SALIVARY C-REACTIVE PROTEIN

ELISA KIT

For Research Use Only

Item No. 1-3302, (Single) 96-Well Kit;

1-3302-5, (5-Pack) 480 Wells

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SALIVARY C-REACTIVE PROTEIN ELISA KIT

Intended Use

The Salimetrics™ CRP kit is an enzyme-linked immunoassay specifically designed and validated for the quantitative measurement of salivary CRP. It is not intended for diagnostic use. It is intended only for research use.

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 false values.

For further information about this kit, its application, or the procedures in this insert, please contact the technical service team at Salimetrics or your local sales representative.

Introduction

C-reactive protein (CRP) is the best-known member of a group of acute-phase proteins, which increase their concentrations during certain inflammatory disorders. CRP is widely used as a bio-marker of inflammation in the body.

Most CRP is produced in the liver, and increased production during the acute phase is induced principally by the cytokine interleukin-6 (IL-6), operating primarily at the level of transcription. (1) IL-6 is released by a

variety of tissues, including activated leukocytes, adipocytes, and endothelial cells. (2,3) In turn, CRP is capable of binding to and modulating the function of monocytes, enhancing their capacity to produce inflammatory cytokines, including IL-6. (4,5) CRP binds to phosphocholine, a common constituent of polysaccharide coatings of bacterial pathogens and of cell membranes. This allows it to function as an opsonin, facilitating phagocytosis of pathogens and dead or dying cells. (1,5) Other functions of CRP include activating the classical complement pathway, activating macrophage tumoricidal activity, and protecting against septic shock. (5)

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. (6,7) Numerous recent research studies investigating serum CRP and its relationship to other diseases have also been carried out. These include hypertension, (8,9) diabetes, (2,10) cancer, (11) and autoimmune disorders. (12) Recent literature suggests possible links between oral health and chronic infection, inflammation, and heart disease. (13) Studies have also linked elevated serum CRP levels to oral contraceptive use. (14,15)

Recent studies have begun to examine 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. (16,17)

Test Principle

A microtitre plate is coated with mouse antibodies to human CRP. CRP in standards and unknowns and goat anti-human CRP antibodies linked to horseradish peroxidase are added. A "sandwich" is formed with the pre-coated antibody on the bottom, the CRP in the middle, and the antibody linked to horseradish peroxidase on the top. After incubation, unbound components are washed away. Bound CRP peroxidase is measured by the reaction of the peroxidase enzyme on the substrate tetramethylbenzidine (TMB). This reaction produces a blue color. A yellow color is formed after stopping the reaction with 2-molar sulfuric acid. Optical density is read on a standard microplate reader at 450 nm. The amount of CRP peroxidase detected is directly proportional to the amount of CRP present. (18)

Safety Precautions

- Liquid stop is a 2-molar solution of sulfuric acid. This solution is caustic; use with care.
- See 'Material Safety Information' at the end of procedure.
- A safety data sheet is available on request.

Storage

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

Materials Supplied with Single Kit

	Item	Quantity/Size
1	**Microtitre Plate** Coated with mouse anti-CRP antibodies.	1/96-well
2	**CRP Standard** Lyophilized. 3000 pg/mL when reconstituted to 1.0 mL. Prepare and serially dilute before use according to Reagent Preparation. Contains: CRP, buffer, preservative.	1 vial
3	**CRP Controls** High, Low. Lyophilized. Reconstitute to 0.5 mL before use according to Reagent Preparation. Contain: CRP, buffer, preservative.	2 vials
4	**Wash Buffer Concentrate (10X)** Dilute before use according to Reagent Preparation. Contains: phosphate buffer, detergent, preservative.	1 bottle/100 mL
5	**CRP Sample Diluent** Ready to use. Contains: phosphate buffer, preservative.	1 bottle/12 mL
6	**CRP Assay Diluent** Ready to use. Contains: phosphate buffer, pH indicator, preservative.	1 bottle/25 mL
7	**CRP Antibody-Enzyme Conjugate** Concentrate. Dilute before use with CRP assay diluent. (See step 6 of procedure.) Contains: CRP conjugated to HRP, preservative.	1 vial/100µL
8	**TMB Substrate Solution** Non-toxic, ready to use.	1 bottle/25 mL
9	**2 M Stop Solution** Contains: sulfuric acid.	1 bottle/12.5 mL

