



SALIVARY C-REACTIVE PROTEIN ELISA KIT

For Research Use Only
Not for use in Diagnostic Procedures

Item No. 1-3302, (Single) 96-Well Kit;
1-3302-5, (5-Pack) 480 Wells



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Intended Use

The Salimetrics® C-Reactive Protein ELISA 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 in humans and some animals. Salimetrics has not validated this kit for serum or plasma samples.

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.

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).



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Test Principle

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 & samples, which is then bound by the anti-CRP detection antibody linked to horseradish peroxidase. After incubation, unbound components are washed away. Bound CRP Antibody Enzyme Conjugate is measured by the reaction of the horseradish peroxidase 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 (18).

Safety Precautions

Read Safety Data Sheets before handling reagents.

Hazardous Ingredients

Liquid Stop Solution is caustic; use with care. We recommend the procedures listed below for all kit reagents.

Handling

Follow good laboratory practices when handling kit reagents. Laboratory coats, gloves, and safety goggles are recommended. Wipe up spills using appropriate 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 call a physician.

The above information is believed to be accurate but is not all-inclusive. This information should be used only as a guide. Salimetrics will not be liable for accidents or damage resulting from misuse of product.

Safety Data Sheets are available by contacting Salimetrics at support@salimetrics.com (See www.salimetrics.com for alternative contact options).



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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 foil pouch with desiccant and used in the frame provided.
- Avoid microbial contamination of opened reagents. Salimetrics recommends using opened reagents within one month. Store all reagents at 2-8°C.
- The quantity of reagent provided with a single kit is sufficient for three 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.
- Do not mix components from different lots of kits.
- 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 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.
- 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 68-74°F (20-23.3°C). Higher or lower temperatures may affect OD values.
- Routine calibration of pipettes and other equipment is critical for the best possible assay performance.
- When mixing plates during assay procedures, avoid speeds that spill the contents of the wells.

Storage

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



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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 SalivaBio Collection Aid (SCA) into a polypropylene vial. Collection protocols/methods are available online at www.salimetrics.com or upon 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 Nos. 1-1302/1-1302-5). Do not use dipsticks, which result in false positive values due to salivary enzymes.

Record the time and date of specimen collection.

Note: Concentrations of CRP may vary depending on the location in the mouth; consistency in collection location is therefore important. We find that placement of an absorbent device from the SalivaBio Oral Swab family (SOS, SCS, SIS) 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 swab 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.



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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. For long term storage, refer to the Salimetrics Collection and Handling Advice Booklet.

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.



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Materials Supplied with Single Kit

	Item	Quantity/Size
1	Microtitre Plate Coated with mouse anti-human 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 / 1 mL
3	CRP Controls High, Low. Lyophilized. Reconstitute to 0.5 mL before use according to Reagent Preparation. Contain: CRP, buffer, preservative.	2 vials / 500 µL ea.
4	CRP Antibody Enzyme Conjugate Concentrate. Dilute before use with assay diluent. (See step 6 of Procedure.) Contains: Goat anti-human CRP antibody conjugated to HRP, preservative.	1 vial / 100 µL
5	CRP Sample Diluent Ready to use Contains: phosphate buffer, preservative.	1 bottle / 12 mL
6	Assay Diluent Contains: phosphate buffer, pH indicator, preservative.	1 bottle / 25 mL
7	Wash Buffer Concentrate (10X) 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
10	Adhesive Plate Covers	2

