ALTA CALIDAD EN PRUEBAS PARA SU LABORATORIO

MICROWELL ELISA

Anti-CCP

Summary of Assay Procedure

Step	(20-25°C Room temp.)	Volume	Incubation time
1	Sample dilution 1:100 = 5 µl / 500 µl		
2	Standards, controls & diluted samples	100 µl	30 minutes
3	Washing buffer (3 times)	350 µl	
4	Enzyme conjugate	100 µl	30 minutes
5	Washing buffer (3 times)	350 µl	
6	TMB Chromogenic Substrate	100 µl	15 minutes
7	Stop solution	100 µl	
8	Reading OD 450 nm		

Name and Intended Use

Anti-CCP ELISA is intendend for the quantitative measurement of IgG class autoantibodies against cyclic citrullinated peptides present in human serum or plasma. It is intended for laboratory use only.

Summary and Explanation of the Test

Rheumatoid arthritis (RA) is an inflammatory rheumatic disorder with a worldwide prevalence of about 0.5-1%. Although the course of RA varies widely among affected individuals, significant number of RA patients present with persistent pain and stiffness, progressive joint destruction, functional decline and premature mortality.

The serum of RA patients contains a variety of antibodies directed against self-antigens. The most widely known of these autoantibodies is the rheumatoid factor (RF) antibody directed against the constant domain of IgG molecules. The presence of RF is one of the American College of Rheumatology's (ACR) criteria for the classification of RA. Although the RF test has good sensitivity for RA, it is not very specific for the disease as it can also be detected in the serum of patients with other rheumatic or inflammatory diseases and even in a substantial percentage of the healthy (elderly) population. For several years it has been recognized that antibodies to anti-perinuclear factor (APF) and anti-keratin (AKA) are highly specific for RA. It was subsequently reported that both of these antibodies reacted with native filaggrin and are now referred to as anti-filaggrin antibodies (AFA). More recently it has been shown that all of these antibodies are directed to citrulline-containing epitopes. 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; In contrast, 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 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 advantage of CCP antibodies is a higher sensitivity and specificity for the diagnosis of rheumatoid arthritis in comparison to the rheumatoid factors alone. Anti-CCP is often found at a very early state of the disease and it has a high predictive value for development of the disease.

Principle of the Test

Purified cyclic citrullinated peptides (CCP) is coated on the surface of microwells. Diluted patient serum is added to wells, and the specific antibody, if present, will binds to the antigen coated on the surface of the reaction well. All unbound materials are washed away. After adding enzyme conjugate, it binds to the antibody-antigen complex. Excess enzyme conjugate is washed off, and TMB Chromogenic Substrate is added. The enzyme conjugate catalytic reaction is stopped at a specific time. The intensity of the color generated is proportional to the amount of anti-CCP IgG antibodies in the sample. The concentration of the anti-CCP IgG antibodies in the sample is calculated through a standard curve.

Material Provided

1. Microwell strips: Cyclical citrullinated peptides coated wells (12 x 8 wells)
2. Sample diluent: 1 bottle (50 ml)
3. Standard set: 1 ml/ vial
   0, 10, 20, 50, 150 and 500 U/ml, in liquid form (ready to use)
4. Control set: 1 ml/ vial
   Range stated on label, in liquid form (ready to use)
6. Washing Concentrate 20x (H). 1 bottle (50 ml)
7. Enzyme Conjugate: Red color solution. 1 vial (12 ml)
8. TMB Chromogenic Substrate: Amber bottle. 1 vial (12 ml)
9. Stop Solution. 1 vial (12 ml)

Storage and Stability

1. Store the kit at 2 - 8 ^{o}C.
2. Always keep microwells tightly sealed in pouch with desiccants. We recommend you use up all wells within 4 weeks after initial opening of the pouch.
3. The reagents are stable until expiration of the kit when stored at 2 - 8 ^{o}C.
4. Do not expose test reagents to heat, sun or strong light during storage or usage.

Warnings and Precautions

1. Potential biohazardous materials:
   The standards and controls contain human source components which have been tested and found nonreactive for hepatitis B surface antigen as well as HIV antibody with FDA licensed reagents. However, as there is no test method that can offer complete assurance that HIV, Hepatitis B virus or other infectious agents are absent, these reagents should be handled at the Biosafety Level 2, as recommended in the Centers for Disease Control/National Institutes of Health manual, "Biosafety in Microbiological and Biomedical Laboratories." 1984
2. Do not pipette by mouth. Do not smoke, eat, or drink in the areas in which specimens or kit reagents are handled.
3. Do not interchange kit components from different lots and products.
4. Do not re-use microplate wells
5. All materials must be at room temperature (20-25 ^{o}C) and gently mix prior to use.
6. This product contains components preserved with sodium azide. Sodium azide may react with lead and copper plumbing to form explosive metal azide. On disposal, flush with a large volume of water.

Specimen Collection and Handling

1. Collect blood specimens and separate the serum.
2. Test serum should be clear and non-hemolyzed.
3. Specimens may be refrigerated at 2 - 8 ^{o}C for up to five days or frozen for up to six months. Avoid repetitive freezing and thawing of serum sample.

Preparation for Assay

1. Prepare 1x washing buffer.

   Prepare washing buffer by adding distilled or deionized water to 20x (H) wash concentrate to a final volume of 1 liter.

2. Sample dilution

   Prepare 1:100 dilutions by adding 5 µl of the patient samples to 500 µl of sample diluent. Mix well.