Materials Needed But Not Supplied

- Precision pipette to deliver 15 µL, 50 µL, 80 µL, 135 µL and 150 µL
- Precision multichannel pipette to deliver 50 µL, 150 µL, and 200 µL
- Vortex
- Plate rotator with 0.08-0.17 inch orbit capable of 500 rpm (If unavailable, tap to mix.)
- Microplate reader with a 450 nm filter
- Computer software for data reduction
- Deionized water
- Reagent reservoirs
- One disposable tube to hold at least 20 mL
- Small disposable tubes for dilution of standard, controls, and samples
- Pipette tips
- Serological pipette to deliver 20 mL

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.

Donors may collect whole saliva by tilting the head forward, allowing the saliva to pool on the floor of the mouth, and then passing the saliva

through the Saliva Collection Aid (SCA), Item No. 5016.02, into a polypropylene vial. Adult samples and samples from children ages 6 and above may also be collected using the SalivaBio Oral Swab (SOS), Item No. 5001.02. Saliva from children under the age of 6 may be collected with the SalivaBio Children's Swab (SCS), Item No. 5001.06. The SalivaBio Infant's Swab (SIS), Item No. 5001.08, is available for use with children under the age of 6 months. Collection protocols are available on request or online at www.salimetrics.com.

Do not use Salivettes, Sorbettes, cotton, or polyester materials to collect samples. The effects of these materials on salivary CRP measurements have not been determined.

Record the time and date of specimen collection.
Concentrations of CRP may vary depending on the location in the mouth; consistency in collection location is therefore important. We find that placement of the SOS underneath the tongue on the floor of the mouth yields results similar to those from whole saliva collected by passive drool. Under certain conditions, however, there is a possibility that the SOS might collect specific glandular saliva. Researchers should be aware of this potential and decide on their collection strategy accordingly.

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.
Samples visibly contaminated with blood should be recollected. Samples may be screened for possible blood contamination, (19,20) using our Blood Contamination EIA Kit (Item No. 1-1302/1-1302-5). Do not use dipsticks, which result in false positive values due to salivary enzymes.

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. We recommend freezing samples at or below -20ºC as soon as possible after collection. Samples are stable for up to 8 hours at room temperature or 4ºC and for 2 months at -20°C or below. Stability beyond these time periods is unknown.

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

Freezing saliva samples will precipitate mucins. On day of assay, thaw completely, vortex, and centrifuge at 1500 x g (@3000 rpm) for 15 minutes. Centrifuging removes mucins and other particulate matter which may interfere with antibody binding, leading to falsely elevated results. Samples should be at room temperature before adding to assay plate. Pipette clear sample into appropriate wells. Re-freeze saliva samples

as soon as possible after adding to the assay plate. Centrifuge/re-centrifuge saliva samples each time that they are thawed. Avoid additional freeze-thaw cycles. CRP levels will drop significantly at 2-8°C beyond 8 hours, but they are minimally affected by freeze-thaw cycles.

Reagent Preparation

- Bring all reagents to room temperature and mix before use. A minimum of 1.5 hours is recommended for the 20 mL of CRP assay diluent used in Step 6 (conjugate dilution) to come to room temperature.
- Bring microtitre 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.***
- Reconstitute each Control vial with 0.5 mL of deionized water. (We recommend sterile water if you plan to store at 2-8°C.) Let sit 20 minutes at room temperature before using. Mix well immediately before use. Use reconstituted controls within 1 month.
- Reconstitute CRP Standard with 1 mL of deionized water. (We recommend sterile water if you plan to store at 2-8°C.) Let sit 20 minutes at room temperature before using. Mix well immediately before use. Use reconstituted standard within 1 month.
- Prepare serial dilutions of the CRP standard as follows:
  - Label five microcentrifuge tubes or other small tubes 2 through 6.
  - Pipette 150 µL of CRP Sample Diluent into tubes 2 through 6.
  - Serially dilute the standard 2X by adding 150 µL of the 3000 pg/mL standard (tube 1) to tube 2. Mix well. After changing pipette tips, remove 150 µL from tube 2 to tube 3. Mix well.

