THERATEST LABS
Innovations in Immunoassays

EL-anti –CCP/2 ™

An enzyme immunoassay for the
detection and measurement of IgG class
antibodies in human serum to cyclic citrullinated peptide (CCP)

For professional use only

Instruction Manual

Catalog Nos.:

104-121 (1 – plate kit)

104-122 (2 - plate kit)

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INTRODUCTION

Intended use

For in vitro diagnostic use

TheraTest EL-anti-CCP/2™ test is intended for use in clinical laboratories as an *in vitro* diagnostic test for the detection and measurement of the IgG class of autoantibodies specific to cyclic citrullinated peptide (CCP) in human serum by enzyme-linked immunosorbent assay (ELISA). It is intended to aid in the diagnosis of Rheumatoid Arthritis (RA) in conjunction with other clinical findings and laboratory tests.

Summary and Explanation

Rheumatoid arthritis (RA) is a common, systemic autoimmune disease affecting 0.5-1.0% of the population (1,2). It is characterized by chronic inflammation of the synovium, which can lead to erosive changes and deformities of the joint (3). The diagnosis of RA has been made with the aid of the 1987 revised American College of Rheumatology criteria for the classification of RA, which include clinical, radiological and immunological features (4). Until recently, the only serological test specified in the ACR criteria was measurement of IgM rheumatoid factor (RF), which can be present in up to 70% of these patients, but the presence of RF is not definitive for RA classification because RF is also found in patients with other autoimmune disorders and infectious diseases and in a small percentage of the apparently healthy population (5, 6). Antikeratin antibodies (AKA) and antiperinuclear factor (APF) have been studied for many years and were found to be highly specific for RA (7). Both AKA and APF have been detected by indirect immunofluorescence with use of the antigenic substrates rat esophagus or buccal mucosal cells, respectively, as antigenic substrates (8,9). Although discovered independently, both AKA and APF are directed to the same antigen, filaggrin (10, 11). During cell differentiation, profilaggrin, present in the keratohyaline granules of human buccal mucosa cells is proteolytically cleaved into filaggrin subunits. At this stage, the protein is dephosphorylated and some arginine residues are converted to citrulline by the enzyme peptidylargine deiminase (12). Schellekens and colleagues examined the binding characteristics of RA sera using citrullinated peptides as the target antigens. They determined that a cyclic peptide form performed better than a linear peptide form in solid phase assays, and in so doing developed the anti-CCP ELISA (13, 14). It has been reported that most citrullinated proteins/peptides are recognized by autoantibodies in RA sera although with differing sensitivities and specificities (7), suggesting an important role for citrullinated antigens in the pathomechanism of RA. To improve sensitivity, dedicated libraries of citrulline-containing peptides were screened with RA sera to obtain novel citrullinated peptides which were incorporated into a second generation CCP test (CCP2). According to recent meta-analyses, the diagnostic sensitivity of the anti-CCP2 assay is 67-68 % in RA, coupled with 95% diagnostic specificity, making the anti-CCP2 test superior to RF (15-17).

The increasing demand for diagnosing and treating RA as early as possible, led to the development of the 2010 American College of Rheumatology/European League Against Rheumatism (ACR/EULAR) classification criteria for RA (18). The new criteria have included the presence of anti-citrullinated protein antibodies (ACPA) as a serologic criterion for RA, besides RF. Anti-CCP2 is the best known and most frequently determined antibody in the ACPA

family. The new criteria also differentiate between low positive and high positive RF and ACPA, assigning higher score to high positive antibody levels.

