Version 03-12/24

User's Manual

C-Reactive Protein ELISA kit

REF **DEIA-XY53**

Σ **96T**

RUO

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

For illustrative purposes only. To perform the assay the instructions for use provided with the kit have to be used.

Creative Diagnostics

Address: 45-1 Ramsey Road, Shirley, NY 11967, USA

Tel: 1-631-624-4882 (USA) 44-161-818-6441 (Europe) **Fax: 1-631-938-8221**

Email: info@creative-diagnostics.com **Web: www.creative-diagnostics.com**

PRODUCT INFORMATION

Intended Use

The Salivary C-Reactive Protein ELISA Kit is an enzyme-linked immunoassay specifically designed and validated for the quantitative measurement of human CRP in oral fluid. It is not intended for diagnostic use. This assay kit was optimized for human salivary research and has not been validated for other human sample types, such as serum or plasma or samples from other species.

Please read the complete kit insert before performing this assay. Failure to follow kit procedure and recommendations for saliva collection and sample handling may result in unreliable values.

General Description

C-Reactive Protein (CRP) circulates in the blood plasma as a stable homopentamer and a prominent member of the acute-phase inflammatory proteins. CRP functions mainly in innate immune defense and levels increase in response to inflammation, infection, tissue damage, necrosis, malignancy and allergic reactions. CRP has diagnostic value as a marker of systemic inflammation in the body and as an independent risk factor for cardiovascular diseases in adults and children.

CRP, also known as pentraxin 1 (PTX1), is a member of the pentraxin family which has several structurally related members. Most CRP is produced by hepatocytes in the liver induced principally by the inflammatory cytokine interleukin-6 (IL-6), but also by IL-1β and TNF.

CRP binds several targets including phosphocholine, a common constituent of polysaccharide coatings of bacterial pathogens and mammalian cell membranes. This allows CRP to function as an opsonin, facilitating phagocytosis of pathogens and dead or dying cells and trigger the classical complement pathway by activating C1q. CRP binding also can stimulate macrophage tumoricidal activity, and protect against septic shock. Another mechanism by which CRP interacts with the innate immune system is through its interaction with Fcγ receptors (FcγRs) on myeloid cells, B lymphocytes, NK cells, and platelets.

Circulating CRP levels in humans are normally quite low, but they increase several hundred fold during the acute-phase response. Elevated serum CRP levels have been associated with the presence of cardiovascular disease. Numerous research studies investigating serum CRP and its relationship to other diseases have also been reported. These include hypertension, diabetes, cancer, autoimmune disorders, obesity and metabolic syndrome. Additional literature suggests possible links between oral health and chronic infection, inflammation, oxidative stress and heart disease. Studies have also linked elevated serum CRP levels to oral contraceptive use.

For CRP there is encouraging research that suggests salivary CRP may be more strongly associated with serum CRP than is true for other inflammatory markers. CRP is prohibitively large to pass from the circulation into the salivary glands by passive diffusion, and it is believed that small amounts of CRP enter whole saliva mainly as a component of the gingival crevicular fluid (GCF) or through salivary glands. Whole saliva is much easier to collect than GCF, which could make saliva the oral fluid of choice for routine assessment of CRP in clinical or field studies.

As with measurements of salivary cytokines, however, the question of local CRP production in the oral cavity must be considered, since it may obscure the relationship between blood and salivary levels and the interpretation of salivary CRP in relation to other analytes of interest. Although the majority of CRP is thought to originate from the liver, CRP and IL-6 mRNAs have been detected in gingival tissue samples from

periodontitis patients and CRP gene expression has been correlated with IL-6 expression.

Several studies have examined the relationship between salivary and serum CRP. One study reported a moderate to strong association between CRP measured in saliva and in serum, while a second longitudinal study found that salivary and plasma CRP were moderately associated cross-sectionally and across two years.

