wash Buffer and then performing the final dilution in Sample Diluent NS. As an example the table below demonstrates the steps suggested to generate a final sample dilution of 1:10,000.

Tube #	Sample to Dilute	Volume to Dilute (μL)	Volume of 1X Wash Buffer (μL)	Volume of Sample Diluent NS (μL)	Starting Dilution	Final Dilution
1	Neat serum/plasma	10	240	0	Neat	1:25
2	Tube #1	10	190	0	1:25	1:500
3	Tube #2	7.5	0	142.5	1:500	1:10,000

12. PLATE PREPARATION

- The 96 well plate strips included with this kit are supplied ready to use. It is not necessary to rinse the plate prior to adding reagents.
- Unused plate strips should be immediately returned to the foil pouch containing the desiccant pack, resealed and stored at 4°C.
- For each assay performed, a minimum of two wells must be used as the zero control.
- For statistical reasons, we recommend each sample should be assayed with a minimum of two replicates (duplicates).
- Differences in well absorbance or “edge effects” have not been observed with this assay.

13. ASSAY PROCEDURE

- **Equilibrate all materials and prepared reagents to room temperature prior to use.**
- **It is recommended to assay all standards, controls and samples in duplicate.**

- 13.1 Prepare all reagents, working standards, and samples as directed in the previous sections.
- 13.2 Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, reseal and return to 4°C storage.
- 13.3 Add 50 µL of all sample or standard to appropriate wells.
- 13.4 Add 50 µL of the Antibody Cocktail to each well.
- 13.5 Seal the plate and incubate for 1 hour at room temperature on a plate shaker set to 400 rpm.
- 13.6 Wash each well with 3 x 350 µL 1X Wash Buffer PT. Wash by aspirating or decanting from wells then dispensing 350 µL 1X Wash Buffer PT into each well. Complete removal of liquid at each step is essential for good performance. After the last wash invert the plate and blot it against clean paper towels to remove excess liquid.
- 13.7 Add 100 µL of TMB Substrate to each well and incubate for 10 minutes in the dark on a plate shaker set to 400 rpm.
- 13.8 Add 100 µL of Stop Solution to each well. Shake plate on a plate shaker for 1 minute to mix. Record the OD at 450 nm. This is an endpoint reading.

Alternative to 13.7 – 13.8: Instead of the endpoint reading at 450 nm, record the development of TMB Substrate kinetically. Immediately after addition of TMB Development Solution begin recording the blue color development with elapsed time in the microplate reader prepared with the following settings:

ASSAY PROCEDURE

Mode:	Kinetic
Wavelength:	600 nm
Time:	up to 15 min
Interval:	20 sec - 1 min
Shaking:	Shake between readings

Note that an endpoint reading can also be recorded at the completion of the kinetic read by adding 100 μ L Stop Solution to each well and recording the OD at 450 nm.

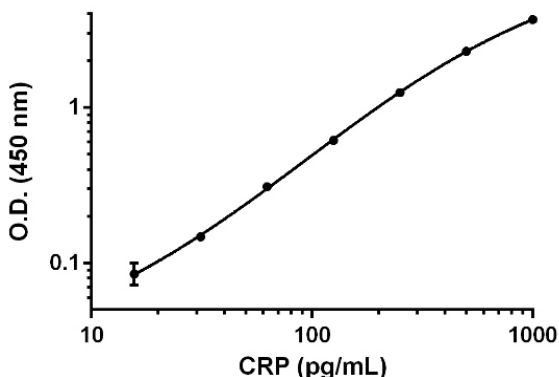
- 13.9 Analyze the data as described below.

14. CALCULATIONS

Subtract average zero standard from all readings. Average the duplicate readings of the positive control dilutions and plot against their concentrations. Draw the best smooth curve through these points to construct a standard curve. Most plate reader software or graphing software can plot these values and curve fit. A four parameter algorithm (4PL) usually provides the best fit, though other equations can be examined to see which provides the most accurate (e.g. linear, semi-log, log/log, 4 parameter logistic). Interpolate protein concentrations for unknown samples from the standard curve plotted. Samples producing signals greater than that of the highest standard should be further diluted and reanalyzed, then multiplying the concentration found by the appropriate dilution factor.

15. TYPICAL DATA

TYPICAL STANDARD CURVE – Data provided for **demonstration purposes only**. A new standard curve must be generated for each assay performed.



Standard Curve Measurements			
Conc. (pg/mL)	O.D. 450 nm		Mean O.D.
	1	2	
0.00	0.096	0.084	0.090
15.63	0.166	0.186	0.176
31.25	0.243	0.232	0.238
62.5	0.402	0.399	0.401
125	0.711	0.701	0.707
250	1.373	1.311	1.343
500	2.435	2.354	2.395
1,000	3.820	3.729	3.775

Figure 1. Example of CRP standard curve. The CRP standard curve was prepared as described in Section 10. Raw data values are shown in the table. Background-subtracted data values (mean \pm SD, $n=2$) are graphed.

