Diagnostic Automation / Cortez Diagnostics, Inc.
IMMUNO DIAGNOSTICS

CE

AccuDiag™ Anti CCP ELISA

Cat# 3175-17

Test	Anti CCP Elisa
Method	Indirect - Method
Principle	Quantitative
Detection Range	1.12 AU/mL
Sample	100 µL
Total Time	~ 105 min
Shelf Life	12-14 Months from the manufacturing date
Specificity	79%
Sensitivity	97%

INTENDED USE

The Diagnostic Automation, Inc. Anti CCP is an (ELISA), intended for the quantitative determination of IgG class antibodies directed against cyclic citrullinated peptides, present in human serum or plasma. Anti CCP kit is intended for laboratory use only.

SUMMARY AND EXPLANATION

Reumathoid arthritis (RA) is one of the most common autoimmune diseases (1-2% European population). The most significant clinical symptom is an inflammation of the synovial membranes which causes a painful swelling of the articulations and the ankylosis.

In order to correctly diagnose RA it is necessary to exclude other forms of arthritis: in such a diagnostic process, the laboratory plays an important role in the determination of IgM, detectable in 60-80% of the patients with RA. The RF antibodies are sensitive but not very specific markers; on the contrary, Anti CCPs are characterized by a specificity of over 90% in patients affected by RA ,and are detectable in a very early asymptomatic stage in the approximately 70% of RA patients whereas only 2% of the control subjects resulted positive.

Therefore, the presence of Anti CCP antibodies can be used in the diagnosis of RA, particularly in the case of erosive arthritis, in childhood in the case of juvenile RA. The test also appears, to be useful in differentiating the collagen pathologies with concomitant arthritis from the RA. The Anti CCP antibodies test has an important prognostic value in the monitoring of articular radiologically detectable damage. The kits quantitative determination is useful in the control and verification of the effects of pharmacological therapy.

The Anti CCP antibody test, together with the determination of RF, increases the ratio of sensitivity/specificity. 20% of the RAs are RF-negative and 15/20% of the RAs are positive only to RF. The simultaneous positive result of a sample to RF and CCP has a positive predictive value of about 100%.

The levels of Anti CCP antibodies are not necessarily correlated to the evolutionary stage of the illness. The advantage of the Anti CCP antibodies is that they are detectable in the patient sera up to 10 years prior to the appearance of symptoms. In addition, in cases of early arthritis a positive test result, according to some studies is related to the development of bone erosive lesions of the articulations.

TEST PRINCIPLE

Anti CCP test is based on the binding of the antibodies present in the sample, to the cyclical citrullinated peptides absorbed on the microplate.

In the first step, the antibodies present in the standards, in the controls or in the prediluted patient samples are bound to the internal surface of the wells. After 60 minutes of incubation, the microplate is washed with a wash buffer to remove the non-reacted serum components. Then a solution of anti-human IgG conjugated with peroxidase recognizes the antibodies of class IgG bound to the immobilized antigens.

After 30 minutes of incubation the excess of enzyme conjugate, that is not specifically bound, is removed by the wash buffer. At this point, a substrate solution containing chromogenic TMB is added to the microplate. After 15 minutes of incubation the color development is stopped by adding the stop solution. The solution turns yellow at this point. The level of developed color is directly proportional to the concentration of the anti CCP IgG antibodies present in the original sample.

The concentration of the anti CCP IgG antibodies in the sample is calculated through a standard curve.

MATERIALS AND COMPONENTS

Materials provided with the test kits

1. Anti-CCP IgG Standards (5 vials, 1.0 mL each)
   STD1
   STD2
   STD3
   STD4
   STD5
2. Control (1 vial, 1,0 mL, ready to use)
   Positive Control
3. Sample Diluent (1 vial, 100 mL)
4. Conjugate (1 vial, 15 mL)
   Sheep anti human-IgG conjugate with horseradish peroxidase (HRP)
5. Coated Microplate
   (1 breakable microplate coated with cyclical citrullinated petides).
6. TMB Substrate (1 vial, 15 mL)
   H_2O_2-TMB (0.26 g/L) (avoid any skin contact)
7. Stop Solution (1 vial, 15 mL)
   Sulphuric acid 0.25M (avoid any skin contact)
8. 10X Conc. Wash Solution (1 vial, 100 mL)

Materials required but not provided

1. Distilled water.

Auxiliary materials and instrumentation

1. Automatic dispenser.

2. Microplate reader (450 nm, 620-630 nm).

Assay Preparation

Preparation of the Standards (S_1…S_5)

For Anti CCP antibodies the system of measurement is calibrated in U/mL. These units show a constant factor of 1:12 in comparison to the Standard WHO Reference W1066 for reumathoid arthritis. The Standards are ready to use and have the following concentrations:

	S_1	S_2	S_3	S_4	S_5
U/mL	1	20	40	400	2000

Once opened, the Standards are stable 6 months at 2-8ºC.