Principles of Testing

This is an indirect sandwich ELISA kit. A "sandwich" is formed when the pre-coated capture anti-CRP antibody present on the plate binds CRP in standards and samples, which is then bound by the anti-CRP detection antibody linked to horseradish peroxidase. After each incubation, unbound components are washed away. Bound anti-CRP Antibody Enzyme Conjugate is then added and the levels are measured by the reaction of the horseradish peroxidase (HRP) enzyme to the substrate tetramethylbenzidine (TMB). This reaction produces a blue color. A yellow color is formed after stopping the reaction with an acidic solution. The optical density is read on a standard plate reader at 450 nm. The amount of CRP Antibody Enzyme Conjugate detected is directly proportional to the amount of CRP present in the sample.

Reagents And Materials Provided

1. CRP Generation II ELISA Microtitre Break-Apart Plate. Coated with mouse anti-human CRP antibodies. 1/96 well
2. CRP Generation II Standard. 800 pg/mL formulated for stability when stored at 4°C. Prepare and serially dilute before use according to Reagent Preparation. Contains: CRP, buffer, preservative. 1 vial / 1 mL
3. CRP Generation II Controls. High and Low. Contain: CRP, buffer, preservative. 2 vials / 500 µL ea.
4. CRP Generation II Antibody Enzyme Conjugate. Concentrate. Dilute before use with CRP Generation II Assay Diluent. (See Procedure.) Contains: Goat anti-human CRP antibody conjugated to HRP, preservative. 1 vial / 100 µL.
5. CRP Generation II Sample Diluent. Ready to use. Contains: phosphate buffer, preservative. 1 bottle / 25 mL.
6. CRP Generation II Assay Diluent. Contains: phosphate buffer, sodium chloride, protein stabilizer, preservative. 1 bottle / 30 mL
7. Wash Buffer Concentrate 10×. Dilute before use according to Reagent Preparation. Contains: phosphate buffer, detergent, preservative. 1 bottle / 100 mL
8. TMB Substrate Solution. Non-toxic, ready to use. 1 bottle / 25 mL
9. Stop Solution. 1 bottle / 12.5 mL

Materials Required But Not Supplied

1. Precision pipettes to deliver 70 µL, 100 µL, 150 µL, 250 µL, and 300 µL
2. Precision multichannel pipette to deliver 50 µL and 100 µL
3. Vortex
4. Plate rotator with 0.08-0.17 inch orbit capable of 500 rpm
5. Microplate reader with capabilities to read 450 nm and 620 to 630 nm

6. Computer software for data reduction
7. Reagent reservoirs
8. Deionized water
9. One disposable polypropylene tube to hold at least 14 mL
10. Small disposable polypropylene tubes for dilution of standard, controls, samples
11. Pipette tips
12. Serological pipette to deliver up to 14 mL
13. Centrifuge capable of 1500 x g

Storage

All unopened components of this kit are stable at 2-8°C until the kit's expiration date.

Specimen Collection And Preparation

Specimen Collection

Avoid sample collection within 60 minutes after eating a major meal or within 12 hours after consuming alcohol. Acidic or high sugar foods can compromise assay performance by lowering sample pH and influencing bacterial growth. To minimize these factors, rinse mouth thoroughly with water 10 minutes before sample is collected.

Collect whole saliva by unstimulated passive drool. Donors may tilt the head forward, allowing the saliva to pool on the floor of the mouth, and then pass the saliva through the Saliva Collection Aid (SCA) into a polypropylene vial.

Samples visibly contaminated with blood should be recollected. Do not use dipsticks, which result in false positive values due to salivary enzymes.

Record the time and date of specimen collection.

CRP does not appear to be flow rate dependent in individuals with CRP levels in the normal range, based on the high correlation ($r(40) = 0.94$, $p < 0.001$, $n = 42$) between measurements in pg/mL and measurements corrected for flow rate. However, the effect of flow rate in individuals with higher levels of CRP has not been determined. It is therefore advisable to collect data on saliva flow in case the correction for flow rate should be necessary, or to allow for future testing of archived samples for additional biomarkers that may be sensitive to flow rate. We recommend you measure the amount of time needed to collect the desired volume of saliva, in order to determine the flow rate (mL/min). The measured concentration should then be multiplied by the flow rate in order to express the result as product measured per unit of time. Protocols for flow-rate conversion are available on request.