16. TYPICAL SAMPLE VALUES

SENSITIVITY –

The calculated minimal detectable (MDD) dose is 4 pg/mL. The MDD was determined by calculating the mean of zero standard replicates (n=29) and adding 2 standard deviations then extrapolating the corresponding concentrations.

RECOVERY –

Three concentrations of CRP were spiked in duplicate to the indicated biological matrix to evaluate signal recovery in the working range of the assay.

Sample Type	Average % Recovery	Range (%)
100% Cell Culture Media (RPMI1640 cont. 15% FBS)	90.1	88.3 – 93.4

LINEARITY OF DILUTION –

Linearity of dilution is determined based on interpolated values from the standard curve. Linearity of dilution defines a sample concentration interval in which interpolated target concentrations are directly proportional to sample dilution.

DATA ANALYSIS

Native CRP was measured in the following biological samples in a 2-fold dilution series. Sample dilutions were made in Sample Diluent NS. Interpolated values below the MDD concentration were not analyzed (NA).

Dilution Factor	Interpolated value	0.0125% Human Serum	0.0125% Human Plasma (Citrate)	0.025% Human Plasma (EDTA)	0.025% Human Plasma (Heparin)
Undiluted	pg/mL	784.30	956.28	363.98	306.11
	% Expected value	100.0	100.0	100.0	100.0
2	pg/mL	361.72	464.76	190.12	155.51
	% Expected value	92.2	97.2	104.5	101.6
4	pg/mL	178.75	231.40	97.14	81.06
	% Expected value	91.2	96.8	106.8	105.9
8	pg/mL	95.95	130.94	49.67	41.75
	% Expected value	97.9	109.5	109.2	109.1
16	pg/mL	47.80	68.68	24.67	20.72
	% Expected value	97.5	114.9	108.4	108.3
32	pg/mL	23.911	36.89	10.02	9.73
	% Expected value	97.6	123.5	88.1	101.7
64	pg/mL	10.72	NA	NA	NA
	% Expected value	87.5	NA	NA	NA

Recombinant CRP was spiked into the following biological samples and diluted in a 2-fold dilution series in Sample Diluent NS.

Dilution Factor	Interpolated value	100% 15F RPMI1640 Media
Undiluted	pg/mL	532.85
	% Expected value	100.0
2	pg/mL	273.98
	% Expected value	102.8
4	pg/mL	139.72
	% Expected value	104.9
8	pg/mL	71.37
	% Expected value	107.2
16	pg/mL	34.63
	% Expected value	104.0

PRECISION –

Mean coefficient of variations of interpolated values from 3 dilutions of human serum within the working range of the assay.

	Intra-Assay	Inter-Assay
n=	5	3
CV (%)	1.5	7.2

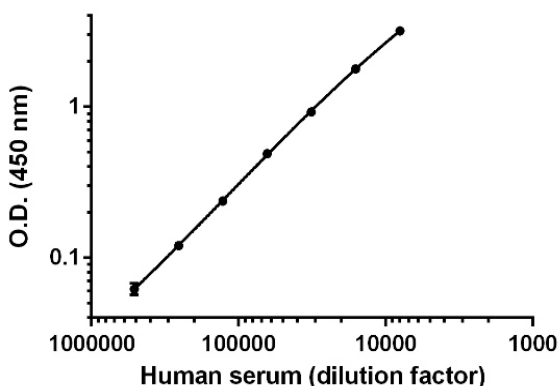


Figure 2. Titration of pooled human serum within the working range of the assay. Background subtracted data values (mean \pm SD, n=2) are graphed.

Serum/Plasma. Pooled human serum and plasma samples were measured in 4 different dilutions in duplicates for CRP using this kit. Means of interpolated sample values adjusted to sample dilution are shown in ng of CRP per mL of serum/plasma in the table below.

Sample Type	CRP (ng/mL)
Human Serum	5,981
Human Plasma - Citrate	7,718
Human Plasma - EDTA	1,530
Human Plasma - Heparin	1,275

