

QUANTA Lite™ CCP 3.1 IgG/IgA ELISA

704550

For *In Vitro* Diagnostic Use

CLIA Complexity: High

Intended Use

The QUANTA Lite™ CCP3.1 IgG/IgA ELISA is a semiquantitative enzyme-linked immunosorbent assay for the detection of IgG and IgA anti-CCP3 (Cyclic Citrullinated Peptide 3) antibodies in patient sera or citrated or EDTA plasma. The presence of these antibodies, when considered in conjunction with other laboratory and clinical findings, is an aid in the diagnosis of rheumatoid arthritis (RA), including RA diagnosed within 2 years of presentation of symptoms.

Summary and Explanation of the Test

Rheumatoid Arthritis (RA) is one of the most common systemic autoimmune diseases, affecting approximately 0.5% of the world population.¹ The diagnosis depends primarily on clinical manifestations of the disease. Until recently, the only serological test routinely used was the determination of the presence of rheumatoid factor (RF), found in approximately 50%-90% of these patients.^{1,2} However, RF is also found in people with infections, other autoimmune diseases and some healthy individuals.^{1,2}

It is important for disease management to diagnose and treat patients with RA as early as possible.³ It has been known for many years that anti-perinuclear autoantibodies, also called anti-keratin autoantibodies, are found in people with RA. Recently, it was discovered that these antibodies recognize an epitope that contains the deimidated form of arginine called citrulline.^{4,5} A circular peptide containing citrulline, called CCP (Cyclic Citrullinated Peptide), was found to be better at discriminating RA patients from other patients than either the perinuclear antibody test or the tests for rheumatoid factor.^{6,7} In a review of the published literature⁸, 68% of patients with RA are positive for anti-CCP, while an average of 5% of non-RA subjects are positive.

An ELISA made with the originally described CCP sequence⁵ was not broadly marketed due to low sensitivity. However, the second generation anti-CCP test (called CCP2 by some companies) is widely available and shows superior performance compared to the original peptide.⁹ INOVA Diagnostics calls its second generation test the CCP IgG ELISA. Importantly, anti-CCP are found in patients with early RA⁸, sometimes predicting the future development of RA better than RF.^{4,7} Antibodies from some RA patients who are negative for anti-CCP2 are reactive with other citrullinated proteins,^{6,7} suggesting that there are additional epitopes that are not present in the second generation CCP antigen sequence. In 2005, kits using a 3rd generation antigen (CCP3) were made available. These 3rd generation peptide kits were shown to be approximately 5% more sensitive compared with kits using CCP2 antigen and equally specific.¹⁰ The antigen used in the QUANTA Lite™ CCP 3.1 kit contains the newer, 3rd generation CCP3 peptide. This kit also uses a conjugate that detects IgA antibodies in addition to the usual IgG antibodies. Sensitivity is enhanced over the CCP 3 kit due to some RA patients having IgA antibodies to CCP 3 in the absence of IgG.¹¹ Additional improvements in the CCP 3.1 ELISA include color-coded breakaway wells and the ability to use either serum or plasma patient samples.

Principles of the Procedure

The antigen used in the QUANTA Lite™ CCP 3.1 IgG/IgA ELISA test is a synthetic, cyclic citrullinated peptide that was found to have high sensitivity and specificity in detecting antibodies in patients with RA. This antigen is bound to the surface of a microwell plate. Pre-diluted controls and diluted patient samples are added to separate wells, allowing any CCP IgG and/or IgA antibodies present to bind to the immobilized antigen. Unbound sample is washed away and an enzyme labeled anti-human IgG/IgA conjugate is added to each well. A second incubation allows the enzyme labeled anti-human IgG and/or IgA to bind to any patient antibodies that have become attached to the microwells. After washing away any unbound enzyme labeled anti-human IgG/IgA, the remaining enzyme activity is measured by adding a chromogenic substrate and measuring the intensity of the color that develops. The assay can be evaluated spectrophotometrically by measuring and comparing the color intensity that develops in the patient wells with the color in the control wells.