Principle of the procedure

The **TheraTest EL-anti-CCP/2 ™ assay** is a solid phase enzyme immunoassay for the detection and measurement of anti-CCP antibodies. Testing is performed using a Standard Curve (semi-quantitative method) or a one point Calibrator (qualitative method). The wells of 96-well polystyrene plates have been coated with a highly purified synthetic cyclic peptide containing modified arginine residues. The wells are incubated with diluted serum specimens, Calibrator(s) and Controls. During the incubation, the antibodies present in the test sample bind to the solid phase antigen. Then the wells are washed, and prediluted, horseradish-peroxidase labeled anti-human IgG (Fcγ specific) is incubated in the wells. Unbound anti-IgG antibody is removed by aspiration and washing. A specific chromogen substrate is added to the wells, and the autoantibody+anti-IgG complex is detected by a resulting color change, which is measured by a spectrophotometric enzyme immunoassay reader. A direct relationship exists between the amount of anti-CCP antibody reactivity in the specimen and the absorbance value detected by the spectrophotometer. Semi-quantitative results, reported as U/ml, are estimated by interpolation from a Standard Curve based on the values of the Calibrators provided. Qualitative results are calculated based on the reference value of the optional one point Calibrator and reported as a ratio by comparison to the normal cut-off value.

WARNINGS AND PRECAUTIONS

For in vitro diagnostic use only

Reagents Containing Human Source Material

Controls and Calibrators contain human serum. Treat as potentially infectious. When tested by FDA-cleared methods for the presence of antibody to HIV (Human Immunodeficiency Virus) and Hepatitis C Virus and for Hepatitis B Surface Antigen (HbsAg), the materials were nonreactive. While these methods are highly accurate, no test method can offer complete assurance that HIV, hepatitis virus or other infectious agents are absent. Therefore these materials and all patient specimens should be handled as though capable of transmitting infectious diseases. Human material should be handled in accordance with good laboratory practices using appropriate precautions as described in the Centers for Disease Control and Prevention Manual, "Biosafety in Microbiological and Biomedical Laboratories", 5th edition, December 2009. Web site: http://www.cdc.gov/biosafety/publications/bmbl5/

Stop Reagent (2 mol/L Phosphoric Acid)

Corrosive! May cause severe burns upon contact with skin. Do not get in eyes, on skin, or on clothing. Do not ingest or inhale fumes. On contact, flush with copious amount of water for at least 15 minutes.

Hazardous Substance Risk & Safety Phrases:

R34 - Causes burns.
S26 - In case of contact with eyes, rinse immediately with plenty of water and seek medical advice.
S36/37/39 - Wear suitable protective clothing, gloves and eye/face protection
S45 - In case of accident or if you feel unwell, seek medical advice immediately (show label where possible).

Chromogen

Irritant! This product contains 3,3',5,5'-tetramethylbenzidine (TMB) (≤0.05%), a chromogenic indicator of horseradish peroxidase activity. It has shown neither mutagenic nor carcinogenic effects in laboratory experiments (19).

Hazardous Substance Risk & Safety Phrases:

R36/37/38 – Irritating to eyes, respiratory system, and skin. Avoid inhalation and direct contact.
S24/25 – Avoid contact with skin or eyes.
S26 – In case of contact with eyes, rinse immediately with plenty of water and seek medical advice.
S36 – Wear suitable protective clothing.
S51 – Use only in well-ventilated areas.

Reagents Containing Sodium Azide

Calibrators and Controls contain sodium azide which can react with lead and copper plumbing to form highly explosive metal azides. On disposal, drain with large quantities of water to prevent azide build-up.

Hazardous Substance Risk & Safety Phrases:

R22 - Harmful if swallowed.
R36/37/38 - Irritating to eyes, respiratory system, and skin. Avoid inhalation and direct contact.
S26 - In case of contact with eyes, rinse immediately with plenty of water and seek medical advice.
S28 - After contact with skin, wash immediately with plenty of water.
S36/37/39 - Wear suitable protective clothing, gloves and eye/face protection
S46 - If swallowed, seek medical advice immediately and show this container label.

