Anti CCP ELISA

GWB-521226

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FOR RESEARCH USE ONLY

Enzyme immunoassay for the quantitative determination of anti CCP in human serum or plasma

1. INTRODUCTION

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 Rheumatoid Factor (RF) antibodies of class 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 kit's 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.

2. INTENDED USE

Anti-CCP kit is an immunoenzymatic test (ELISA), intended for the quantitative determination of IgG class antibodies directedagainst cyclic citrullinated peptides, present in human serum or plasma.Anti CCP kit is intended for laboratory use only.

3. PRINCIPLE OF THE ASSAY

The anti-CCP IgG test is based on the binding of the antibodies present in the sample, to the cyclical citrullinated peptides absorbed on the microplate. Any present antibodies in calibrators, controls or prediluted patient samples bind to the inner surface of the wells. After a 60 minutes incubation the microplate is washed with wash buffer for removing non-reactive Serum or Plasma components. An anti-human-IgG horseradish peroxidase conjugate solution recognize IgG class antibodies bound to the immobilized antigens. After a 30 minutes incubation any excess enzyme conjugate, which is not specifically bound is washed away with was buffer. A chromogenic substrate solution containing TMB is dispensed into the wells. After 15 minutes of incubation the color development is stopped by adding the stop solution. The solution color change into yellow. The amount of color is directly proportional to the concentration of IgG antibodies present in the original sample. The concentration of anti CCP IgG antibodies in the sample is calculated through a calibration curve.

4. MATERIALS

4.1. Reagents supplied

Anti CCP Coated Wells: 12 breakapart 8-well snap-off strips coated with human CCP; in resealable aluminium foil.
Stop Solution: 1 bottle containing 15 ml sulphuric acid, 0.25 mol/l (avoid any skin contact), ready to use
Conjugate: 1 bottle containing 15 ml with anti h-IgG conjugated with horseradish peroxidase (HRP)
TMB Substrate Solution: 1 bottle containing 15 ml 3, 3´, 5, 5´-tetramethylbenzidine (H_2O_2-TMB 0.26 g/l) (avoid any skin contact), ready to use
Sample diluent: 1 bottle containing 100ml, Phosphate buffer
Wash solution: 1 bottle containing 50 ml (10x conc.)
anti-CCP Standards: 5 bottles, 1.0 ml each, ready to use
Standard 1: 1 U/ml
Standard 2: 20 U/ml
Standard 3: 40 U/ml
Standard 4: 400 U/ml
Standard 5: 2000 U/ml
Positive Control: 1 bottle containing 1.0 ml, ready to use

4.2. Materials supplied

1 Strip holder
1 Cover foils
1 Test protocol
1 Distribution and identification plan

4.3. Materials and Equipment needed

ELISA microwell plate reader, equipped for the measurement of absorbance at 450 nm
Manual or automatic equipment for rinsing wells
Pipettes to deliver volumes between 10 and 1000 µl
Vortex tube mixer
Distilled water
Disposable tubes
Timer

5. STABILITY AND STORAGE

The reagents are stable up to the expiry date stated on the label when stored at 2...8 °C in the dark.

6. REAGENT PREPARATION

It is very important to bring all reagents, samples and standards to room temperature (22…28°C) before starting the test run!

6.1. Coated snap-off Strips

The ready to use break apart snap-off strips are coated with human CCP. Store at 2…8 °C. Open the bag only when it is at room temperature. *Immediately after removal of strips, the remaining strips should be resealed in the aluminium foil along with the desiccant supplied and stored at 2…8 °C; stability until expiry date. Do not remove the adhesive sheets on the unused strips.*

6.2. anti-CCP Standards

For anti-CCP antibodies the system of measurement is calibrated in express arbitrary relative units, U/mL. These units show a constant factor of 1:12 in comparison to the Standard WHO Reference W1066 for reumathoid arthritis. The standards have the following approximate concentrations:

	S1	S2	S3	S4	S5
U/mL	1	20	40	400	2000

6.3. TMB Substrate Solution

The bottle contains 15 ml of a tetramethylbenzidine/hydrogen peroxide system. The reagent is ready to use and has to be stored at 2...8°C in the dark. *The solution should be colourless or could have a slight blue tinge. If the substrate turns into blue, it may have become contaminated and should be thrown away*.

6.4. Stop Solution

The bottle contains 15 ml 0.15 M sulphuric acid solution (R 36/38, S 26). This ready to use solution has to be stored at 2...8°C.

6.5. Wash Solution

Before use, dilute the content of the bottle of the "10X Conc. Wash Solution" with distilled water to 1,000 mL. To prepare smaller volumes keep the dilution ratio of 1:10. The diluted washing solution is stable at 2-8°C for at least 30 days. Crystals may be observed in the concentrated wash solution; in this case shake at room temperature to complete dissolution of the crystals. For increased precision, dilute the entire bottle of concentrated wash solution to 1,000 mL, taking care also to transfer crystals completely, then mix until crystals are completely dissolved.

7. SPECIMEN COLLECTION AND PREPARATION

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, which can be performed by adding 10 ul of sample into 990 ul of dilution buffer. 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 lipidsor hemolysis .The Controls are ready to use.