Reagents

1. Polystyrene microwell ELISA plate coated with purified, synthetic CCP 3 antigen (12-1 x 8 wells), with holder in foil package containing a desiccant
2. ELISA Negative Control, 1 vial of buffer containing preservative and human serum with no human antibodies to CCP, prediluted, 1.2mL
3. CCP 3.1 IgG/IgA ELISA Low Positive, 1 vial of buffer containing preservative and human serum antibodies to CCP, prediluted, 1.2mL
4. CCP 3.1 IgG/IgA ELISA High Positive/Calibrator A, 1 vial of buffer containing preservative and human serum antibodies to CCP, prediluted, 1.2mL
5. CCP 3.1 IgG/IgA ELISA Calibrator B, 1 vial of buffer containing preservative and human serum antibodies to CCP, prediluted, 1.2mL
6. CCP 3.1 IgG/IgA ELISA Calibrator C, 1 vial of buffer containing preservative and human serum antibodies to CCP, prediluted, 1.2mL
7. CCP 3.1 IgG/IgA ELISA Calibrator D, 1 vial of buffer containing preservative and human serum antibodies to CCP, prediluted, 1.2mL

8. CCP 3.1 IgG/IgA ELISA Calibrator E, 1 vial of buffer containing preservative and human serum antibodies to CCP, prediluted, 1.2mL
9. HRP Sample Diluent, 1 vial – colored pink containing Tris-buffered saline, Tween 20, protein stabilizers and preservative, 50mL
10. High Specificity Wash Concentrate, 1 vial of 10x concentrate - colored red containing Tris-buffered saline and Tween 20, 100mL. Refer to the Methods Section for dilution instructions.
11. HRP CCP 3.1 IgG/IgA Conjugate, (goat), anti-human IgG/IgA, 1 vial – colored light yellow containing buffer, protein stabilizers and preservative, 10mL
12. TMB Chromogen, 1 vial containing stabilizers, 10mL
13. HRP Stop Solution, 0.344M Sulfuric Acid, 1 vial – colorless, 10mL

Warnings

1. **WARNING:** This product contains a chemical (0.02% chloramphenicol) in the sample diluent, controls, and conjugate known to the State of California to cause cancer.
2. All human source material used in the preparation of controls for this product has been tested and found negative for antibody to HIV, HBsAg, and HCV by FDA cleared methods. No test method however can offer complete assurance that HIV, HBV, HCV or other infectious agents are absent. Therefore, the CCP 3.1 IgG/IgA ELISA Low Positive, CCP 3.1 IgG/IgA ELISA High Positive/Calibrators and ELISA Negative Control should be handled in the same manner as potentially infectious material.¹²
3. Sodium Azide is used as a preservative. Sodium Azide is a poison and may be toxic if ingested or absorbed through the skin or eyes. Sodium azide may react with lead or copper plumbing to form potentially explosive metal azides. Flush sinks, if used for reagent disposal, with large volumes of water to prevent azide build-up.
4. The HRP conjugate contains a dilute poisonous/corrosive chemical, which may be toxic if ingested in large amounts. To prevent possible chemical burns, avoid contact with skin and eyes.
5. TMB Chromogen contains an irritant, which may be harmful if inhaled, ingested or absorbed through the skin. To prevent injury, avoid inhalation, ingestion or contact with skin and eyes.
6. The HRP Stop Solution consists of a dilute sulfuric acid solution. Avoid exposure to bases, metals, or other compounds, which may react with acids. Sulfuric acid is a poison and corrosive, which may be toxic if ingested. To prevent chemical burns, avoid contact with skin and eyes.
7. Use appropriate personal protective equipment while working with the reagents provided.
8. Spilled reagents should be cleaned up immediately. Observe all federal, state and local environmental regulations when disposing of wastes.

