Anti-CCP

Intended use
The Anti-CCP IgG assay is an enzyme-linked immunosorbent assay (ELISA) for the in vitro quantitative determination of cyclic citrullinated peptide antibodies (Anti-CCP) of IgG type in human serum or plasma.

Summary
Cyclic citrullinated peptide antibodies (CCP) are autoantibodies (antibodies to an individual's own proteins) that are directed against peptides and proteins that are citrullinated. They are present in the majority of patients with rheumatoid arthritis. Clinically, cyclic citrullinated peptides (CCP) are frequently used to detect these antibodies in patient serum or plasma (then referred to as anti–citrullinated peptide antibodies).
During inflammation, arginine amino acid residues can be enzymatically converted into citrulline residues in proteins such as vimentin, by a process called citrullination. If their shapes are significantly altered, the proteins may be seen as antigens by the immune system, thereby generating an immune response.
Anti-CCP have proved to be powerful biomarkers that allow the diagnosis of rheumatoid arthritis (RA) to be made at a very early stage.

Test principle
Indirect method, total duration of assay: 70 minutes.
The Anti-CCP IgG ELISA employs solid phase, indirect ELISA method for detection of antibodies to Anti-CCP IgG in two-step incubation procedure. Polystyrene microwell strips are pre-coated with highly immunoreactive human Anti-CCP antigens. During the first incubation step, anti-Anti-CCP IgG specific antibodies, if present, will be bound to the solid phase pre-coated Anti-CCP antigens. The wells are washed to remove unbound serum proteins, and rabbit anti-human IgG antibodies (anti-IgG) conjugated to the enzyme horseradish peroxidase (HRP-Conjugate) are added. During the second incubation step, these HRP-conjugated antibodies will be bound to any antigen-antibody(IgG) complexes previously formed and the unbound HRP- conjugate is then removed by washing. Chromogen solutions containing Tetramethylbenzidine (TMB) and urea peroxide are added to the wells and in presence of the antigen-antibody-anti-IgG (HRP) immunocomplex, the colorless Chromogens are hydrolyzed by the bound HRP conjugate to a blue-colored product. The blue color turns yellow after stopping the reaction with sulfuric acid. The amount of color intensity can
be measured and it is proportional to the amount of antibody captured in the wells, and to the amount of antibody in the sample respectively.

Reagents
Materials provided
• **Coated Microplate** - symbol CCP PLATE , 8 x 12 strips, 96 wells. Pre-coated with human TG antigen.
• **Calibrators** - symbol CCP CAL A-F , 6 vials, 1 mL each, ready to use; Concentrations: 0(A), 10(B), 25(C), 50(D), 150(E) and 300(F) U/mL* *'s reference value
• **Enzyme Conjugate** - symbol CCP CONJ , 1 vial, 11.0 mL of HRP (horseradish peroxidase) labeled rabbit anti-human IgG antibodies (anti-IgG) in Tris-NaCl buffer containing BSA (bovine serum albumin). Contains 0.1% ProClin300 preservative.
• **Sample Diluent** - symbol CCP DILUT 1 vial, 11mL. Ready to use. Containing buffer salts and a dye
• **Substrate** - symbol SUBSTRATE 1 vial, 11mL, ready to use, (tetramethylbenzidine) TMB.
• **Stop Solution** - symbol STOP 1 vial, 6.0 mL of 1 mol/L sulfuric acid.
• **Wash Solution Concentrate** - symbol WASH 40X 1 vial, 25 mL (40X concentrated), PBS-Tween wash solution.
• **IFU:** 1 copy.
• **Plate Lid:** 1 piece.

Materials required (but not provided)
• Microplate reader with 450nm and 620nm wavelength absorbent capability.
• Microplate washer.
• Incubator.
• Plate shaker.
• Micropipettes and multichannel micropipettes delivering 50 µl with a precision of better than 1.5%.
• Absorbent paper.
• Distilled water