General Precautions and Information

1. Handle patient sera and kit reagents with appropriate precautions.
2. Do not pipette by mouth.
3. Do not eat, drink, or smoke in designated work areas.
4. Work in a well ventilated area when using kit reagents.
5. The Stop Reagent can irritate eyes and mucous membranes.
6. Wash hands thoroughly after using specimens and kit reagents.
7. Do not use test components beyond the expiration date.
8. Avoid microbial contamination of the reagents. If solutions become turbid, they should not be used.
9. Avoid exposing reagents to excessive heat or light during storage.
10. Do not allow the Chromogen to come in contact with metal or oxidizing agents.
11. Use disposable glassware and plasticware or wash all material thoroughly according to standard laboratory practice.
12. Calibrators, Controls and IgG enzyme conjugate are lot specific and therefore are not interchangeable among kits of different lot numbers.
13. Dispose of containers and unused kit reagents in accordance with federal, state and local regulatory requirements

STORAGE AND HANDLING

1. Store all reagents at 2° – 8°C when received. Avoid freezing reagents.
2. All reagents must be brought to room temperature (18° – 25°C) for 30 minutes prior to use.
3. Avoid direct sunlight.

4. **Important**: When stored at 2º – 8º C, the 10X Wash Buffer may form crystals. The crystals must be dissolved prior to dilution of the 10X concentrate when only a portion of the concentrate is being diluted. If all of the bottled contents are transferred at once to a 1-L graduated cylinder, be sure to rinse the bottle multiple times with water to dissolve and transfer any crystallized salts. When stored at 2 – 8 ºC, the 10X Wash Buffer is stable until kit expiration, the 1X Wash Buffer is stable for 8 weeks.

SPECIMEN REQUIREMENTS

Collection and Storage of Serum

A whole blood specimen should be obtained using accepted medical techniques to avoid hemolysis. The blood should be clotted and the serum separated by centrifugation within 24 h of collection. Grossly hemolyzed, lipemic or icteric serum is not acceptable, since it may affect the results of the test. Serum may be stored at 2º - 8 °C for up to 14 days prior to testing. If testing cannot be completed within 14 days of collection, the separated serum must be stored at –20°C. Do not use serum that has been thawed more than once or which has been heat inactivated. The performance of plasma samples has not been evaluated; therefore plasma should not be used in the test.

PROCEDURE

Before starting the assay, read the product insert carefully. Instructions should be followed exactly as they appear in this kit insert to ensure valid results.

Materials Provided

1. **CCP coated wells in 96-well plate format, 2 Plates:** For single use only**!** All wells are coated with cyclic citrullinated peptide. Ready for use. The unused wells and the frame may be stored and used at a later date. They are returned to their desiccant-containing metallized pouch, placed in the resealable pouch which is then sealed carefully and stored dry at 2º - 8°C until the expiration date.
2. **10X Wash Buffer, 100 mL:** 10X concentrated buffer with preservative.
3. **CCP Specimen Diluent**: Buffer with bovine protein, preservative and yellow dye. Ready for use.
4. **Set of five CCP Calibrators: Calibrator 1 – Calibrator 5, 1.5 mL each:** Calibrators contain human serum with various concentrations of IgG antibodies to CCP and preservative in stabilizing buffer. See attached Data Sheet for performance characteristics.
5. **CCP Positive Control, 0.35 mL:** Human serum containing IgG antibodies to CCP and preservative in stabilizing buffer. See attached Data Sheet for performance characteristics.
6. **CCP Negative Control, 0.35 mL:** Human serum without IgG antibodies to CCP and preservative in stabilizing buffer. See attached Data Sheet for performance characteristics.
7. **CCP Anti-IgG Enzyme Conjugate, 27 mL:** Goat anti-human IgG (Fcγ specific) conjugated with horseradish peroxidase, with preservative in stabilizing buffer and green dye.
8. **Chromogen, 27 mL:** 3, 3’5,5’ tetramethybenzidine (TMB) in buffer with hydrogen peroxide.
9. **Stop Reagent, 27 mL:** 2 mol/L phosphoric acid.

10. **Resealable pouch**.