Precautions

1. This product is for *In Vitro* Diagnostic Use.
2. Substitution of components other than those provided in this system may lead to inconsistent results.
3. Incomplete or inefficient washing and insufficient liquid removal from the ELISA well strips will cause poor precision and/or high background.
4. Adaptation of this assay for use with automated sample processors and other liquid handling devices, in whole or in part, may yield differences in test results from those obtained using the manual procedure. It is the responsibility of each laboratory to validate that their automated procedure yields test results within acceptable limits.
5. A variety of factors influence the assay performance. These include the starting temperature of the reagents, the ambient temperature, the accuracy and reproducibility of the pipetting technique, the thoroughness of washing and liquid removal from the wells of the ELISA strips, the photometer used to measure the results, and the length of the incubation times during the assay. Careful attention to consistency is required to obtain accurate and reproducible results.
6. Strict adherence to the protocol is recommended.
7. Incomplete resealing of the zip-lock pouch containing microwell strips and desiccant will result in antigen degradation and poor precision.
8. Unacceptably low absorbencies may be observed following **two** or more uses from a single bottle of HRP conjugate over a period of time. It is important to follow all recommended HRP conjugate handling procedures to prevent this occurrence.
9. Chemical contamination of the HRP conjugate can result from improper cleaning or rinsing of equipment or instruments. Residues from common laboratory chemicals such as formalin, bleach, ethanol or detergent will cause degradation of the HRP conjugate over time. Thoroughly rinse all equipment or instruments after the use of chemical cleaners/disinfectants.

Storage Conditions

1. Store all the kit reagents at 2-8°C. Do not freeze. Reagents are stable until the expiration date when stored and handled as directed.
2. Unused antigen coated microwell strips should be resealed securely in the foil pouch containing desiccant and stored at 2-8°C.
3. Diluted wash buffer is stable for 1 week at 2-8°C.

Specimen Collection

This procedure should be performed with either a serum specimen or citrated plasma or EDTA plasma specimen, since these samples yielded identical results. Lithium and heparin plasma should not be used because they may yield results different than serum. Addition of azide or other preservatives to the test samples may adversely affect the results. Microbially contaminated, heat-treated, or specimens containing visible particulate should not be used. Grossly hemolyzed or lipemic serum or specimens should be avoided.

Following collection, the serum should be separated from the clot or the plasma should be stored as described below. NCCLS Document H18-A3 recommends the following storage conditions for samples: 1) Store samples at room temperature no longer than 8 hours. 2) If the assay will not be completed within 8 hours, refrigerate the sample at 2-8°C. 3) If the assay will not be completed within 48 hrs, or for shipment of the sample, freeze at -20°C or lower. Frozen specimens must be mixed well after thawing and prior to testing.

Procedure

Materials provided

- 1 CCP 3.1 IgG/IgA ELISA microwell plate (12-1 x 8 wells), with holder
- 1 1.2mL prediluted ELISA Negative Control
- 1 1.2mL prediluted CCP 3.1 IgG/IgA ELISA Low Positive
- 1 1.2mL prediluted CCP 3.1 IgG/IgA ELISA High Positive/Calibrator A
- 1 1.2mL prediluted CCP 3.1 IgG/IgA ELISA Calibrator B
- 1 1.2mL prediluted CCP 3.1 IgG/IgA ELISA Calibrator C
- 1 1.2mL prediluted CCP 3.1 IgG/IgA ELISA Calibrator D
- 1 1.2mL prediluted CCP 3.1 IgG/IgA ELISA Calibrator E
- 1 50mL HRP Sample Diluent
- 1 100mL High Specificity Wash Concentrate, 10x concentrate
- 1 10mL HRP CCP 3.1 IgG/IgA Conjugate, (goat), anti-human IgG/IgA
- 1 10mL TMB Chromogen
- 1 10mL HRP Stop Solution, 0.344M Sulfuric Acid

Additional Materials Required But Not Provided

Micropipets to deliver 5, 100, 200-300 and 500µL

Disposable micropipet tips

Test tubes for patient sample dilutions, 4mL volume

Distilled or deionized water

1L container for diluted High Specificity Wash Concentrate

Microwell plate reader capable of measuring OD at 450nm (and 620nm for dual wavelength readings)