Assay Procedure

1. Place the desired number of coated strips into the holder.
2. Dispense 100 µl of standards, controls and pre-diluted patient samples into the appropriate wells. For the reagent blank, dispense 100 µl sample diluent in 1A well position. Tap the holder to remove air bubbles from the liquid and mix well. Incubate for 30 minutes at room temperature.
3. Remove liquid from all wells. Repeat washing three times with washing buffer.
4. Dispense 100 µl of enzyme conjugate to each well and incubate for 30 minutes at room temperature.
5. Remove enzyme conjugate from all wells. Repeat washing three times with washing buffer.
6. Dispense 100 µl of TMB Chromogenic Substrate to each well and incubate for 15 minutes at room temperature.
8. Add 100 µl of Stop solution to stop reaction.

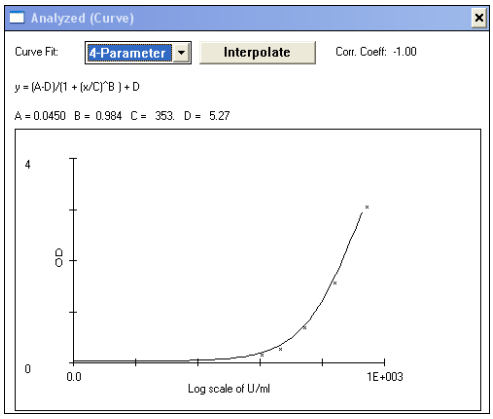
Make sure there are no air bubbles in each well before reading

9. Read O.D. at 450 nm with a microwell reader.

Calculation of Results

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

	U/ml	OD (450nm)	OD (450nm)	Average OD
Standard 1	0	0.050	0.051	0.051
Standard 2	10	0.197	0.196	0.197
Standard 3	20	0.321	0.320	0.328
Standard 4	50	0.728	0.727	0.728
Standard 5	150	1.612	1.610	1.611
Standard 6	500	3.100	3.090	3.095



Quality Control

The test run may be considered valid provided the following criteria are met:
1. The O.D. value of the reagent blank against air from a microwell reader should be less than 0.150.
2. If the O.D. value of the standard 6 is lower than 1.0, the test is not valid and must be repeated.
3. The concentration of controls should be in the range stated on the labels.
4. The samples having an OD value higher the Standard 6 (500 U/mL) should be subsequently diluted and the concentration of Anti CCP antibodies should be calculated applying the dilution factor.

Interpretation

In a normal range study with samples from 183 healthy blood donors the following ranges have been established with this ELISA assay:

Cut-off: 10 U/ml
Negative: < 10 U/ml
Positive: ≥ 10 U/ml

Linearity

Patient samples containing high levels of specific antibody were serially diluted in sample diluent to demonstrate the dynamic range of the assay and the upper/ lower end of linearity.

Sample	Diluetion	Observed U/ml	Expected U/ml	O/E %
1	1:100	353.6	350	101%
	1:200	151.7	175	87%
	1:400	89.2	87.5	102%
	1:800	45.24	43.75	103%
	1:1600	21.76	21.88	99%
2	1:100	291.4	300	97%
	1:200	150.5	150	100.3%
	1:400	71.79	75	96%
	1:800	37.37	37.5	99.7%
	1:1600	19.38	18.75	103%

Detection Limit

The analytical sensitivity (lower detection limit, 0 + 2SD) was established to be 1.9 U/ml.

Reproducibility

Intra-assay Precission:
Coefficient of variation (CV) was calculated for each of three samples from the results of 24 determinations in a single assay. Results are shown in the table below.

Sample	Average (U/ml)	CV %
1	58.15	4.05
2	60.51	3.74
3	75.04	6.13

Inter-assay Precission:
Coefficient of variation (CV) was calculated for each of three samples from the results of 4 determinations in 5 different assay. Results are shown in the table below.

Sample	Average (U/ml)	CV %
1	5.58	2.61
2	47.51	1.84
3	132.93	3.52

Performance Characteristics

Sensitivity and Specificity:
Sensitivity, specificity and accuracy were evaluated using a commercial available ELISA kit on 191 specimens. The correlation results are summarized in the following table:

		Reference ELISA		
		N	P	Total
MICROWELL ELISA	N	183(D)	2 (B)	185
	P	0 (C)	6 (A)	6
	Total	183	8	191

Sensitivity = A / (A+B)= 6/(6+2)=75%

Specificity = D/(C+D) = 183/183 = 100%

Accuracy (Overall agreement) = (A+D) / (A+B+C+D) = 189/191=98.9%

High Dose Hook

High dose hook is a phenomenon whereby very high level specimens may read within the dynamic range of the assay. For the Anti-CCP ELISA assay, no high dose hook effect was observed when a sample containing approximately 6000 U/mL of anti-CCP antibody was assayed.

Interference

Potential Interfering Substance	No Interference Found up to the Following Concentration
Bilirubin	40 mg/dl
Hemoglobin	200 mg/dl

Limition of Procedure

This assay is a diagnostic aid. A definite clinical diagnosis should not be based on the results of a single test, but should be made by the physician after all clinical and laboratory findings have been evaluated concerning the entire clinical picture of patient. Also every decision for therapy should be taken individually.
The above pathological and normal reference ranges for antibodies in patient samples should be regarded as recommendations only. Each laboratory should establish its own ranges according to an applicable laboratory guidelines.

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