Materials required but not provided

1. Calibrated precision micropipettes with disposable plastic tips that deliver 10µL, 100 µL and 1 mL.
2. Calibrated adjustable multichannel pipettes (8- or 12-channel).
3. Disposable Pipette tips.
4. Microtubes, polypropylene (dilution tubes or cluster tubes) with a rack of 96-well format.
5. Timer.
6. Pipettes (1 mL, 5 mL, and 10 mL).
7. Pipette reagent reservoirs (to accommodate multichannel pipettes).
8. Deionized or distilled water.
9. Single (450 nm) or dual (450 nm test, 620-690 nm reference) wavelength spectrophotometer (ELISA plate reader) for 96-well microtiter plates.
10. Clean wash bottle and automated plate washer (optional).

Reagent preparation:

1. Coated Wells

Testing is performed using the Standard Curve (semi-quantitative method) or the one point Calibrator (qualitative method); suggested plate arrangements of wells are shown on the attached Data Sheet. The entire plate or strip (or strips) may be employed, or individual wells may be used as desired.

2. Wash Solution

The 10X Wash Buffer must be diluted 1:10 with deionized or distilled water prior to use. Prepare 1X Wash Buffer by pouring the contents of the 10X Wash Buffer into a clean one liter volumetric container. Rinse the bottle with deionized or distilled water to remove residual buffer and redissolve any existing crystals. Add the rinse to the one liter container. Add deionized or distilled water until a total volume of 1.0 L is reached; mix thoroughly. Diluted Wash Buffer is stable for 8 weeks at 2º - 8 °C.

3. Specimens, Positive Control, **Negative Control**

Specimens and Controls must be diluted 1:101 in the provided CCP Specimen Diluent prior to being tested. Use high accuracy pipettes. For example, pipette 10 µL of serum into 1 mL of CCP Specimen Diluent. Discard any unused diluted Specimens and Controls after the test procedure is completed.

4. Calibrators

Calibrators are provided pre-diluted. They are ready to use. Testing is performed using all 5 Calibrators for the semi-quantitative test, or only Calibrator 5 for the qualitative test; suggested plate arrangements of wells are shown on the attached Data Sheet.

Assay Procedure

1. Allow all reagents and patient sera to equilibrate to room temperature prior to use (18º-25°C). Plates should equilibrate to room temperature in their sealed metallized pouch to prevent condensation.

2. Mark the position of the samples (i.e., Calibrator(s), Positive Control, Negative Control, and Specimens) on a worksheet, and arrange dilution tubes accordingly in a rack. A suggested plate arrangement is shown on attached Data Sheet. In the semi-quantitative method all five Calibrators are run. In the qualitative method only Calibrator 5 is run.
3. Determine the number of wells needed. The remaining unused wells should be returned to their desiccant-containing metallized pouch, placed in the resealable pouch which is then sealed carefully and stored dry at 2º - 8°C for later use.
4. Dispense 1 mL of CCP Specimen Diluent into each dilution tube.
5. Dilute all serum Specimens and Controls 1:101 (e.g. add 10 µL of serum to 1 mL CCP Specimen Diluent) and mix well. Do not dilute Calibrators.
6. Pipette 100 µL of the Calibrator(s), diluted samples and Controls into the appropriate wells. For best results pipette all materials within 5 minutes from the start of the assay. This step is facilitated by the use of multichannel pipettes.
7. Incubate the plate for 30 - 35 minutes at room temperature (18º - 25 °C).
8. Aspirate or decant the contents of the wells and wash the plate 3 times with 300 µL of 1X Wash Buffer. An automated plate washer may be used for this step. Remove all residual liquid from the wells by inverting and blotting the plate on absorbent paper.
9. Immediately pipette 100 µL of CCP Anti-IgG Enzyme Conjugate into the wells.
10. Incubate plate(s) for 30 - 35 minutes at room temperature (18º - 25 °C).
11. Aspirate or decant Enzyme Conjugate from all wells and wash the plate as in Step 8 above.
12. Immediately dispense 100 µL of Chromogen into each well. Incubate the plate(s) for 15(±1) min. at room temperature (18º - 25 °C).
13. Pipette 100 µL of Stop Reagent into each well and mix by gently tapping the side of the plate. The blue color changes to yellow.
14. Determine the absorbance of each well at 450 nm using a single or dual wavelength spectrophotometer (ELISA plate reader). Absorbance values should be read within 30 minutes of completing the assay. For a dual wavelength spectrophotometer, set test wavelength at 450 nm with the reference between 620 and 690 nm.