Preparation of the Sample

The samples for the determination of the Anti CCP antibodies are human serum or plasma. **All samples of serum or plasma must be prediluted 1:100 with sample diluents**; for example 10 µL of sample can be diluted with 990 µL of sample diluent.

Draw the blood through venous collection in a vacutainer and separate the serum (after clot formation) or the plasma from the cells by centrifugation.

The samples can be stored refrigerated at 2-8 °Cs for up to 3 days. For longer periods of storage, the samples should be frozen to -20°C. To avoid repeated freezing and thawing, the samples should be fractioned. Avoid the use of samples with high levels of lipids or hemolysis. The Controls are ready to use.

Preparation of the Wash Solution

Dilute the contents of each vial of the buffered wash solution concentrate (10X) with distilled water to a final volume of 1000 mL prior to use For smaller volumes respect the 1:10 dilution ratio. The diluted wash solution is stable for 30 days at 2-8º.

In concentrated wash solution it is possible to observe the presence of crystals. In this case, mix at room temperature until complete dissolution of crystals is observed. For greater accuracy dilute the whole bottle of concentrated wash solution to 1000 mL taking care also to transfer the crystals completely, then mix until the crystals are completely dissolved.

ASSAY PROCEDURE

1. **Allow all reagents to reach room temperature (22-28°C) for at least 30 minutes.** At the end of the assay store immediately the reagents at 2-8°C: avoid long exposure to room temperature.
2. Unused coated microwell strips should be released securely in the foil pouch containing desiccant and stored at 2-8°C**.**
3. To avoid potential microbial and/or chemical contamination, unused reagents should never be transferred into the original vials.
4. As it is necessary to perform the determination in duplicate in order to improve accuracy of the test results, prepare two wells for each point of the standard curve (S_1-S_5), two for each Control, two for each sample, one for Blank.

Reagent	Standard	Sample/ Control	Blank
Standard S_1-S_5	**100 µL**		
Controls		**100 µL**	
Diluted Sample		**100 µL**	
Incubate for 60 minutes at room temperature (22-28ºC). Remove the content from each well, wash the wells 3 times with 300 µL of the diluted wash solution. **Important note:** during each washing step, gently shake the plate for 5 seconds and remove excess solution by tapping the inverted plate on an absorbent paper towel. **Automatic washer:** If you use automated equipment, wash the wells at least 5 times.			
Conjugate	**100 µL**	**100 µL**	
Incubate for 30 minutes at room temperature (22-28 ºC). Remove the content from each well, wash the wells 3 times with 300 µL of diluted wash solution. **Washing:** follow the same indications of the previous point.			
TMB Substrate	**100 µL**	**100 µL**	**100 µL**
Incubate for 15 minutes in the dark at room temperature (22-28C).			
Stop Solution	**100 µL**	**100 µL**	**100 µL**
Shake the microplate gently. Read the absorbance (E) at 450 nm against a reference wavelength of 620-630 nm or against Blank within 5 minutes.			

RESULTS

Validating the Results

The samples having an OD value higher the Standards5 (2000 U/mL) should be subsequently diluted and the concentration of Anti CCP antibodies should be calculated applying the dilution factor.

Standard curve

For the Anti CCP test, the method of choice for treatment of results is a 4-parameter-fit with axes Lin-Log for optical density and concentration, respectively. Also, it is possible to use a smoothed spline approximation and coordinated Lin-Log. However, it is recommended to use a Lin-Log curve.

First calculate the average optical density with standards. Use a sheet of paper with Lin-Log axes and plot averaged optical density of each standards versus their concentration. Draw the best fitting curve approximating the path of all standard points. The standard points may also be connected with straight line segments. The concentration of unknown ay then be estimated from the standard curve by interpolation.

Typical results (only as an example)
The below reported table shows the typical results for the Anti CCP test. The data are to be considered as example only and not be used for the calculation of the results.

N	OD1	OD2	Mean	U/mL
STD 1	0.037	0.043	0.040	1
STD2	0.304	0.285	0.295	20
STD3	0.514	0.551	0.533	40
STD4	1.771	1.589	1.680	400
STD5	2.631	2.284	2.458	2000
Patient 1	1.024	1.019	1.022	103

REFERENCE VALUES

In a study of the normal values performed with samples of serum from healthy donors, the followings intervals of normality with the Anti CCP IgG test have been determined.

Anti CCP (U/mL)	
Normal	< 30
Positive	≥ 30

Please pay attention to the fact that the determination of a range of expected values for a "normal" population in a given method is dependent on many factors, such as specificity and sensitivity of the method used and type of population under investigation. Therefore, each laboratory should consider the range given by the Manufacturer as a general indication and produce their own range of expected values based on the indigenous population where the laboratory operates.

The positive results should be verified relative to the clinical state of the patient. In addition, every decision related to the therapy should be taken on an individual patient basis. Each laboratory should establish its own normal and pathological intervals of serum Anti CCP.