Method

Before you start

1. Bring all reagents and samples to room temperature (20-26°C) and mix well.
2. Dilute the High Specificity Wash Concentrate 1:10 by adding the contents of the High Specificity Wash Concentrate bottle to 900mL of distilled or deionized water. If the entire plate will not be run within this period, a smaller quantity can be prepared by adding 10.0mL of the concentrate to 90mL of distilled or deionized water for every 16 wells that will be used. The diluted buffer is stable for 1 week at 2-8°C.
3. Prepare a 1:101 dilution of each patient sample by adding 5µL of sample to 500µL of HRP Sample Diluent. Diluted samples must be used within 8 hours of preparation. **DO NOT DILUTE** the CCP 3.1 IgG/IgA ELISA Low Positive, CCP 3.1 IgG/IgA ELISA High Positive and ELISA Negative Control.
4. Determination of the presence or absence of anti-CCP 3.1 antibodies using arbitrary units requires two wells for each of the three controls and one or two wells for each patient sample. It is recommended that samples be run in duplicate.
5. If desired, results can be quantitated by using a 5-point standard curve. For points A through E of the 5-point standard curve, use **PREDILUTED** CCP 3.1 Calibrators A through E directly from the vial. The five point standard curve has the following values:

Point		Units
A	Prediluted CCP 3.1 IgG/IgA Calibrator A	250.0
B	Prediluted CCP 3.1 IgG/IgA Calibrator B	125.0
C	Prediluted CCP 3.1 IgG/IgA Calibrator C	62.5
D	Prediluted CCP 3.1 IgG/IgA Calibrator D	31.25
E	Prediluted CCP 3.1 IgG/IgA Calibrator E	15.62

Assay procedure

1. **ALL REAGENTS MUST BE BROUGHT TO ROOM TEMPERATURE (20-26°C) PRIOR TO BEGINNING THE ASSAY.** Place the required number of microwells/strips in the holder. **Immediately return unused strips to the pouch containing desiccants and seal securely to minimize exposure to water vapor.**

2. Add 100µL of the **prediluted** CCP 3.1 IgG/IgA ELISA Low Positive, the CCP 3.1 IgG/IgA ELISA High Positive, Calibrators B through E if desired, the ELISA Negative Control and the diluted patient samples to the wells. Cover the wells and incubate for 30 minutes at room temperature on a level surface. The incubation time begins after the last sample addition.
3. Wash step: Thoroughly aspirate the contents of each well. Add 200-300µL of the **diluted** HRP Wash buffer to all wells, then aspirate. Repeat this sequence twice more for a total of three washes. Invert the plate and tap it on absorbent material to remove any residual fluid after the last wash. It is important to completely empty each well after each washing step. Maintain the same sequence for the aspiration as was used for the sample addition.
4. Add 100µL of the HRP CCP 3.1 IgG/IgA Conjugate to each well. Conjugate should be removed from the bottles using standard aseptic conditions and good laboratory techniques. Remove only the amount of conjugate from the bottle necessary for the assay. **TO AVOID POTENTIAL MICROBIAL AND/OR CHEMICAL CONTAMINATION, NEVER RETURN UNUSED CONJUGATE TO THE BOTTLE.** Incubate the wells for 30 minutes as in step 2.
5. Wash step: Repeat step 3.
6. Add 100µL of TMB Chromogen to each well and incubate **in the dark** for 30 minutes at room temperature.
7. Add 100µL of HRP Stop Solution to each well. Maintain the same sequence and timing of HRP Stop Solution addition as was used for the TMB Chromogen. Gently tap the plate with a finger to thoroughly mix the wells.
8. Read the absorbance (OD) of each well at 450nm within one hour of stopping the reaction. If bichromatic measurements are desired, 620nm can be used as a reference wavelength.