Procedural Notes

1. Storage

Place unused strips in the open metallized pouch (with desiccant) for light protection and place this assembly into the provided resealable pouch and store at 2-8 °C.

2. Pipetting

To avoid cross-contamination and sample carryover, pipette the Calibrator(s), Positive Control, Negative Control, and Specimens using separate pipette tips. A multi-channel pipette may be used to pipette the Enzyme Conjugate, Wash Solution, Chromogen and Stop Reagent.

3. Washing

Each column of wells may be washed using a multi-channel pipette. The wells may be aspirated using an appropriate vacuum apparatus, fitted with a Pasteur pipette, or their contents may be dumped into a disposal container. Alternatively, commercial semi-automated washing systems may be used. When using either washing technique, the plate should be inverted and blotted against absorbent paper after the last wash. Use reagent grade water only (CAP type 1 or USP grade) for preparing the 1X Wash Buffer.

4. Measurement of Absorbance Values

Absorbance values should be measured within 30 minutes after completion of assay.

When using the semi-quantitative method, specimens with absorbance values that exceed that of Calibrator 5 are outside the range of the assay, and should be reported as >… U/mL. If exact determination is desired, the specimen should be pre-diluted (example: 1:10) with the provided CCP Specimen Diluent, and should be retested. The retest result should be multiplied by the dilution factor (for example, if the specimen was prediluted 1:10, the units obtained should be multiplied by 10).

RESULTS

Calculation of Results

Most ELISA readers are computer compatible and data may be calculated with the help of computer programs. Check periodically that the program chosen yields the same results as obtained by manual calculations.

The autoantibody unit value is unique for each Calibrator. The autoantibody units for the Calibrators are reported on the lot specific Data Sheet accompanying the kit.

1. Semi-quantitative method with Standard Curve:

Plot the absorbance values of Calibrator 1 to Calibrator 5 against their respective concentrations. Plot the concentrations in U/mL on the X axis and the absorbance values on the Y axis, and draw the best fit curve. Determine the concentrations of the patient samples from the curve against their corresponding absorbance values. Alternatively, use a curve fitting computer software; a 4-parameter logistic curve fit is recommended.

2. Qualitative method with one point Calibrator:

Antibody activity is calculated as follows:

$$\text{Conversion Factor} = \frac{\text{Reference value of Calibrator 5}}{\text{Absorbance (OD) value of Calibrator 5}}$$

$$\text{Specimen Result} = \frac{\text{Conversion Factor} \times \text{Absorbance value of Specimen}}{5}$$

With the one point Calibrator method, reactivity is related to the quantity of antibody present in a non-linear fashion. Increases and decreases in patient antibody concentrations are reflected in a corresponding rise or fall in reactivity, but the change is not proportional.

Interpretation of Results

Semi-quantitative Method	**Qualitative Method**
Negative: ≤ 5.0 U/mL	Negative: ≤ 1.0
Equivocal range: 5.1 - 6.9 U/mL	Equivocal range: 1.1 – 1.3
Positive: ≥ 7.0 U/mL	Positive: ≥ 1.4

The above ranges are suggested values only. The reference range should be validated by each laboratory to reflect the characteristics of the population they serve. When the results are equivocal it is recommended to report them as equivocal, and repeat the test with a sample from another bleeding.