Sensitivity / Specificity
The obtained results have shown a 79% clinical sensitivity and a 97% specificity for the diagnosis of rheumatoid arthritis.

Detection Limit
The lowest concentration of anti CCP that can be distinguished from Standard zero is 1.12 U/mL with a 98% confidence limit.

Intra-assay and inter-assay
Intra-Assay
The within run variation was determined by 12 replications of two different sera with values in the range of standard curve. The within assay variability is ≤ 5.5%.

Inter-Assay.
The between run variation was determined by replicating the measurements of one control serum with different lots of kits and/ or different mix of lots of reagents. The between assay variability is ≤ 6.8%.

WASTE MANAGEMENT
Reagents must be disposed off in accordance with local regulations.

PRECAUTION

1. Please adhere strictly to the sequence of pipetting steps provided in this protocol. The performance data represented here were obtained using specific reagents listed in this Instruction For Use.
2. All reagents should be stored refrigerated at 2-8 ºC in their original container. Any exceptions are clearly indicated. The reagents are stable until the expiry date when stored and handled as indicated.
3. Allow all kit components and specimens to reach room temperature (22-28 ºC) and mix well prior to use.
4. Do not interchange kit components from different lots. The expiry date printed on box and vials labels must be observed. Do not use any kit component beyond their expiry date.
5. **WARNING: the conjugate reagent is designed to ensure maximum dose sensitivity and may be contaminated by external agents if not used properly:** therefore, it is recommended to use disposable consumables (tips, bottles, trays, etc.) For divided doses, take the exact amount of conjugate needed and do not re-introduce any waste product into the original bottle. In addition, **for doses dispensed with the aid of automatic and semi-automatic devices,** before using the conjugate, it is advisable to clean the fluid handling system, ensuring that the procedures of washing, deproteinization and decontamination are effective in avoiding contamination of the conjugate; this procedure is highly recommend when the kit is processed using analyzers which are not equipped with disposable tips.
   For this purpose, Diagnostic Automation, Inc. supplies a separate decontamination reagent for cleaning needles
6. If you use automated equipment, the user has the responsibility to make sure that the kit has been appropriately tested.
7. The incomplete or inaccurate liquid removal from the wells could influence the assay precision and/or increase the background. To improve the performance of the kit on automatic systems is recommended to increase the numbers of washes.
8. It is important that the time of reaction in each well is held constant for reproducible results. Pipetting of samples should not extend beyond ten minutes to avoid assay drift. If more than 10 minutes are needed, follow the same order of dispensation. If more than one plate is used, it is recommended to repeat the dose response curve in each plate.
9. Addition of the TMB Substrate solution initiates a kinetic reaction, which is terminated by the addition of the Stop Solution. Therefore, the TMB Substrate and the Stop Solution should be added in the same sequence to eliminate any time deviation during the reaction.
10. Observe the guidelines for performing quality control in medical laboratories by assaying controls and/or pooled sera.
11. Maximum precision is required for reconstitution and dispensing of the reagents.
12. Samples which are microbiologically contaminated, highly lipemeic or haemolysed should not be used in the assay.
13. Plate readers measure vertically. Do not touch the bottom of the wells.

WARNINGS

1. This kit is intended for in vitro use by clinical professional only. Not for internal or external use in Humans or Animals.
2. Use appropriate personal protective equipment while working with the reagents provided.
3. Follow Good Laboratory Practice (GLP) for handling blood products.
4. All human source material used in the preparation of the reagents has been tested and found negative for antibody to HIV 1 & 2, HbsAg, and HCV. No test method however can offer complete assurance that HIV, HBV, HCV or other infectious agents are absent. Therefore, Standards and Controls should be handled in the same manner as potentially infectious material.
5. Material of animal origin used in the preparation of the kit has been obtained from animals certified as healthy and the bovine protein has been obtained from

countries not infected by BSE, but these materials should be handled as potentially infectious.

6. Some reagents contain small amounts of Sodium Azide (NaN3) or Proclin 300R as preservatives. Avoid the contact with skin or mucosa.
7. Sodium Azide may be toxic if ingested or absorbed through the skin or eyes; moreover it may react with lead or copper plumbing to form potentially explosive metal azides. If you use a sink to remove the reagents, allow scroll through large amounts of water to prevent azide build-up.
8. The TMB Substrate 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.
9. The Stop Solution consists of a diluted sulphuric acid solution. Sulphuric acid is poisonous and corrosive and can be toxic if ingested. To prevent chemical burns, avoid contact with skin and eyes.
10. Avoid the exposure of reagent TMB/H_2O_2 to directed sunlight, metals or oxidants. Do not freeze the solution.

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ISO 13485
ISO 9001

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Date Adopted	Cat # 3175-17
2015-01-01	AccuDiag™- Anti CCP ELISA -2015
EC REP	CEpartner4U, Esdoornlaan 13, 3951DB Maarn. The Netherlands. www.cepartner4u.eu
Revision D Date: 2015-10-07	