Continue for tubes 4, 5, and 6. The final concentrations of standards for tubes 1 through 6 are, respectively, 3000 pg/mL, 1500 pg/mL, 750 pg/mL, 375 pg/mL, 187.5 pg/mL, and 93.75 pg/mL. Standard concentrations in pmol/L are 130.43, 65.22, 32.61, 16.30, 8.15 and 4.08 pmol/L, respectively.

- Prepare 1X wash buffer by diluting wash buffer concentrate 10-fold with room-temperature deionized water (100 mL of 10X wash buffer to 900 mL of deionized water. ***Dilute only the amount needed for current day's use, and discard any leftover reagent.*** (If precipitate has formed in the concentrated wash buffer, heat to 40°C for 15 minutes to dissolve crystals. Cool to room temperature before use in assay.)

General Kit Use Advice

- This kit uses break-apart microtitre strips. You may run less than a full plate. Unused wells must be stored at 2-8°C in the sealed foil pouch with desiccant and used in the frame provided.
- The quantity of reagent provided with this kit is sufficient for three individual runs. The volume of diluent and conjugate used for assays using less than a full plate should be scaled down accordingly, keeping the same dilution ratio.
- Do not mix components from different lots of kits.
- When using a multichannel pipette, reagents should be added to duplicate wells at the same time. Follow the same sequence when adding additional reagents so that incubation time with reagents is the same for all wells.
- 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.

- When running multiple plates, or multiple sets of strips, a standard curve must be run with each individual plate and/or set of strips.
- The temperature of the laboratory may affect assays. Salimetrics' kits have been validated at 68-74ºF (20-23.3ºC). Higher or lower temperatures will cause an increase or decrease in OD values, respectively. Salimetrics cannot guarantee test results outside of this temperature range.
- Avoid microbial contamination of opened reagents. Salimetrics recommends using opened reagents within one month.
- Routine calibration of pipettes is critical for the best possible assay performance.

Procedure

Step 1: Determine your plate layout. Here is a suggested layout.

	1	2	3	4	5	6	7	8	9	10	11	12
A	3000 Std	3000 Std	C-L	C-L								
B	1500 Std	1500 Std	Unk 1	Unk 1								
C	750 Std	750 Std	Unk 2	Unk 2								
D	375 Std	375 Std	Unk 3	Unk 3								
E	187.5 Std	187.5 Std	Unk 4	Unk 4								
F	93.75 Std	93.75 Std	Unk 5	Unk 5								
G	Zero	Zero	Unk 6	Unk 6								
H	C-H	C-H	Unk 7	Unk 7								

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

Step 3: Pipette 20 mL of CRP assay diluent into a disposable tube. (Scale down proportionally if not using the full plate.) Set aside for Step 5.

Step 4:

- Dilute saliva 10X in CRP sample diluent using 15 µL saliva to 135 µL of CRP sample diluent. ***Do not dilute samples in CRP assay diluent.***
- ***Dilute only saliva samples. Do not pre-dilute controls.***

Step 5:

- Pipette 50 µL of standards, controls, and diluted unknown samples into appropriate wells. Standards, controls, and unknown samples should be assayed in duplicate.
- Pipette 50 µL of CRP sample diluent into two wells to serve as the zero.

Step 6: Dilute the enzyme conjugate 1:250 by adding 80 µL of the conjugate to the 20 mL of CRP assay diluent prepared in Step 3. (Scale down proportionally if not using the full 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 150 µL to each well using a multichannel pipette.

Step 7: Cover plate with adhesive cover provided. Incubate at room temperature for 2 hours, mixing constantly at 500 rpm.

Step 8: 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 decanting the liquid into a sink. After each wash, blot plate on paper towels before turning upright. ***If using a plate washer, blotting is still recommended after the last wash.***

Step 9: Add 200 µL of TMB solution to each well with a multichannel pipette.

