



**ImmuLisa™**  
**Cyclic Citrullinated Peptide (CCP)**  
**ELISA**

For in vitro diagnostic use

**IVD**

A microtitre plate based enzyme  
immunoassay for detection of  
Rheumatoid Arthritis specific autoantibodies  
(anti-CCP)  
Catalogue No. 8001  
For in vitro diagnostic use

**CE**

**PIE8001**

## BRIEF INFORMATION

The ImmuLisa Cyclic Citrullinated Peptide (CCP) ELISA is an enzyme immunoassay (EIA) for the in vitro detection of antibodies in human serum or plasma that react with synthetic peptides containing citrulline residues. Because of its high specificity the test can be used as an aid to the diagnosis of Rheumatoid Arthritis (RA). With this EIA kit 96 analyses can be performed. The anti-CCP ELISA can be performed as a qualitative test or a quantitative assay (using a calibration curve). Calibrators and controls are measured in duplicate; samples can be measured either singular or in duplicate. The EIA contains all the reagents required to perform the test.

The Immunoscan RA is intended for professional use within a laboratory.

## INTRODUCTION

Rheumatoid Arthritis (RA) is one of the most common systemic autoimmune diseases. The aetiology of the disease, which affects up to 1-2% of the world population, is unknown. The diagnosis of RA depends primarily on clinical manifestation of the disease. The only serological test routinely used is the determination of the presence of rheumatoid factors (RF) in the serum. RF are antibodies directed to the constant region of immunoglobulins of the IgG class. However, these antibodies are also present in relatively high percentages in other autoimmune diseases, infections and in up to 15% of healthy individuals.

Antibodies of a more specific nature have also been found in sera of RA patients (see (1) for an overview). Anti-perinuclear factor (APF) antibodies are reported to be present in around 50% of RA patients with a specificity of over 70% (2). A number of cyclic synthetic peptides not related to filaggrin or other known proteins were described which are specifically recognized by autoantibodies in sera from RA patients (3). These peptides were subsequently used in an EIA for the detection of RA-specific autoantibodies (3). Clinical evaluation studies showed that the EIA was positive in a significant number of well-defined RA patient sera with an excellent specificity against disease controls (3-8). A diagnostic and prognostic value for the measurement of the anti Cyclic Citrullinated Peptides (anti-CCP) antibodies was found in relation to joint involvement and radiological damage in early RA (7, 9-14). Anti-CCP antibodies can be detected years before the development of clinical symptoms (14). A prospective cohort study showed that 93% of the anti-CCP positive patients with undifferentiated arthritis finally developed rheumatoid arthritis, demonstrating the strong positive predictive value of these antibodies (14). The ImmuLisa CCP assay offered by IMMCO Diagnostics is based on highly purified synthetic peptides containing citrulline residues and is a valuable addition to the diagnosis of RA. This anti-CCP kit contains improved synthetic peptides selected on the basis of superior performance in the detection of RA autoantibodies (8-14).

## PRINCIPLE OF THE TEST

The anti-CCP antibody kit is based on an ELISA method. The test utilizes microtitre plate wells coated with citrullinated synthetic peptides (antigen). Diluted patient serum or plasma is applied to the wells and incubated. If specific antibodies are present, they will bind to the antigen in the wells. Unbound material is washed away and any bound antibody is detected by adding horse radish peroxidase (HRP) labelled anti-human IgG, followed by a second washing step and an incubation with substrate.

The presence of reacting antibodies will result in the development of colour, which is proportional to the quantity of bound antibody, and this is determined photometrically.

## SPECIFICITY AND SENSITIVITY

The Immulisa CCP measures antibodies against synthetic peptides with citrulline residues (anti-CCP). The anti-CCP assay is calibrated in the quantitative assay in relative units using a positive patient serum pool. The standard curve ranges from 25 -1600 units/mL. These values have been chosen arbitrarily by Euro-Diagnostica since no generally recognised (international) standard exists for expressing the titre of anti-CCP antibodies. The specificity and sensitivity were evaluated in clinical studies with 311 RA patients, 942 diseased non-RA patients (including other autoimmune and wide range of infectious diseases) and 330 healthy controls. The sensitivity was 70%. The specificity was 97% with diseased non-RA patients and 99% with healthy individuals.

## HANDLING AND STORAGE

- Store the kit at + 2° C to + 8° C in a dark place.
- Do not use reagents beyond their expiration date.
- It is advisable to unpack the sealed microtitre plate immediately before use.
- Any direct action of light on the chromogen solution should be avoided.
- If the following phenomena are observed, this may indicate a degeneration of the reagents:
  - A blue colouring of the chromogen solution before putting it into the wells.
  - A weak or absent colour reaction of the first calibrator A (1600 units/mL)  $E_{450nm} < 0,9$ .