Sample Handling and Preparation

After collection, it is important to keep samples cold in order to avoid bacterial growth (and loss of CRP) in the specimen. Refrigerate sample within 30 minutes, and freeze at or below -20°C within 4 hours of collection. Samples may be stored at -20°C for up to 6 months.

Do not add sodium azide to saliva samples as a preservative, as it may cause interference in the immunoassay.

On day of assay, thaw the saliva samples completely, vortex, and centrifuge at 1500 x g for 15 minutes. Freezing saliva samples will precipitate mucins. Centrifuging removes mucins and other particulate matter which may interfere with antibody binding and affect results. Samples should be at room temperature before making dilutions. Pipette clear sample into appropriate dilution tubes. Re-freeze saliva samples as soon as possible after running assay. Re-centrifuge saliva samples each time that they are thawed. Avoid multiple freeze-thaw cycles. CRP levels will drop significantly at 2-8°C beyond 8 hours, but they are minimally affected by freeze-thaw cycles.

Saliva samples must be diluted for this assay. See Procedure for details.

Reagent Preparation

1. Bring all reagents to room temperature and mix before use. A minimum of 30 minutes is recommended for the 30 mL of CRP Assay and Sample Diluents to come to room temperature.
2. Bring Microtiter Plate to room temperature before use. It is important to keep the foil pouch with the plate strips closed until warmed to room temperature, as humidity may have an effect on the coated wells.
3. Prepare 1× wash buffer by diluting Wash Buffer Concentrate (10×) 10-fold with roomtemperaturedeionized water (100 mL of Wash Buffer Concentrate (10×) to 900 mL of deionized water). Dilute only enough for current day's use and discard any leftover reagent. (If precipitate has formed in the concentrated wash buffer, it may be heated to 40°C for 15 minutes. Cool to room temperature before use in assay.)
4. Prepare serial dilutions of the CRP Generation II Standard as follows:

   Label six polypropylene microcentrifuge tubes or other small tubes 2 through 7.

   Pipette 250 µL of CRP Generation II Sample Diluent into tubes 2 through 7.

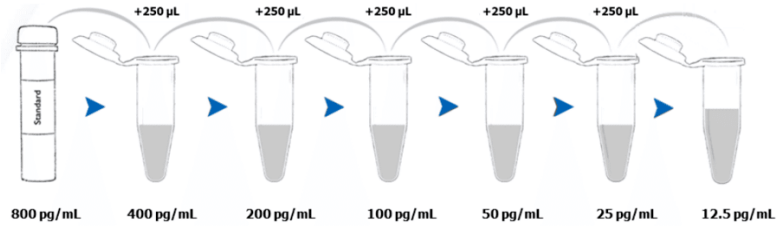
   Serially dilute the standard 2X by adding 250 µL of the 800 pg/mL standard (tube 1) to tube 2. Mix well.

   After changing pipette tips, remove 250 µL from tube 2 to tube 3. Mix well.

   Continue for tubes 4, 5, 6 and 7.

   The final concentrations of standards for tubes 1 through 7 are, respectively, 800 pg/mL, 400 pg/mL, 200 pg/mL, 100 pg/mL, 50 pg/mL, 25 pg/mL and 12.5 pg/mL. Standard concentrations in pmol/L are 7.6 pmol/L, 3.8 pmol/L, 1.9 pmol/L, 0.95 pmol/L, 0.48 pmol/L, 0.24 pmol/L, and 0.12 pmol/L, respectively.

   CRP Generation II Sample Diluent is used as the Zero Standard.
5. Dilute CRP Generation II High Control and Low Control 2x by pipetting 150 µL of each into 150 µL of CRP Sample Diluent, respectively.



Assay Procedure

Step 1: Read and prepare reagents according to the Reagent Preparation section before beginning assay. Determine your plate layout. Here is a suggested layout. (Standards, controls, and saliva samples should be

assayed in duplicate.)