Precautions and warnings
• For in vitro diagnostic use only.
• Exercise the normal precautions required for handling all laboratory reagents.
• Disposal of all waste material should be in accordance with local guidelines.
• Do not use reagents beyond the labeled expiry date.
• Do not mix or use components from kits with different batch codes.
• All the specimen and reaction wastes should be considered potentially biohazard. The handling of specimens and reaction wastes should be in accordance with the local regulations and guidelines.
• The Stop Solution contains sulfuric acid, which can cause severe burns. In the event of contact with eyes, rinse immediately with plenty of water and seek medical advice.´
• Neutralized acids and other liquid waste should be decontaminated by adding a sufficient volume of sodium hypochlorite to obtain a final concentration of at least 1.0%. Exposure to 1.0% sodium hypochlorite for 30 minutes may be necessary to ensure effective decontamination.
• Some reagents contain 0.05% or 0.1% ProClin 300 which may cause sensitization by skin contact, which must therefore be avoided. Reagents and their containers must be disposed of safely. If swallowed, seek medical advice immediately and show this container or label.
• Substrate has an irritant effect on skin and mucosa. In case of possible contact, wash eyes with an abundant volume of water and skin with soap and abundant water. Wash contaminated objects before reusing them. If inhaled, take the person to open air.
• For information on hazardous substances included in the kit please refer to the Materials Safety Data Sheet (MSDS), which is available on request.
• Do not smoke, drink, eat or apply cosmetics in the work area.
• Do not pipette by mouth. Wear protective clothing, disposable gloves and eye/face protection when handling samples and reagents. Wash hands after use.

Storage and stability
• Store at 2-8°C.
• Don't freeze.
• Seal and return unused reagents to 2-8°C, under which conditions the stability will be retained for 2 months, or until the labeled expiry date, whichever is earlier.

Specimen collection and preparation
• Human serum or plasma is recommended for this assay.
• Cap and store the samples at 18-25 °C for no more than 8 hours. Stable for 7 days at 2-8°C, and 1 month at -20°C. Freeze only once.
• Do not use heat-inactivated samples.
• Sediments and suspended solids in samples may interfere with the test result which should be removed by centrifugation. Ensure that complete clot formation in serum samples has taken place prior to centrifugation.
• Avoid grossly hemolytic, lipemic or turbid samples.

Wash solution (40X dilution)
Add deionized water to the 40X concentrated Wash Solution Concentrate. Dilute 25 mL of Wash Solution Concentrate with 975 mL of deionized water to a final volume of 1000 mL. Stable for 2 months at room temperature.

Quality control
Each laboratory should have assay controls at levels in the low, normal, and elevated range for monitoring assay performance. The controls should be treated as unknowns and values determined in every test procedure performed. The recommended controls requirement for this assay are to purchase trueness control materials separately and test them together with the samples within the same run. The result is valid if the control values fall within the concentration ranges printed on the labels.

Test procedure
Ensure the patients' samples and reagents are at ambient temperature (18-25 °C) before measurement. Mix all reagents through gently inverting prior to use.
• Use only the number of wells required and format the microplate wells for each calibrator and sample to be assayed.
• Add 100 µL of calibrators to each well.
• Add 100 µL of Sample Diluent (Green color) to each well Except the Calibrator-wells.
• Add 10 µL of Sample to each Sample Diluent well (NOTE: Reagents in Wells will turn Blue color from Green), then shake 30 seconds.
• Cover the plate with a plate lid and incubate at 37 °C for 30 minutes.
• Discard the contents of the micro plate by decantation or aspiration. If decanting, tap and blot the plate dry with absorbent paper.
• Add 350 µL of wash solution, decant (tap and blot) or aspirate. Repeat 4 additional times for a total of 5 washes. At the end of washing, invert the plate and tap out any residual wash solution onto absorbent paper.
• Add **100 µL** of **enzyme conjugate**,
• Cover the plate with a plate lid and incubate at **37 °C for 30 minutes**.
• Add **350 µL** of **wash solution**, decant (tap and blot) or aspirate. Repeat 4 additional times for a total of **5 washes**. At the end of washing, invert the plate and tap out any residual wash solution onto absorbent paper.
• Add 100 µL of Substrate to each well. Cover and incubate at ambient temperature (18-25°C) in the dark for reaction for 10 minutes. Do not shake the plate after substate addition.
• Add 50 µL of stop solution to each well.
• Shake for 15-20 seconds to mix the liquid within the wells. It is important to ensure that the blue color changes to yellow completely.
• Read the absorbance of each well at 450 nm (using 620 to 630 nm as the reference wavelength to minimize well imperfections) in a micro plate reader. The results should be read within 30 minutes of adding the stop solution.