Quality Control

1. The CCP 3.1 IgG/IgA ELISA Low Positive, the CCP 3.1 IgG/IgA ELISA High Positive and the ELISA Negative Control should be run with every batch of samples to ensure that all reagents and procedures perform properly.
2. Note that since the CCP 3.1 IgG/IgA ELISA Low Positive, the CCP 3.1 IgG/IgA ELISA High Positive and the ELISA Negative Control are prediluted, they do not control for procedural methods associated with dilution of specimens.
3. Additional controls may be tested according to guidelines or requirements of local, state and/or federal regulations or accrediting organizations. Additional suitable control sera may be prepared by aliquoting pooled human serum specimens and storing at $\leq -20^{\circ}\text{C}$.
4. In order for the test results to be considered valid, all of the criteria listed below must be met. If any of these are not met, the test should be considered invalid and the assay repeated.
 - a. The absorbance of the prediluted CCP 3.1 IgG/IgA ELISA High Positive must be greater than the absorbance of the prediluted CCP 3.1 IgG/IgA ELISA Low Positive, which must be greater than the absorbance of the prediluted ELISA Negative Control.
 - b. The prediluted CCP 3.1 IgG/IgA ELISA High Positive must have an OD greater than 1.0 while the prediluted ELISA Negative Control absorbance cannot be over 0.2.
 - c. The CCP 3.1 IgG/IgA ELISA Low Positive absorbance must be more than twice the ELISA Negative Control or over 0.25.
 - d. The ELISA Negative Control and CCP 3.1 IgG/IgA ELISA High Positive are intended to monitor for substantial reagent failure. The CCP 3.1 IgG/IgA ELISA High Positive will not ensure precision at the assay cutoff.
 - e. The user should refer to CLSI (NCCLS) Document C24-A3 for additional guidance on appropriate QC practices.

Calculation of Semi-Quantitative Results - Ratio Method

The average OD for each set of duplicates is first determined. The reactivity for each sample can then be calculated by dividing the average OD of the sample by the average OD of the CCP 3.1 IgG/IgA ELISA Low Positive. The result is multiplied by the number of units assigned to the CCP 3.1 IgG/IgA ELISA Low Positive found on the label.

$$\text{Sample Value (units)} = \frac{\text{Sample OD}}{\text{CCP 3.1 IgG/IgA ELISA Low Positive OD}} \times \text{CCP 3.1 IgG/IgA ELISA Low Positive (units)}$$

Reactivity is related to the quantity of antibody present in a non-linear fashion. While increases and decreases in patient antibody concentrations will be reflected in a corresponding rise or fall in reactivity, the change is not proportional (i.e. a doubling of the antibody concentration will not double the reactivity).

Optional Method of Calculation: Semi-Quantitative Results using a Standard Curve

Calculation of results with the optional standard curve

1. Determine a mean value for all duplicate readings.
2. Plot the mean absorbance (OD) of the samples in the standard curve against their values in Units. Use a linear/linear Cubic Spline (third order polynomial) or a point-to-point line fit to draw the curve.
3. Determine the unknown anti-CCP 3.1 concentration in Units from the "Y" axis by reading the corresponding absorbance on the "X" axis. Calculate the unknown Units.

- Note: The values calculated with the ratio method will be different than those calculated by the standard curve. High values will differ more than low values. The CCP 3.1 IgG/IgA High Positive will not yield a value of 250 Units with the ratio method.

Interpretation of Results

The ELISA assay is very sensitive to technique and is capable of detecting even small differences in patient populations. The values shown below are suggested values only. Each laboratory should establish its own normal range based upon its own techniques, controls, equipment and patient population according to their own established procedures.

The sample can then be classified as negative, weak positive, moderate positive or strong positive according to the table below.

	Units
Negative	<20
Weak Positive	20 – 39
Moderate Positive	40 – 59
Strong Positive	≥60

- A positive result indicates the presence of IgG and/or IgA anti-CCP 3 antibodies and suggests the possibility of RA.
- A negative result indicates no CCP 3 antibodies or levels below the negative cut-off of the assay.
- It is suggested that the results reported by the laboratory should include the statement: "The following results were obtained with the INOVA QUANTA Lite™ CCP 3.1 IgG/IgA ELISA. Anti-CCP values obtained with different manufacturers' assay methods may not be used interchangeably. The magnitude of the reported IgG or IgA levels cannot be correlated to an endpoint titer."

Limitations of the Procedure

- The presence of immune complexes or other immunoglobulin aggregates in the patient sample may cause an increased level of non-specific binding and produce false positives in this assay.
- Not all RA patients are positive for anti-CCP.
- Results of this assay should be used in conjunction with clinical findings and other serological tests.
- The assay performance characteristics have not been established for matrices other than serum, EDTA plasma and citrated plasma.
- The diagnostic value of anti-CCP 3 in Juvenile Rheumatoid Arthritis patients has not been determined.
- Interfering substances: High levels of hemoglobin, bilirubin, cholesterol or triglycerides do not cause false negative or false positive results.