	1	2	3	4	5	6	7	8	9	10	11	12
A	800 Std	800 Std	Ctrl-L	Ctrl-L								
B	400 Std	400 Std	Ctrl-H	Ctrl-H								
C	200 Std	200 Std	SMP-1	SMP-1								
D	100 Std	100 Std	SMP-2	SMP-2								
E	50 Std	50 Std	SMP-3	SMP-3								
F	25 Std	25 Std	SMP-4	SMP-4								
G	12.5 Std	12.5 Std	SMP-5	SMP-5								
H	0 Std	0 Std	SMP-6	SMP-6								

Step 2: Keep the desired number of strips in the strip holder and place the remaining strips back in the foil pouch. Reseal the foil pouch with unused wells and desiccant. Store at 2-8°C.

Step 3: Pipette 14 mL of CRP Generation II Assay Diluent into the disposable tube. (Scale down proportionally if using less than the entire plate.) Set aside for Step 7.

Step 4:

Dilute saliva 2X in CRP Generation II Sample Diluent using 150 µL saliva to 150 µL of CRP Generation II Sample Diluent.

Do not dilute samples in CRP Generation II Assay Diluent.

Step 5:

Pipette 100 µL of Standards, diluted Controls (see page 10) and diluted saliva samples into appropriate wells.

Pipette 100 µL of CRP Generation II Sample Diluent into two wells to serve as the Zero Standard.

Place adhesive cover provided over plate. Mix plate on a plate rotator continuously at 500 rpm for 2 hours at room temperature.

Step 6: Wash the plate 4 times with 1X wash buffer. A plate washer is recommended. However, washing may be done by gently squirting wash buffer into each well with a squirt bottle, or by pipetting 300 µL of wash buffer into each well and then discarding the liquid over a sink. After each wash, the plate should be thoroughly blotted on paper towels before turning upright. If using a plate washer, blotting is still recommended after the last wash.

Step 7: Dilute the CRP Generation II Antibody Enzyme Conjugate 1:200 by adding 70 µL of the conjugate to the 14 mL tube of CRP Generation II Assay Diluent. (Scale down proportionally if not using the entire plate.) Conjugate tube may be centrifuged for a few minutes to bring the liquid down to the tube bottom. Immediately mix the diluted conjugate solution and add 100 µL to each well using a multichannel pipette.

Step 8: Place adhesive cover provided over plate. Mix plate on a plate rotator continuously at 500 rpm for 2 hours at room temperature.

Step 9: Wash the plate 4 times with 1X wash buffer. A plate washer is recommended. However, washing may be done by gently squirting wash buffer into each well with a squirt bottle, or by pipetting 300 µL of wash buffer into each well and then discarding the liquid over a sink. After each wash, the plate should be thoroughly blotted on paper towels before turning upright. If using a plate washer, blotting is still

recommended after the last wash.

Step 10: Add 100 µL of TMB Substrate Solution to each well with a multichannel pipette.

Step 11. Incubate the plate in the dark (covered) at room temperature for 30 minutes, mixing for 5 minutes on a plate rotator at 500 rpm.

Step 12: Add 50 µL of Stop Solution with a multichannel pipette.

Step 13:

Mix on a plate rotator for 3 minutes at 500 rpm. If green color remains, continue mixing until green color turns to yellow. Be sure all wells have turned yellow. Caution: Spillage may occur if mixing speed exceeds 600 rpm.

Wipe off bottom of plate with a water-moistened, lint-free cloth and wipe dry.

Read in a plate reader at 450 nm. Read plate within 10 minutes of adding Stop Solution. (For best results, a secondary filter correction at 620 to 630 nm is recommended.)

Quality Control

The CRP High and Low Controls should be run with each assay. The control ranges established at CD are to be used as a guide. Each laboratory should establish its own range. Variations between laboratories may be caused by differences in techniques and instrumentation.