Calculation
• Record the absorbance obtained from the printout of the microplate reader.
• Calculate the mean absorbance of any duplicate measurements and use the mean for the following calculation.
• Plot the common logarithm of absorbance against concentration in U/mL for each calibrator.
• Draw the best-fit curve through the plotted points on linear graph paper. Point-to-Point method is suggested to generate a calibration curve.

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The following data is for demonstration only and cannot be used in place of data generations at the time of assay

Sample	Value (U/mL)	Absorbance
Calibrator A	0	0.014
Calibrator B	10	0.117
Calibrator C	25	0.515
Calibrator D	50	1.094
Calibrator E	150	1.982
Calibrator F	300	2.971
Control 1	6.09	0.095
Control 2	60.47	1.187
Sample	106.64	1.597

Limitations – interference

• Assay results should be utilized in conjunction with other clinical and laboratory data to assist the clinician in making individual patient management decisions.
• The assay is unaffected by icterus (bilirubin < 427 µmol/L or < 25 mg/dL), hemolysis (Hb < 0.311 mmol/L or < 0.5 g/dL), lipemia (Intralipid < 1500 mg/dL), and biotin (< 123 nmol/L or < 30 ng/mL).
• Samples should not be taken from patients receiving therapy with high biotin doses (i.e. > 5 mg/day) until at least 8 hours following the last biotin administration.
• Interference was observed from rheumatoid factor above a concentrations of 150 U/mL.
• Autoantibodies are heterogeneous and this gives rise to non-linear dilution phenomena for certain individual samples.
• There is no high-dose hook effect at anti-CCP concentrations up to 5000 U/mL.
• For diagnostic purposes, the results should always be assessed in conjunction with the patient's medical history, clinical examination and other findings.

Measuring range

1.0-300 U/mL (defined by the lower detection limit and the maximum of the master curve). Values below the lower detection limit are reported as < 1.0 U/mL. Values above the measuring range are reported as > 300 U/mL.

Lower limits detection

1.0 U/mL
The lower detection limit represents the lowest analyte level that can be distinguished from zero. It is calculated as the value lying two standard deviations above that of the lowest standard (master calibrator, standard 1+2 SD, repeatability study, n=21).

Expected values

In an external study using the Anti-CCP assay on samples from 218 asymptomatic healthy individuals, 467 confirmed RA patients and 398 patients with other rheumatic and non- rheumatic disorders, an optimal cutoff of 15 U/mL was determined; samples with a concentration ≥ 15 U/mL being considered positive for anti-CCP.

Each laboratory should investigate the transferability of the expected values to its own patient population and if necessary determine its own reference ranges.

Specific performance data

Representative performance data are given below. Results obtained in individual laboratories may differ.

Precision

Precision was determined using reagents, pooled human sera, and controls in a modified protocol (EP5-A) of the CLSI (Clinical and Laboratory Standards Institute): 2 times daily for 20 days (n = 40). The following results were obtained:

		Repeatability		Intermediate precision	
Sample	Mean (U/mL)	SD (U/mL)	CV%	SD (U/mL)	CV%
Human Serum 1	6.53	0.47	7.15	0.53	8.15
Human Serum 2	58.19	3.31	5.68	3.62	6.22
Human Serum 3	174.88	10.79	6.17	11.93	6.82
Control 1	5.98	0.45	7.49	0.50	8.41
Control 2	60.58	3.73	6.16	3.98	6.57

Clinical Sensitivity and specificity

In an external study using the Anti-CCP assay on samples from 218 asymptomatic healthy individuals, 467 confirmed RA patients and 398 patients with other rheumatic and non- rheumatic disorders, an optimal cutoff of 10 U/mL was determined. At this cutoff the sensitivity was calculated to be 89.5 % with a specificity of 98.0 %.

References

1. Puszczewicz M, Iwaszkiewicz C (May 2011). "Role of anti-citrullinated protein antibodies in diagnosis and prognosis of rheumatoid arthritis". Arch Med Sci. 7 (2): 189–94. doi:10.5114/aoms.2011.22067. PMC 3258718. PMID 22291756.
2. Raptopoulou A, Sidiropoulos P, Katsouraki M, Boumpas DT (2007). "Anti-citrulline antibodies in the diagnosis and prognosis of rheumatoid arthritis: evolving concepts". Crit Rev Clin Lab Sci. 44 (4): 339–63. doi:10.1080/10408360701295623. PMID 17558653. S2CID 1773519.

CODIGO: RSET113-3