8. ASSAY PROCEDURE

8.1. Test Preparation

Please read the test protocol carefully **before** performing the assay. Result reliability depends on strict adherence to the test protocol as described. Prior to commencing the assay, the distribution and identification plan for all specimens and controls should be carefully established on the result sheet supplied in the kit. Select the required number of microtiter strips or wells and insert them into the holder. Please allocate at least:

1 well (e.g. A1) for the substrate blank 2 wells (e.g. B1+C1) control
negative 2 wells (e.g. D1+E1) for standard 1 2 wells (e.g. F1+G1)
for standard 2 2 wells (e.g. H1+A2) for standard 3 2 wells (e.g.
B2+C2) for standard 4 2 wells (e.g. D2+E2) for standard 5

Reagent	Standard	Sample or Control	Blank
Standard S1-S5	100 µL		
Controls		100 µL	
Diluted Sample		100 µL	
Incubate for 60 minutes at room temperature (22-28°C). Remove the content from each well, wash the wells three times with 300 µL of the diluted wash solution.			
Conjugate	100 µL	100 µL	
Incubate for 30 minutes at room temperature (22-28°C). Remove the content from each well, wash the wells three times with 300 µL of diluted wash solution.			
TMB substrate	100 µL	100 µL	100 µL
Incubate for 15 minutes in the dark at room temperature (22-28°C).			
Stop solution	100 µL	100 µL	100 µL
Shake the microplate gently. Read the absorbance (E) at 450 nm against Blank within 30 minutes from addition of the stop solution.			

9.1. Validating the Results

The samples having an OD value higher the Standard 5 should be subsequently diluted and the concentration of anti-CCP antibodies should be calculated applying the dilution factor.

9.2. 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 calibrators. Use a sheet of paper with Lin-Log axes and plot averaged optical density of each calibrator versus their concentration. Draw the best fitting curve approximating the path of all calibrator points. The calibrator points may also be connected with straight line segments. The concentration of unknowns may then be estimated from the calibration curve by interpolation.

Typical results (to consider 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
STD1	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

10. SPECIFIC PERFORMANCE CHARACTERISTICS

10.1.Precision and reproducibility

Intraasay:Within run variation was determined by replicate 12 times two different sera with values in the range of standard curve. The withinassay variability is ≤5.5%

Interassay:Between run variation was determined by replicate the measurements of one control serum with different lots of kits and/or differentmix of lots of reagents. The between assay variability is ≤6.8%.

10.2. Detection Limits

The lowest concentration of anti CCP that can be distinguished from the standard zero is approximately 1.12 U/mL with 98% confidence limit.

10.3. Analytic Sensitivity / Specificity

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

11. PRECAUTIONS AND WARNINGS

WARNINGS

This kit is intended for research use by professional persons only. Use appropriate personal protective equipment while working with the reagents provided. All human source material used in the preparation of standards and controls for this product 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, the Standard and the Controls should be handled in the same manner as potentially infectious material. 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. Some reagents contain small amounts of Sodium Azide (NaN3) or Proclin 300R as preservatives. Avoid the contact with skin or mucosa. 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. 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. 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. Avoid the exposure of reagent TMB/H2O2 to directed sunlight, metals or oxidants.

PRECAUTIONS

Please adhere strictly to the sequence of pipetting steps provided in this protocol. All reagents should be stored refrigerated at 2-8°C in their original container. Any exceptions are clearly indicated. Allow all kit components and specimens to reach room temperature (22-28°C) and mix well prior to

use. Do not interchange kit components from different lots. The expiry dates printed on the labels of the box and of the vials must be observed. Do not use any kit component beyond their expiry date. 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 recommended when the kit is processed using analyzers which are not equipped with disposable tips.If you use automated equipment is your responsibility to make sure that the kit has been appropriately tested. The incomplete or inaccurate liquid removal from the wells could influence the assay precision and/or increase the back ground.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 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. Observe the guidelines for performing quality control in medical laboratories by assaying controls and/or pooled sera. Maximum precision is required for reconstitution and dispensation of the reagents. Samples microbiologically contaminated should not be used in the assay. Highly lipemeic or haemolysed specimens should similarly not be used Plate readers measure vertically. Do not touch the bottom of the wells.

12.1. Disposal Considerations

Residues of chemicals and preparations are generally considered as hazardous waste. The disposal of this kind of waste is regulated through national and regional laws and regulations. Contact your local authorities or waste management companies which will give advice on how to dispose hazardous waste.

13. LITERATURE

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4 del Val del Amo N, Ibanez Bosch R, Fito Manteca C, et al. Anti-cyclic citrullinated peptide antibody in rheumatoid arthritis: relation with disease aggressiveness. Clin Exp Rheumatol. 2006;24(3):281-6

5 Samanci N, Ozdem S, Akbas H, et al. Diagnostic value and clinical significance of anti-CCP in patients with advancedrheumatoid arthritis. J Natl Med Assoc. 2005;97(8):1120-6

6 Matsui T, Shimada K, Ozawa N, et al. Diagnostic utility of anti-cyclic citrullinated peptide antibodies for very early rheumatoid arthritis. J Rheumatol. 2006;33(12):2390-7

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8 Kroot EJ, De Jong BA, Van Leeuwen MA, Swinkels H, Van den Hoogen FH, Van't Hof M, et al. The prognostic value of anti-cyclic citrullinate peptide antibody in patients with recent-onset rheumatoid arthritis. Arthritis Rheum 2000; 43: 1831-5.

SCHEME OF THE ASSAY

Anti-CCP

Test Preparation

Prepare reagents and samples as described.Establish the distribution and identification plan for all specimens and controls on the resultsheet supplied in the kit.

Select the required number of microtiter strips or wells and insert them into the holder.

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Stop solution	100 µL	100 µL	100 µL
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