Step 10: Incubate in the dark at room temperature for 30 minutes with constant mixing at 500 rpm.

Step 11: Add 50 µL of stop solution with a multichannel pipette.

Step 12:

- Mix on a plate rotator for 3 minutes at 500 rpm (or tap to mix). Be sure all wells have turned yellow. If green color remains, continue mixing until green color turns to 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 microplate reader at 450 nm. Read plate within 10 minutes of adding stop solution. (Correction at 630 nm is desirable.)

Assay Summary

1. Bring all reagents to room temperature and mix before use.
2. Bring plate to room temperature and prepare for use.
3. Reconstitute CRP Standard and Controls. Let sit for 20 minutes before using.
4. Prepare tube with 20 mL of CRP assay diluent for conjugate dilution, which will be made later.
5. Prepare 1X wash buffer.
6. Serially dilute CRP standard using CRP sample diluent.
7. Dilute saliva samples 10X using CRP sample diluent. Do not dilute controls.
8. Pipette 50 µL of standards, controls, and diluted unknowns into appropriate wells.

9. Pipette 50 µL of CRP sample diluent into zero wells.
10. Make 1:250 dilution of conjugate (80 µL into 20 mL CRP assay diluent), mix, and immediately pipette 150 µL into each well.
11. Place adhesive cover over plate, then incubate at room temperature for 2 hours, mixing constantly at 500 rpm.
12. Wash plate 4 times with 1X wash buffer. Blot.
13. Add 200 µL TMB solution to each well.
14. Incubate in dark at room temperature for 30 minutes with constant mixing at 500 rpm.
15. Add 50 µL stop solution to each well. Mix for 3 minutes at 500 rpm.
16. Wipe plate bottom clean and read within 10 minutes of adding stop.

Calculations

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 optical density on the Y axis.

3. Using the average optical density values of the controls and unknowns, determine the corresponding concentration of CRP in pg/mL from the standard curve. We recommend using a linear curve fit.

4. Multiply the calculated concentrations by the dilution factor of 10 to obtain final CRP concentrations in pg/mL.

5. Samples with CRP values greater than 3000 pg/mL should be diluted with CRP sample diluent and rerun for accurate results. If an additional dilution of the sample is used, multiply the results by the dilution factor.

   When running multiple plates, or multiple sets of strips, a standard curve must be run with each individual plate and/or set of strips.

Quality Control

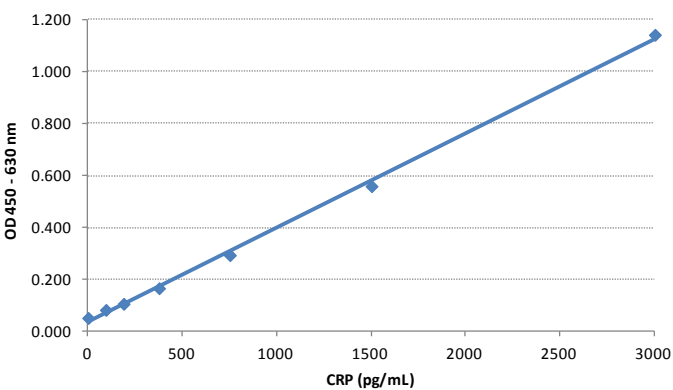
The Salimetrics' high and low salivary CRP controls should be run with each assay. The control ranges established at Salimetrics are to be used as a guide. Each laboratory should establish its own range. Variations between laboratories may be caused by differences in techniques and instrumentation.

Typical Results

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

Well	Standard	Average OD	CRP (pg/mL)
A1,A2	S1	1.1420	3000
B1,B2	S2	0.5590	1500
C1,C2	S3	0.2935	750
D1,D2	S4	0.1660	375
E1,E2	S5	0.1060	187.5
F1,F2	S6	0.0825	93.75
G1,G2	0	0.0515	NA

Example: CRP Linear Curve Fit



Material Safety Information*

Hazardous Ingredients

Liquid stop solution is caustic; use with care. We recommend the procedures listed below for all kit reagents. MSDS sheets are available from Salimetrics upon request.