## KIT CONTENTS

### Contents EIA-kit:

- 1 Sealed (12x8 wells) peptide-coated microtitre plate. Ready to use.
- 5 Vials containing calibrators (positive human serum pool) (1.2 mL). Ready to use (blue).
- 1 Vial containing reference control human serum (1.2 mL). Ready to use (blue).
- 1 Vial containing positive control human serum (1.2 mL). Ready to use (blue).  
(Value on label in units/mL).
- 1 Vial containing negative control human serum (1.2 mL). Ready to use (blue).
- 1 Vial containing conjugate solution (peroxidase conjugated to anti human IgG antibodies) (15 mL) Ready to use (red).
- 1 Vial containing substrate solution TMB (15 mL). Ready to use.
- 2 Vials containing dilution buffer (35 mL). Ready to use (blue).
- 1 Vial containing stop solution (15 mL). Ready to use.
- 2 Vials containing wash buffer (35 mL) 20 x concentrated.

## PRECAUTIONS

1. The stop solution contains 0.5 M sulphuric acid. Do not allow the reagent to get into contact with the skin.
2. Avoid contact of all biological materials with skin and mucous membranes.
3. Do not pipette by mouth.
4. Controls and calibrators contain serum of human origin. Although tested against and confirmed negative for HIV 1+2, HCV, HbsAg and HIV-1 Ag, this material must be treated as potentially infectious.
5. TMB (3, 3', 5, 5'-tetramethylbenzidine) is toxic by inhalation, in contact with skin and if swallowed. Observe care when handling the substrate.
6. Do not use components past the expiration date and do not intermix components from different lots.
7. Each well is ultimately used as an optical cuvette. Therefore, do not touch the under-surface of the wells and prevent damage and dirt.
8. Optimal results will be obtained by strict adherence to this protocol. Careful pipetting and washing throughout this procedure are necessary to maintain precision and accuracy.
9. Calibrators, controls and diluent buffer contain 0.09% sodium azide.

## SAMPLE PREPARATION

This test is performed on serum or citrated/heparinised plasma specimens. For serum samples collect venous blood specimens and allow clotting to completion. Store samples for a maximum of 48 hr at 4-8° C. For prolonged storage freeze at -20° C. Dilute patient sample 1:50. (Mix 10 µL sample in a tube with 490 µL dilution buffer. Use 100 µL in the test. (See assay protocol, page 7).

## PREPARATION AND HANDLING OF REAGENTS

Before beginning the test, the microtitre plate and reagents should be brought to room temperature. Do not open the plate sealing until the plate has reached room temperature. Mix reagents thoroughly before use.

The reagents included in the kit are sufficient to carry out 96 analyses (including Calibrator and control analyses).

Calibrators and controls are analysed in duplicate.

Buffer concentrates and TMB may contain salt crystals, which should be dissolved at 37° C.

1. Store all reagents immediately after use in the dark at 2-8° C.
2. Peptide-coated microtitre plate. Ready to use.  
Re-seal surplus strips in foil with desiccant and store at 2-8° C.
3. Wash buffer (35 mL):  
The wash buffer is delivered 20 times concentrated. Prepare dilutions before use.  
Add 35 mL wash buffer to 665 mL distilled water and mix thoroughly.
4. Substrate solution TMB (15 mL):  
Ready to use reagent. Keep in the dark. The substrate solution may precipitate at 4° C.  
See above.
5. Dilution buffer (35 mL): Ready to use.
6. Conjugate solution (15 mL): Ready to use.
7. Stop solution (15 mL): Ready to use.
8. Calibrator A-E (1.2 mL).  
Five diluted positive human serum calibrators, with values expressed in relative units. Calibrator A contains 1600 units/mL, B 800 units/mL, C 200 units/mL, D 50 units/mL and E 25 units/mL. Calibrators are ready to use.
9. Reference control (1.2 mL). Diluted human serum, ready to use.
10. Negative control (1.2 mL). Diluted human serum, ready to use.
11. Positive control ( 1.2 mL). Diluted human serum, ready to use.