Materials Needed But Not Supplied

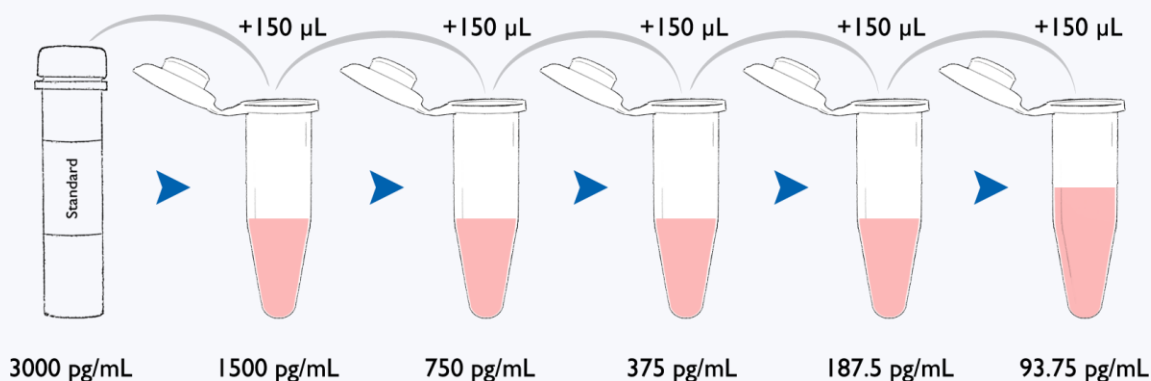
- Precision pipette to deliver 15 μ L, 50 μ L, 80 μ L, 135 μ L, 150 μ L, 500 μ L and 1 mL
- Precision multichannel pipette to deliver 50 μ L, 150 μ L, and 200 μ L
- Vortex
- Plate rotator with 0.08-0.17 inch orbit capable of 500 rpm
- Plate reader with 450 nm and 620 to 630 reference filters
- Computer software for data reduction
- Deionized water
- Reagent reservoirs
- One disposable polypropylene tube to hold at least 20 mL
- Small disposable polypropylene tubes for dilution of standard & samples
- Pipette tips
- Serological pipette to deliver up to 20 mL
- Centrifuge capable of 1500 x g



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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 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.***
- Prepare 1X wash buffer by diluting Wash Buffer Concentrate (10X) 10-fold with room-temperature deionized water (100 mL of Wash Buffer Concentrate (10X) 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.)
- Reconstitute each CRP 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 polypropylene microcentrifuge tubes or other small tubes 2 through 6.
 - Pipette 150 μ L of CRP Sample Diluent into tubes 2 through 6.
 - Serially dilute the standard 2X by adding 150 μ L of the 3000 pg/mL standard (tube 1) to tube 2. Mix well.
 - After changing pipette tips, remove 150 μ L 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.
 - CRP Sample Diluent is used as the Zero Standard.



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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	3000 Std	3000 Std	Ctrl-L	Ctrl-L								
B	1500 Std	1500 Std	SMP-1	SMP-1								
C	750 Std	750 Std	SMP-2	SMP-2								
D	375 Std	375 Std	SMP-3	SMP-3								
E	187.5 Std	187.5 Std	SMP-4	SMP-4								
F	93.75 Std	93.75 Std	SMP-5	SMP-5								
G	0 Std	0 Std	SMP-6	SMP-6								
H	Ctrl-H	Ctrl-H	SMP-7	SMP-7								

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 20 mL of Assay Diluent into the disposable tube. (Scale down proportionally if using less than the entire plate.) Set aside for Step 6.

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 Assay Diluent.***
- ***Dilute only saliva samples. Do not pre-dilute controls.***

Step 5:

- Pipette 50 µL of standards, controls, and diluted saliva samples into appropriate wells.
- Pipette 50 µL of CRP Sample Diluent into two wells to serve as the Zero Standard.

Step 6: Dilute the CRP Antibody Enzyme Conjugate 1:250 by adding 80 µL of the conjugate to the 20 mL tube of 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 150 µL to each well using a multichannel pipette.



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Step 7: Place adhesive cover provided over plate. Mix plate on a plate rotator ***continuously*** at 500 rpm for 2 hours at room temperature.

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 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 9: Add 200 µL of TMB Substrate Solution to each well with a multichannel pipette.