Expected Values from Clinical Samples

The ability of the QUANTA Lite™ CCP3.1 IgG/IgA ELISA to detect IgG/IgA CCP3 antibodies was evaluated by comparison of 942 samples to an ELISA that measures IgG anti-CCP2. Included were 495 patients with a clinical diagnosis of RA from both internal (186) and external (309) studies. Sera from 275 random blood donors were also tested. Of the 272 where age and sex were available, 97 were female and the average age was 42.4 years. Disease controls included 74 people with other rheumatic diseases (ORD) such as systemic lupus erythematosus (34), Sjögren's Syndrome (16) and scleroderma (24), as well as an additional 98 samples with antibodies to infectious diseases (ID) such as hepatitis C (58), herpes simplex virus (15), cytomegalovirus (14), toxoplasmosis (6) and rubella (5) in one large internal study.

Clinical Sensitivity and Specificity

The following table summarizes the findings of all our clinical studies.

Samples N=942	CCP3.1 ELISA		CCP ELISA	
	+	-	+	-
RA (Total N=495)	348	147	320	175
RA – within 2 years (N=86)	55	31	47	39
Total Controls (N=447)	10	437	8	439
Blood Donors (N=275)	2	273	3	272
ORD (N=74)	6	68	4	70
Infectious Disease (N=98)	2	96	1	97

Clinical Sensitivity [95% Confidence Interval (CI)] =	CCP3.1 = 70.3% (66-74%)
	CCP = 64.6% (60-69%)
In RA within 2 years =	CCP3.1 = 64.0% (52-74%)
	CCP = 54.7% (43-65%)
Clinical Specificity (95% CI) =	CCP3.1 = 97.8% (96-99%)
	CCP = 98.2% (97-99%)

Method Comparison

N=942	CCP IgG ELISA		Total	% Agreement (95% CI)
	Positive	Negative		
CCP3.1 IgG/IgA ELISA	Positive	320	358	Pos. % Agree=97.6% (95.3-98.9%)
	Negative	8**	584	Neg. % Agree=93.8% (91.6-95.6%)
	Total	328	942	Tot. % Agree=95.1%

*33 of these samples are diagnosed RA.

**5 of these samples are diagnosed RA.

Cross-Reactivity

To assess potential cross-reactivity of CCP3.1 antigen with other autoantibodies, the QUANTA Lite™ CCP3.1 ELISA was evaluated with 16 samples, all having high levels of various other autoantibodies. Included in this group were 2 samples each that reacted with SS-A, SS-B, Sm, RNP, Scl-70, Jo-1, Ribo-P and dsDNA. All samples were negative for anti-CCP3.1. The above study shows no significant cross-reactivity of the CCP3.1 antigen with a variety of other autoantibodies.

Precision and Reproducibility

The between-run assay variation was measured by running duplicates of negative, low positive, and strong positive samples in 6 separate assays on 5 different days. The within-run assay variation was measured by running 9 samples 9 or 10 times each on the same ELISA plate. Representative results are shown below.

Between-run Variation

	Negative 1		Low 1		Low 2		High 1	
	1 pt.*	5 pt.**	1 pt.	5 pt.	1 pt.	5 pt.	1 pt.	5 pt.
Mean	6 U	4 U	22 U	23 U	28 U	30 U	69 U	85 U
SD	0.4	0.5	0.7	0.6	0.8	1.2	1.7	1.2
CV%	8%	11%	3%	3%	3%	4%	2%	1%

Within-Run Variation

	Negative 1		Low 1		Low 2		High 1	
	1 pt.	5 pt.	1 pt.	5 pt.	1 pt.	5 pt.	1 pt.	5 pt.
Mean	4 U	2 U	22 U	24 U	26 U	30 U	68 U	81 U
SD	0.2	0.2	0.9	1.1	0.9	1.0	1.9	3.2
CV%	4%	13%	4%	5%	3%	3%	3%	4%

*Ratio method test results

**Standard Curve test results

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