Calculation

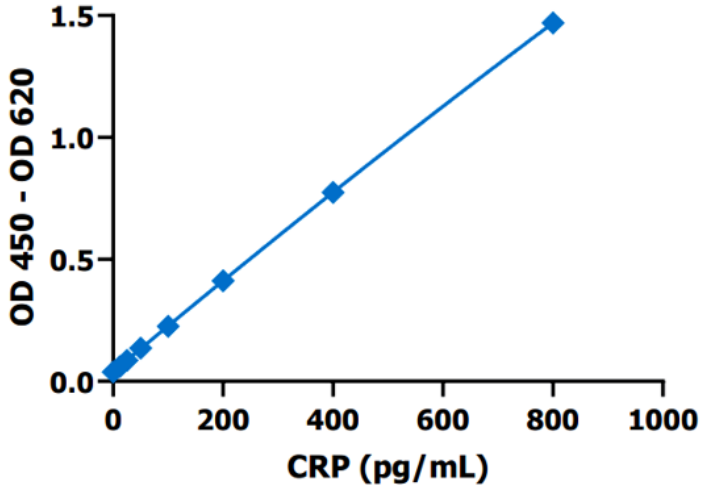
1. Compute the average optical density (OD) for all duplicate wells.
2. Plot the reference standard concentrations on the X axis and the corresponding average OD on the Y axis.
3. Determine the concentrations of the controls and saliva samples by interpolation using data reduction software. We recommend using a 4-parameter non-linear regression curve fit.
4. Multiply the calculated concentrations of the saliva samples only by the dilution factor of 2 to obtain final CRP sample concentrations in pg/mL.
5. Samples (diluted 2X) with CRP values greater than 800 pg/mL (or >1600 pg/mL after multiplying by the dilution factor of 2) should be diluted further with CRP Generation II Sample Diluent and rerun for accurate results. If a dilution of the sample is used, multiply the results by the additional dilution factor. **A new Standard Curve must be run with each full or partial plate.**

Typical Standard Curve

The results shown below are for illustration only and should not be used to calculate results from another assay.

Well	Standard	Average OD	CRP (pg/mL)
A1,A2	S1	1.469	800
B1,B2	S2	0.774	400
C1,C2	S3	0.412	200
D1,D2	S4	0.226	100
E1,E2	S5	0.135	50
F1,F2	S6	0.086	25
G1,G2	S7	0.061	12.5
H1,H2	Zero	0.038	0

Example:



Reference Values

Example Ranges: To be used as a guide only. Each laboratory should establish its own range.

Group	N	Range (pg/mL)	Mean (pg/mL)	Std Dev of Mean (pg/mL)
Adults	62	62.34 - 3072.55	733.84	695.24

Precision

The intra-assay precision was determined from the mean of 20 replicates each.

Saliva Sample	N	Mean (pg/mL)	Standard Deviation (pg/mL)	Coefficient of Variation (%)
1	20	1197.17	20.78	2%
2	20	740.24	9.48	1%
3	20	208.39	9.50	2%
4	20	148.37	4.45	2%
5	20	95.77	2.32	1%

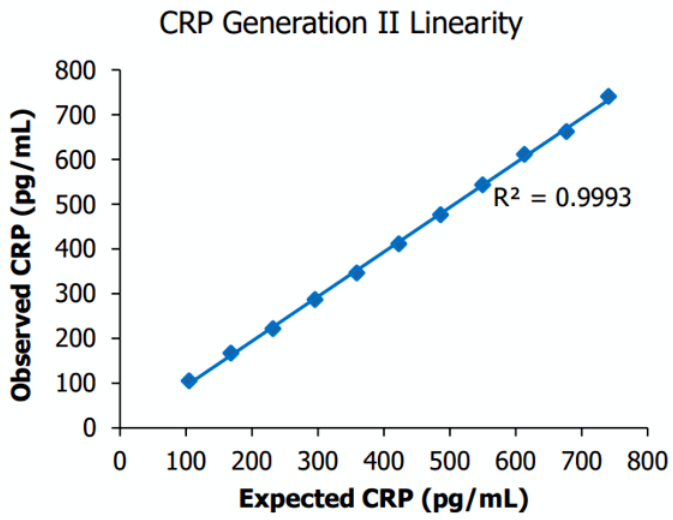
The inter-assay precision was determined from the mean of average duplicates for 10 separate runs.

Saliva Sample	N	Mean (pg/mL)	Standard Deviation (pg/mL)	Coefficient of Variation (%)
1	20	1186.76	30.69	3%
2	20	730.35	21.81	3%
3	20	209.61	5.94	3%
4	20	155.64	9.91	6%
5	19	110.35	7.35	7%

Linearity

Two saliva samples were diluted with each other proportionately and assayed.