## ASSAY PROCEDURE

### Rinsing protocol

In EIA's unbound components have to be removed efficiently between each immunological incubation step. This is achieved by appropriate rinsing. It should be clear that each rinsing procedure must be carried out with care to guarantee good results. Rinsing can be carried out manually or with automatic plate washing equipment as follows:

#### Manual rinsing

1. Empty the contents of each well by turning the microtitre plate upside down followed by a firm short vertical movement.
2. Fill all the wells with 300  $\mu$ L wash buffer.
3. This rinsing cycle (1 and 2) should be carried out 3 times.
4. Turn the plate upside down and empty the wells by a firm short vertical movement.
5. Place the inverted plate on absorbent paper towels and tap the plate firmly to remove residual washing solution in the wells.
6. Continue immediately to next reagent addition step.

#### Rinsing with automatic microtitre plate washing equipment

When using automatic plate washing equipment, check that all wells can be aspirated completely and that the wash buffer is correctly dispensed reaching the rim of each well during each rinsing cycle. The washer should be programmed to execute three rinsing cycles. Continue immediately to next reagent addition step.

#### Assay Protocol

Prepare samples according to section sample preparation, page 6 (i.e. dilute 1:50 in dilution buffer) and reagents according to preparation and handling of reagents, page x. The microtitre plate is ready to use, do not wash! Patient samples can be tested either singular or in duplicate.

#### Assay Protocol

Prepare samples according to section sample preparation, page 6 (i.e. dilute 1:50 in dilution buffer) and reagents according to preparation and handling of reagents, page x. The microtitre plate is ready to use, do not wash! Patient samples can be tested either singular or in duplicate.

#### Quantitative protocol

1. Pipette 100  $\mu$ L dilution buffer in duplicate (wells A<sub>1</sub>, A<sub>2</sub>: blank).
2. Pipette 100  $\mu$ L of each calibrator in duplicate (wells B<sub>1</sub>, B<sub>2</sub> - F<sub>1</sub>, F<sub>2</sub>).
3. Pipette 100  $\mu$ L of negative and positive control in duplicate (wells G<sub>1</sub>, G<sub>2</sub>- H<sub>1</sub>, H<sub>2</sub>).
4. Pipette 100  $\mu$ L of diluted patient samples into their respective wells of the microtitre plate. The total time for pipetting in steps 1-4 should not exceed 15 minutes.
5. Incubate for 60 min. at room temperature (18-25° C).
6. Discard the solution from the microtitre plate and wash according to the rinsing protocol.
7. Pipette 100  $\mu$ L conjugate solution into each well.
8. Incubate for 30 min. at room temperature (18-25° C).
9. Discard the conjugate solution from the microtitre plate and wash according to the rinsing protocol.
10. Pipette 100  $\mu$ L substrate solution into each well.
11. Incubate for 30 min. at room temperature (18-25° C).
12. Add 100  $\mu$ L stop solution to each well.
13. Read absorbance values immediately at 450 nm.

### Qualitative protocol

Run as described in the quantitative protocol with one exception: Replace the calibrator set (A-E) with the reference control.

### VALIDATION

For the quantitative protocol calibrator A (1600 unit/mL) should have an OD of  $>0.9$ .

Calculate the mean of duplicate wells for each calibrator and control. The value of the controls should then be calculated as in interpretation of results, see below.

The result of the positive control should be within the range stated on the label in units/mL and the negative control should be  $<25$  units/mL. If this is not achieved, the test results are not valid and the test should be repeated.

For the qualitative protocol the ratio of the positive control versus the reference control should be within the range stated on the label. The ratio of the negative control versus the reference control should be  $<1.0$ .

### INTERPRETATION OF RESULTS

#### Quantitative protocol

Subtract the mean absorbance value of the wells  $A_1$  and  $A_2$  from the individual absorbance of the wells containing the calibrators, controls and samples. The absorbance values of the five calibrators (mean values of the duplicates) can be plotted manually on the linear y-axis versus the units on a logarithmic x-axis. The calibration curve is close to linearity in the range 25-1600 units/mL. The antibody titre is expressed in units determined using the calibrator sera by reading the unit's value corresponding to the net mean absorbance of sample on the calibration curve. Alternatively, a software program using a 4-parameter curve fit can be used for the calculation.

The five calibrators (A - E) have been assigned a value of 1600 units/mL (A), 800 units/mL (B), 200 units/mL (C), 50 units/mL (D) and 25 units/mL (E). These values have been chosen arbitrarily by Euro-Diagnostica, since no generally recognised (inter)national standard exists for expressing the titre of anti-CCP antibodies. Samples reading higher than the calibrator A (1600 units/mL) can be retested at higher sample dilution. At present there is no evidence that the units obtained, can be used as a measure of the severity of the disease. Antibodies from different patients may have different affinities, which means that the autoantibody immunoreactivity rather than the concentration is measured.