QUALITY CONTROL

1. Ensure that adequate maintenance and calibration of the plate-reader is performed according to the manufacturer's instructions, and that the correct wavelength is employed.
2. Positive and Negative Controls

Positive and Negative Controls should be run in each assay. The Controls should be tested as unknowns. The Positive and Negative Control values should fall within the ranges provided on the enclosed Data Sheet. If the values are not in agreement with those on the Data Sheet, the assay is not valid and the results should not be reported.

3. If repeating the assay, prepare a fresh dilution of each Control and sample.

LIMITATIONS OF THE PROCEDURE

1. The Positive Control and the Calibrator(s) for a specific antibody may contain other antibodies, i.e. they may not be monospecific.
2. The **TheraTest EL-anti-CCP/2™** assay should not be performed on grossly hemolyzed, lipemic, icteric or microbially contaminated samples. This method has been tested using serum samples only. The performance using other types of specimens has not been determined.
3. Diagnosis should not be made solely on the basis of a positive test result. The results must be interpreted in conjunction with all clinical information and laboratory data available to the physician (i.e. history, physical exam, and other diagnostic procedures).
4. This assay has not been evaluated on the pediatric population.
5. The value of anti-CCP in juvenile arthritis has not been determined.
6. The clinical effectiveness of monitoring CCP autoantibody levels as an indication of progression/remission of Rheumatoid Arthritis has not been defined.
7. Equivocal results should be repeated with a sample from another bleeding.

EXPECTED VALUES

100 serum samples from asymptomatic apparently healthy blood bank donors with an age range of 16-70 years, comprising approximately equal numbers of males (n=49) and females (n=51), were tested with the TheraTest EL-anti-CCP/2™ test.

Results should be considered normal and abnormal (positive) as follows:

Test	Percentile	Normal	Equivocal	Abnormal
Semi-quantitative	99-100	≤ 5.0 U/ml	5.1 - 6.9 U/ml	≥ 7.0 U/ml
Qualitative	99-100	≤ 1.0	1.1 - 1.3	≥ 1.4

PERFORMANCE CHARACTERISTICS

Comparative studies

The **TheraTest EL-anti-CCP/2™** assay was compared to another commercially available ELISA anti-CCP test for a total of 319 specimens: 100 Blood Bank Donors, 119 patients with rheumatoid arthritis (RA) and 100 patient samples sent by a rheumatologist to a reference lab for anti-CCP testing.

- The 100 Blood Bank Donors were represented by 50 females and 50 males with a median age of 45 and a range from 16 to 81. The race distribution was 15% Hispanic, 12% African American, 4% Asian and 69% others.
- The 119 patients with RA, (meet ACR criteria), were represented by 100 females and 19 males with a median age of 52 and a range from 15 to 87
- The 100 patient samples sent by a rheumatologist to a reference lab for anti-CCP testing were represented by at least 60 females and 30 males with a median age of 59 and a range from 18 to 95.

Samples below the limit of detection and above the upper limit of claimed linearity were excluded from method comparison analysis leaving 177 samples for comparison. The results are presented in the Table 1 below:

Table 1. TheraTest EL-anti-CCP/2 ™ *versus another anti-CCP ELISA test (n=177)*

		Another ELISA anti-CCP assay		
		Positive	**Negative**	**Total**
EL-anti-CCP/2™	**Positive**	46	3	49
	Equivocal	2	1	3
	Negative	0	125	125
	Total	48	129	177

Equivocal results are treated as positive:

Percent Positive Agreement: (48/48)100 = 100% (95% CI: 93 - 100)
Percent Negative Agreement: (125/129)100 = 97% (95% CI: 92 - 99)
Percent Total Agreement: (173/177)100 = 98% (95% CI: 94 -99)

Equivocal results are treated as negative:

Percent Positive Agreement: (46/48)100 = 96% (95% CI: 86 - 99)
Percent Negative Agreement: (126/129)100 = 98% (95% CI: 93 - 100)