Handling

Follow good laboratory procedures when handling kit reagents. Laboratory coats, gloves, and safety goggles are recommended. Wipe up spills using standard absorbent materials while wearing protective clothing. Follow local regulations for disposal.

Emergency Exposure Measures

In case of contact, immediately wash skin or flush eyes with water for 15 minutes. Remove contaminated clothing. If inhaled, remove individual to fresh air. If individual experiences difficulty breathing, give oxygen and call a physician.

*The above information is believed to be accurate but is not all-inclusive. This information should only be used as a guide. Salimetrics shall not be liable for accidents or damage resulting from contact with reagents.

Salivary CRP ELISA Kit Performance Characteristics

Precision

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

Sample	N	Mean (pg/mL)	Standard Deviation (pg/mL)	Coefficient of Variation (%)
High	20	1992.54	38.76	1.9
Low	20	178.77	10.52	5.9

The inter-assay precision was determined from the mean of average duplicates for 14 separate runs.

Sample	N	Mean (pg/mL)	Standard deviation (pg/mL)	Coefficient of Variation (%)
High	14	2167.14	80.38	3.7
Low	14	238.11	26.67	11.2

Sensitivity

The limit of sensitivity was determined by interpolating the mean optical density plus 2 SDs for 10 sets of duplicates at the 0 pg/mL standard. The minimal concentration of CRP that can be distinguished from 0 is 10 pg/mL.

Sample Dilution Recovery

Two saliva samples were diluted with CRP Sample Diluent and assayed.

Sample	Dilution Factor	Expected (pg/mL)	Observed (pg/mL)	Recovery (%)
1			1259.61	
	1:2	629.80	609.68	96.8
	1:4	314.9	288.05	91.5
	1:8	157.45	158.68	100.8
	1:16	78.73	76.66	97.4
2			1627.90	
	1:2	813.95	788.82	96.9
	1:4	406.97	365.49	89.8
	1:8	203.49	196.14	96.4
	1:16	101.74	101.47	99.7

Recovery

Saliva samples containing different levels of an endogenous CRP were spiked with known quantities of CRP and assayed.

Sample	Endogenous (pg/mL)	Added (pg/mL)	Expected (pg/mL)	Observed (pg/mL)	Recovery (%)
1	1544.63	1000	2544.63	2685.88	105.6
2	1463.34	200	1663.34	1523.24	91.6
3	1463.34	50	1513.34	1389.34	91.8
4	1266.43	1000	2266.43	2423.10	106.9
5	1199.78	200	1399.78	1352.03	96.6
6	1299.76	50	1349.76	1362.27	100.9

Specificity of Antiserum

Compound	Spiked Concentration (ng/mL)	% Cross-reactivity in Salivary CRP EIA
Human Albumin	10,000	ND
Human Alpha 1-Antitrypsin	10,000	ND
Lysozyme	10,000	ND
Human IL-6	10,000	ND

ND = None detected (<0.004)

Salivary CRP Example Values from Healthy Adults, Aged 20-54 Years* (16)

N	Mean (pg/mL)	Std Error of Mean (pg/mL)	Range (pg/mL)
51	1293.28	140.61	113.69 - 6131.40

*To be used as a guide for research purposes only. Each laboratory should establish its own range.

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Seller's Limited Warranty

"Seller warrants that all goods sold hereunder will be free from defects in material and workmanship. Upon prompt notice by Buyer of any claimed defect, which notice must be sent within thirty (30) days from date such defect is first discovered and within three months from the date of shipment, Seller shall, at its option, either repair or replace the product that is proved to Seller's satisfaction to be defective. All claims should be submitted in written form. This warranty does not cover any damage due to accident, misuse, negligence, or abnormal use. Liability, in all cases, will be limited to the purchased cost of the kit.

It is expressly agreed that this limited warranty shall be in lieu of all warranties of fitness and in lieu of the warranty of merchantability. Seller shall not be liable for any incidental or consequential damages that arise out of the installation, use or operation of Seller's product or out of the breach of any express or implied warranties."