#### Qualitative protocol

Subtract the mean absorbance value of the wells A<sub>1</sub> and A<sub>2</sub> from the individual absorbance of the wells containing the controls and samples.

Calculate the absorbance (optical density) ratio for the control and for each sample.

$$\text{Absorbance ratio} = \frac{\text{Control or Sample OD}}{\text{Reference control OD}}$$

#### EVALUATION CRITERIA

##### Quantitative protocol

Samples with results < 25 units/mL are defined as negative. Samples ≥ 25 units/mL are defined as positive. Patients with a result (≥ 25 units/mL < 50) are considered as weakly positive and should be re-tested at a later date.

##### Qualitative protocol

Users should calculate a cut-off between positive and negative samples that is specific to their patient populations. Results from the patient populations used in the Euro-Diagnostica clinical trial suggest the following cut-off:

Absorbance ratio	Result Interpretation
< 0.95	Negative
≥ 0.95 to ≤ 1.0	Borderline - recommend repeat testing
> 1.0	Positive

## PERFORMANCE CHARACTERISTICS

### Reproducibility

Table 1.

8 replicates of each of 3 sera were assayed to evaluate Intra-assay reproducibility.

	High		Medium		Low	
	Units/mL	OD	Units/mL	OD	Units/mL	OD
Mean	1007.4	1.918	240.1	1.179	95.7	0.686
S.D.	128.5	0.05	15.5	0.04	6.7	0.03
%C.V.	12.8	2.8	6.5	3.3	7.0	4.9

Table 2.

8 replicates of each of 3 sera were assayed on 3 lots to evaluate Inter-Lot reproducibility.

	High		Medium		Low	
	Units/mL	OD	Units/mL	OD	Units/mL	OD
Mean	1116.6	2.049	255.1	1.266	99.4	0.723
S.D.	136.6	0.10	22.8	0.08	8.7	0.05
%C.V.	12.2	5.1	8.9	6.0	8.7	6.4

Table 3.

8 replicates of each of 3 sera were assayed on 3 separate occasions to evaluate Inter-assay reproducibility.













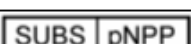

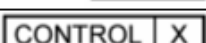
	High		Medium		Low	
	Units/mL	OD	Units/mL	OD	Units/mL	OD
Mean	1105.9	1.955	257.4	1.205	93.1	0.673
S.D.	129.5	0.05	20.2	0.04	5.6	0.04
%C.V.	11.7	2.6	7.9	3.4	6.0	6.5

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### Explanation of Symbols

	Utgångsdatum. Expiration date. Verfalldatum. Date d'expiration. Fecha de caducidad. La data di scadenza.
	Biologiskt material. Biological material. Biologisches Material. Matériel biologique. Material biológico. Il materiale biologico.
	Förvaringstemperatur. Store at. Lagerung bei. Conserver à. Almacenar a. La conservatione.
	Tillverkare. Manufacturer. Hersteller. Fabricant. El fabricante. Il fabbricante.
	Lot number. Batch number. Lot Nummer. Numéro de lot. El número de la serie. Il numero di lotto.
	Medicinteknisk produkt för in vitro diagnostik. In vitro diagnostic medical device. In Vitro diagnostische medizinische Vorrichtung. Dispositif médical pour diagnostic in vitro. En vitro artefacto médico diagnóstico. In vitro diagnostico medico congegno.
	Produkt nummer. Product number. Produktzahl. Référence. El número del producto. Il numero di prodotto.
	Se bruksanvisning. See instruction for use. Siehe Anweisung zum Gebrauch. Voir notice d'emploi. Vea la instrucción para el uso. Vedere l'istruzione per l'uso.
	Antigen. Antigene. Antigène. Antigeno. L'antigene.
	Spädningsbuffert. Diluent. Probenverdünnungspuffer. Diluant. Diluyente. Il diluente.
	Konjugat. Conjugate. Conjugué. Conjugado. Conjugato.
	Tvättbuffert 30x konc. Washsolution 30x conc. Waschpuffer 30x konc. Solution lavage conc. 30x. Solución de lavado conc. 30x. Soluzione di lavaggio 30x conc.
	Substrat pNPP. Substrate pNPP. pNPP Substrat. Sustrato pNPP. Substrato pNPP.
	Kalibrator. Calibrator. Etalon. Calibrador. Calibratore.
	Kontroll. Control. Kontrolle. Contrôle. Controllo.

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