Step 10. Incubate the plate in the dark (covered) at room temperature for 30 minutes mixing ***constantly*** on a plate rotator 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. 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 Salimetrics' High and Low 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.



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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 saliva samples, 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 of the saliva samples by the dilution factor of 10 to obtain final CRP concentrations in pg/mL.
5. Samples (diluted 10X) with CRP values greater than 3000 pg/mL (or >30,000 pg/mL after multiplying by the dilution factor of 10) 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.

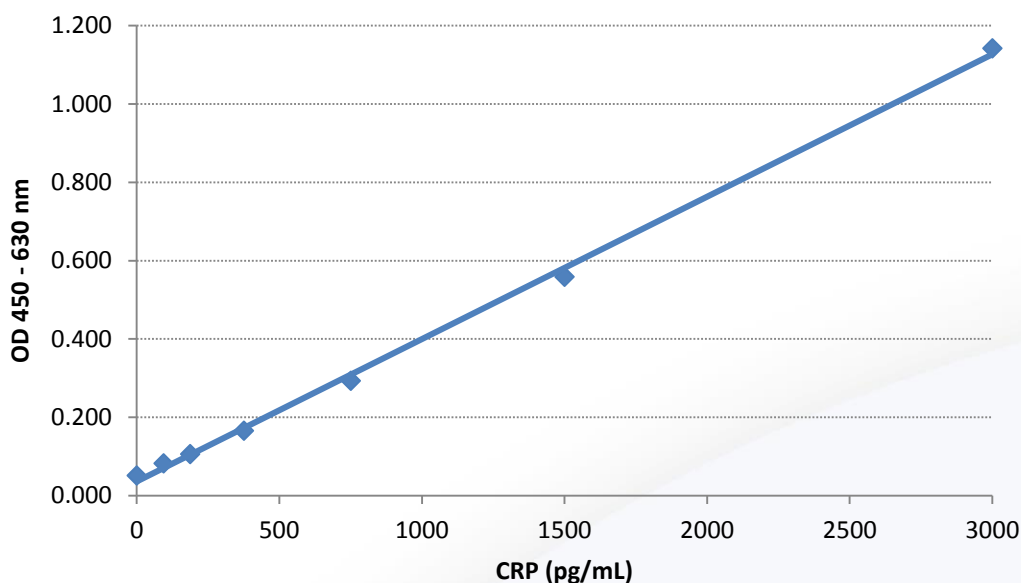
A new Standard Curve must be run with each full or partial plate.

Typical Results

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	1.1420	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	Zero	0.0515	0

Example: CRP Linear Curve Fit



Limitations

- Samples (diluted 10X) with CRP values greater than 3000 pg/mL (or >30,000 pg/mL after multiplying by the dilution factor of 10) 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.
- See "Specimen Collection" recommendations to ensure proper collection of saliva specimens and to avoid interfering substances.
- Samples collected with sodium azide are unsuitable for this assay.
- Any quantitative results indicating abnormal CRP levels should be followed by additional testing and evaluation.

Salivary CRP Example Ranges* (16)

Group	N	Range (pg/mL)	Mean (pg/mL)	Std Error of Mean (pg/mL)
Healthy Adults Aged 20-55	51	113.69 - 6131.40	1293.28	140.61

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



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Salivary CRP EIA Kit Performance Characteristics

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 (%)
H	20	1992.54	38.76	1.9
L	20	178.77	10.52	5.9

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

Saliva Sample	N	Mean (pg/mL)	Standard Deviation (pg/mL)	Coefficient of Variation (%)
H	14	2167.14	80.38	3.7
L	14	238.11	26.67	11.2

Recovery

Six saliva samples containing different levels of an 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	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



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Sensitivity

The lower 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 samples were serially diluted with CRP Sample Diluent and assayed.

Saliva 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

Antibody Specificity

Compound	Spiked Concentration (ng/mL)	% Cross-reactivity in Salivary CRP ELISA
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)



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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."

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