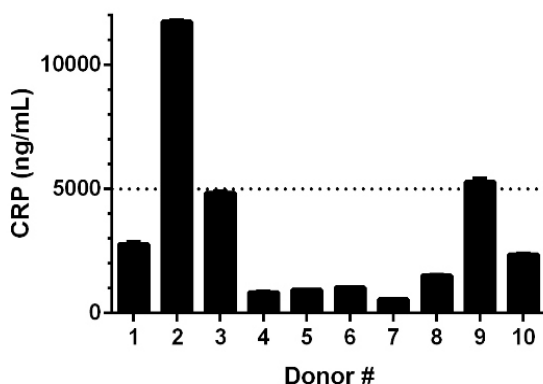


Figure 3. CRP concentrations in 10 individual human serum donors. 10,000X diluted sera from 10 apparently healthy male donors were measured using this kit. Interpolated data values corrected for sample dilution are graphed in ng of CRP per mL of serum (mean \pm SD, $n=2$). Nine out of ten individual human sera tested within or at reported Human serum range ($< 5,000$ ng/mL, dotted line). The mean of CRP concentration of these nine individual sera was determined to be 2,240 ng/mL with a range of 559 – 5,285 ng/mL. Note that one individual human serum sample (donor # 2) tested substantially higher, 11,737 ng/mL.

17. ASSAY SPECIFICITY

This kit recognizes both native and recombinant human CRP protein.

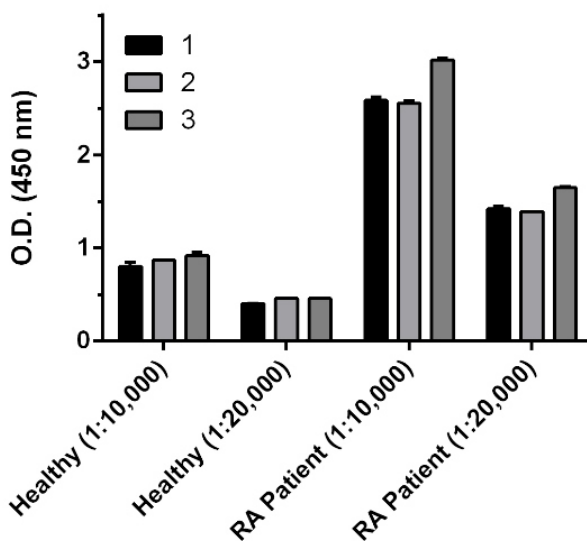


Figure 4. Comparison of CRP signals in three healthy and three rheumatoid arthritis patient sera. Background subtracted data values of two serum dilutions (as indicated in parenthesis) are graphed (mean \pm SD, $n=2$).

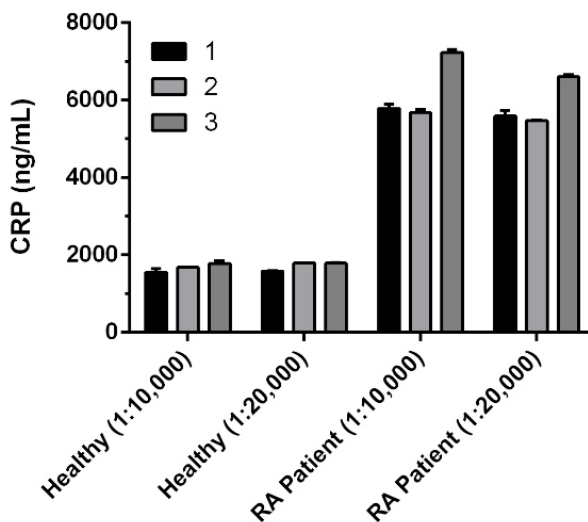


Figure 5. Quantification of CRP concentrations in three healthy and three rheumatoid arthritis (RA) patient sera. The concentrations of CRP were interpolated from data values shown in Figure 4 using CRP standard curve, corrected for sample dilution, and graphed in ng of CRP per mL of serum. As expected, note that the CRP serum concentrations are increased in RA patients.

18. SPECIES REACTIVITY

This kit recognizes CRP protein in the human plasma, serum and cell culture supernatant samples only.

Mouse and rat samples have not been tested with this kit.

Please contact our Technical Support team for more information.

19. TROUBLESHOOTING

Problem	Cause	Solution
Poor standard curve	Inaccurate Pipetting	Check pipettes
	Improper standard dilution	Prior to opening, briefly spin the stock standard tube and dissolve the powder thoroughly by gentle mixing
Low Signal	Incubation times too brief	Ensure sufficient incubation times; increase to 2 or 3 hour standard/sample incubation
	Inadequate reagent volumes or improper dilution	Check pipettes and ensure correct preparation
	Incubation times with TMB too brief	Ensure sufficient incubation time until blue color develops prior addition of Stop solution
Large CV	Plate is insufficiently washed	Review manual for proper wash technique. If using a plate washer, check all ports for obstructions.
	Contaminated wash buffer	Prepare fresh wash buffer
Low sensitivity	Improper storage of the ELISA kit	Store your reconstituted standards at -80°C, all other assay components 4°C. Keep TMB substrate solution protected from light.
Precipitate in Diluent	Precipitation and/or coagulation of components within the Diluent.	Precipitate can be removed by gently warming the Diluent to 37°C.

20. NOTES

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