Saliva Sample	Dilution	Expected (pg/mL)	Observed (pg/mL)	Recovery (%)
1		740.66	740.66	100%
	1:9	677.08	662.15	98%
	2:8	613.50	611.15	100%
	3:7	549.92	543.26	99%
	4:6	486.35	476.26	98%
	5:5	422.77	411.21	97%
	6:4	359.19	345.95	96%
	7:3	295.61	286.42	97%
	8:2	232.04	221.81	96%
	9:1	168.46	167.18	99%
2		104.88	104.88	100%



Recovery

Spike and Recovery

Three saliva samples containing different levels of endogenous CRP were spiked with known quantities of CRP and assayed.

Saliva Sample	Endogenous (pg/mL)	Added (pg/mL)	Expected (pg/mL)	Observed (pg/mL)	Recovery (%)
1	1223.76	84.37	1176.21	1186.73	101%
		7.97	1099.80	1133.95	103%
		0.00	1091.84	1113.03	102%
2	741.35	84.37	762.40	776.50	102%
		7.97	686.00	707.20	103%
		0.00	678.03	704.74	104%
3	147.60	84.37	223.01	227.72	102%
		7.97	146.61	156.86	107%
		0.00	138.64	142.97	103%

Sample Dilution Recovery

Three saliva samples containing different levels of endogenous CRP were diluted in Sample Diluent and assayed.

Saliva Sample	Dilution	Expected (pg/mL)	Observed (pg/mL)	Recovery (%)
1	Neat		1147.39	
	x2	573.70	561.08	98%
	x4	286.85	285.40	99%
	x8	143.42	139.41	97%
2	Neat		745.85	
	x2	372.93	386.97	104%
	x4	186.46	206.65	111%
	x8	93.23	109.85	118%
3	Neat		703.32	
	x2	351.66	352.42	100%
	x4	175.83	188.03	107%
	x8	87.92	97.10	110%

Precautions

General Kit Use Advice

1. This kit uses break-apart microtiter strips. You may run less than a full plate. Unused wells must be stored at 2-8°C in the foil pouch with desiccant and used in the frame provided.
2. Avoid microbial contamination of opened reagents. Salimetrics recommends using opened reagents within one month. Store all reagents at 2-8°C.
3. The quantity of reagent provided with a single kit is sufficient for two partial runs. The volumes of wash buffer and enzyme conjugate prepared for assays using less than a full plate should be scaled down accordingly, keeping the same dilution ratio.
4. Do not mix components from different lots of kits.
5. We recommend saving all reagents until data analysis has confirmed a successful run to facilitate troubleshooting if necessary.
6. Prior to sample addition, please label each strip to assure plate orientation and sample order when data is acquired on plate reader.
7. To ensure highest quality assay results, pipetting of samples and reagents must be done as quickly as possible (without interruption) across the plate. Ideally, the process should be completed within 20 minutes or less.
8. When using a multichannel pipette to add reagents, always follow the same sequence when adding all reagents so that the incubation time is the same for all wells.
9. When running multiple plates, or multiple sets of strips, a standard curve must be run with each individual plate and/or set of strips.
10. The temperature of the laboratory may affect assays. Salimetrics' kits have been validated at 68-74°F (20-23.3°C). Higher or lower temperatures may affect OD values.
11. Routine calibration of pipettes and other equipment is critical for the best possible assay performance.
12. When mixing plates during assay procedures, avoid speeds that spill the contents of the wells.

Limitations

1. Samples (diluted 2X) with CRP values greater than 800 pg/mL (or >1600 pg/mL after multiplying by the dilution factor of 2) should be diluted further with CRP Sample Diluent and rerun for accurate results. If a dilution of the sample is used, multiply the results by the additional dilution factor.
2. See "Specimen Collection" recommendations to ensure proper collection of saliva specimens and to avoid interfering substances.
3. Samples collected with sodium azide are unsuitable for this assay.
4. Any quantitative results indicating abnormal CRP levels should be followed by additional testing and evaluation.

Tel: 1-631-624-4882 (USA)
Tel: 44-161-818-6441 (Europe)
Fax: 1-631-938-8221
Email: info@creative-diagnostics.com