Percent Total Agreement: (172/177)100 = 97% (95% CI: 94 - 99)

Interference

Potential interference from disease specimens with 58-256 IU/ml of RF or 630 IU/ml of dsDNA (but negative for CCP) was evaluated. Normal specimens and specimens containing anti-CCP antibodies were spiked with sera containing anti-dsDNA antibodies (630 IU/mL) and anti-RF-IgM antibodies (58 IU/mL, 163 IU/ml and 256 IU/ml). For spiked normal specimens, the change in unit value from normal alone was 0 to $\pm$ 1 unit, and all results remained in the normal range. For spiked CCP positive specimens, the change in unit value from CCP positive alone was 0 to $\pm$ 10%, and all results remained in the abnormal range.

Precision

The between-run/total precision and within-run precision/repeatability was measured by testing 8 specimens in triplicates in 20 separate assays, 1 to 2 assays per day. The precision for quantitative results is presented in the following Table 2.

Table 2. EL-anti-CCP/2™ Semi-quantitative method:

	Mean (U/ml)	Within-run Repeatability CV%	Between-run Total CV%
Specimen 1	3.2	6.3	9.0
Specimen 2	4.1	4.2	6.2
Specimen 3	7.6	4.5	6.5
Specimen 4	15.5	3.6	5.5
Specimen 5	21.3	4.1	5.7
Specimen 6	35.4	4.8	8.4
Specimen 7	56.4	4.3	6.6
Specimen 8	100.6	4.1	8.9

Results on the same specimens according to the qualitative calculation are presented in the following Table 3.

Table 3. EL-anti-CCP/2™ Qualitative method:

	Quantitative Result (U/ml)	Target Qualitative Result	Obtained Qualitative Result (number) (total n=60)		
			+	equivocal	-
Specimen 1	3.2	-	0	0	60
Specimen 2	4.1	-	0	0	60
Specimen 3	7.6	+	19	41	0
Specimen 4	15.5	+	60	0	0
Specimen 5	21.3	+	60	0	0
Specimen 6	35.4	+	60	0	0
Specimen 7	56.4	+	60	0	0
Specimen 8	100.6	+	60	0	0

Lower limit of detection

A series of sixty results on a blank sample collected from seventeen assays on nine different days and a series of sixty results on five very low-level samples collected from 16 different assays on eight different days were prepared. The limit of blank (LoB) was estimated as the 95th percentile of the ordered distribution. This point and the standard deviation of the low sample measurements were used to find the concentration where measurements are very likely to exceed the highest value of the blanks.

LoB = 0.0465 Units/ml

LoD = 0.135 Units/ml

Linearity

The highest calibrator, Calibrator 5 and two other specimens positive for anti-CCP (at two levels) were each diluted with Zero Calibrator in five dilutions, from 1:1 to 1:20 and tested in duplicate in the EL-anti-CCP/2™ assay using the Semi-quantitative Method. Results from these three specimens were pooled together and graphed, (Expected vs Measured Units/ml) and regression analysis using data points above the LoD was performed. For the area examined, 2.6 - 123.2 Units/ml, $y = 1.001x + 1.499$, $R^2 = 0.988$.

An additional three specimens positive for anti-CCP (at three levels) were each diluted with Zero Calibrator in ten dilutions, from 1:1 to 1:10 and tested in duplicate in the EL-anti-CCP/2™ assay using the Semi-quantitative Method. Results from these three specimens were pooled together and graphed, (Expected vs Measured Units/ml) and regression analysis using data points above the LoD was performed. For the area examined, 3.7 - 109.5 Units/mL, $y = 1.021x + 2.014$, $R^2 = 0.985$.

High concentration hook effect

High dose hook effect is a phenomenon whereby extremely high level specimens produce false negative or low values. High positive specimens with absorbance values beyond the calibration range were diluted and retested. The original concentration of the specimens was calculated by multiplying the unit value of the diluted specimens with the dilution factor. No low absorbance values were observed when the specimens were tested undiluted. For the **TheraTest EL-anti-CCP/2 ™** assay no hook effect was observed when samples containing up to approximately 1,700 U/mL were assayed.

TROUBLESHOOTING

Problem	Possible Causes	Solution
Control values out of range.	1. Incorrect temperature, timing or pipetting; reagents not mixed.	1. Check that temperature was correct. Check that time was correct. See "Poor Precision" (below) No. 2-4. Repeat test.
	2. Cross-contamination of controls.	2. Pipette carefully.
	3. Improper dilution.	3. Repeat test.
	4. Optical pathway not clean.	4. Check for moisture or dirt. Wipe bottom and reread.
	5.Wavelength of filter incorrect.	5. Change filter to 450 ± 5 nm.
All test results negative.	1. One or more reagents not added, or added in wrong sequence.	1. Recheck procedure. Check for unused solutions. Repeat test.
	2. Improper dilution of wash buffer.	2. Repeat test.
	3. Antigen coated plate inactive.	3. Check for obvious moisture in unused wells. Rerun test with controls only for activity.
All test results yellow.	1. Contaminated chromogen.	1. Check absorbance of unused chromogen.
Scattered false positives	2. Contaminated buffers/reagents.	2. Check all solutions for turbidity.
	3. 1X Wash Buffer contaminated.	3. Use clean container. Check quality of water used to prepare buffer.
	4. Improper dilution of serum.	4. Repeat test.
	5. Contaminated pipette	5. Use felt-plugged tips for chromogen
Poor precision.	1. Pipettor delivery CV greater than 5%.	1. Check calibration of pipettor. Use reproducible technique.
	2. Serum or reagents not mixed sufficiently; reagents not at room temperature prior to addition.	2. Mix all reagents gently but thoroughly and equilibrate to room temperature.
	3. Reagent addition taking too long; inconsistency in timing intervals, air bubbles.	3. Develop consistent uniform technique and avoid splashing or use multi-channel device or autodispenser to decrease time.
	4. Air currents blowing over plate during incubations.	4. Cover plate or place in chamber.
	5. Optical pathway not clean.	5. Wipe bottom of plate with soft tissue. Check instrument light source and detector for dirt.
	6. Instrument not equilibrated before readings were taken.	6. Check instrument manual for warm up procedure.
	7. Washing not consistent; trapped bubbles; liquid left in wells at end of wash cycle.	7. Use only acceptable washing devices. Lengthen timing delay on washing devices. Check that all wells are filled.
	8. Improper pipetting.	8. Avoid air bubbles in pipette tips.

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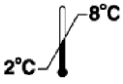

(GB)(USA)(CDN) Store at between (D)(A)(B)(CH) Lagerung bei zwischen (F)(B)(CH)(CDN) Conserver à entre (I)(CH) Conservare a tra (E) Conservar a temp. entre (P) Armazene a entre (NL) Bewaar bij tussen (DK) Opbevares mellem (S) Förvaras vid

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Abbreviated Test Procedure

1. **Dilute Controls and Specimens 1:101 with CCP Specimen Diluent.**
2. **Pipette 100 µL of Calibrator(s), diluted Controls and Specimens into appropriate wells (see Data Sheet for configuration).**
3. **Incubate for 30-35 minutes at room temperature (18º - 25ºC).**
4. **Wash the wells three times with 1X Wash Buffer.**
5. **Add 100 µL of the Anti-IgG Enzyme Conjugate into appropriate wells.**
6. **Incubate for 30-35 minutes at room temperature (18º - 25ºC).**
7. **Wash the wells three times with 1X Wash Buffer.**
8. **Add 100 µL of Chromogen into each well.**
9. **Incubate for 15±1 minutes at room temperature (18º- 25ºC).**
10. **Add 100 µL of Stop Reagent into each well.**
11. **Read the absorbance at 450 nm (reference wavelength 620-690 nm) within 30 minutes.**

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