INSTRUCTION FOR USE

IMMUNOSCAN CCPlus®

FOR RESEARCH USE ONLY.
NOT FOR USE IN DIAGNOSTIC PROCEDURES.

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REF **RA-96 PLUS RUO**

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INTENDED USE

The Immunoscan CCPlus® test kit is an enzyme-linked immunosorbent assay (ELISA) for qualitative and semi-quantitative determination of IgG antibodies to Cyclic Citrullinated Peptides (CCP) in human sera. The assay is used to detect antibodies in a single serum specimen. The results of the assay shall not be used for clinical diagnosis or patient management. The analysis should be performed by trained laboratory professionals.
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SUMMARY AND EXPLANATION

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 Immunoscan CCPlus® assay offered by Svar Life Science is based on highly purified synthetic peptides containing citrulline residues. 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 RA PEPTIDE EIA

The anti-CCP antibody kit is based on an ELISA method. The test utilizes microtitre plate wells coated with citrullinated synthetic peptides (antigen). Diluted serum sample 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.

PRECAUTIONS

FOR RESEARCH USE ONLY. Not for use in diagnostic procedures.

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. - The Centers for Disease Control and Prevention and National Institutes of Health recommended that potentially infectious agents be handled at the Biosafety Level 2.
5. TMB (3, 3', 5, 5'-tetramethylbenzidin) 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.
10. It has been reported that sodium azide may react with lead and copper in plumbing to form explosive compounds. When disposing, flush drains with water to minimize build-up of metal azide compounds.

Product Safety Data Sheet is available on request from Svar Life Science.

KIT CONTENTS

Contents kit:
- 1 Sealed (96 wells) CCP 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).
- 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.

MATERIALS OR EQUIPMENT REQUIRED BUT NOT PROVIDED

-   Microplate reader with filter 450 nm.
-   Automatic microtitre plate washer.

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 a weak or absent colour reaction of the first calibrator A (3200 U/mL) E450 nm <0.9, is observed, the test result is invalid.

SAMPLE PREPARATION

This test is performed on serum specimens. For serum samples, allow clotting to completion. Store samples for a maximum of 48 hrs at 4-8° C. For prolonged storage freeze at -20° C. Dilute sample 1:50. (Mix 10 µL sample in a tube with
490 µL dilution buffer. Use 100 µL in the test. (See assay protocol).

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 may contain salt crystals, which should be dissolved at room temperature (18-25° C).
1.   Store all reagents immediately after use in the dark at 2-8° C.
2.   CCP peptide-coated microtitre plate. Ready to use.
       Re-seal surplus wells 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.
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 3200 U/mL, B 800 U/mL, C 200 U/mL,
       D 50 U/mL and E 25 U/mL. Calibrators are ready to use.
9.   Reference control (1.2 mL). Diluted human serum, 25 U/mL, 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 µL 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, (i.e. dilute 1:50 in dilution buffer) and reagents according to preparation and handling of reagents. The microtitre plate is ready to use, do not wash! samples can be tested either singular or in duplicate.

Semi-Quantitative protocol
1. Pipette 100 µL dilution buffer in duplicate (wells A_1, A_2: blank).
2. Pipette 100 µL of each calibrator in duplicate (wells B_1, $B_2 – F_1$, F_2).
3. Pipette 100 µL of negative and positive control in duplicate (wells G_1, G_2- H_1, H_2).
4. Pipette 100 µL of diluted 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. ± 5 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 µL conjugate solution into each well.
8. Incubate for 30 min. ± 5 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 µL substrate solution into each well.
11. Incubate for 30 min. ± 5 min. at room temperature (18-25° C).
12. Add 100 µL stop solution to each well.
13. Read absorbance values within 10 min. at 450 nm.

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

QUALITY CONTROL

For the semi-quantitative protocol calibrator A (3200 U/mL) should have an OD of $\geq$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.
Please refer to positive control label for valid positive control range.Negative control should be <25 U/mL.
For the qualitative protocol, please refer to positive control label for valid ratio of positive control versus reference control.   The ratio of negative control versus reference control should be <0.95.
If any of the values are not within their respective ranges, the test should be considered invalid and it should be repeated.

INTERPRETATION OF RESULTS

Semi-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 25-2962 U/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 3200 U/mL (A),
800 U/mL (B), 200 U/mL (C), 50 U/mL (D) and 25 U/mL (E). These values have been chosen arbitrarily by Svar Life Science, since no generally recognised (inter)national standard exists for expressing the titre of anti-CCP antibodies. Samples reading higher than the calibrator A (3200 U/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 subjects may have different affinities, which means that the autoantibody immunoreactivity rather than the concentration is measured.

The calibration curve cannot be used for absorbance values below calibrator E (25 U/mL). Values should be reported as <25 U/mL.

Qualitative protocol
Subtract the mean absorbance value of the wells A_1 and A_2 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.

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

EVALUATION CRITERIA

Semi-Quantitative protocol
Samples with results < 25 U/mL are defined as negative. Samples $\geq$25 U/mL are defined as positive.

Qualitative protocol
Users should calculate a cut-off between positive and negative samples that is specific to their subject selections. Results from the subject selection used in the Svar Life Science performance evaluation study suggest the following cut-off:

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

Expected Results
The anti-CCP EIA measures antibodies against synthetic peptides with citrulline residues. The anti-CCP EIA is calibrated in the semi-quantitative assay in relative units using a positive serum pool. The standard curve ranges from 25-3200 U/mL. These values have been chosen arbitrarily by Svar Life Science since no generally recognised international standard exists for expressing the titre of anti-CCP antibodies. The specificity and sensitivity were evaluated in previous studies with 311 RA samples, 942 diseased non-RA samples (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 samples and 99% with healthy subjects. (15)

PERFORMANCE CHARACTERISTICS

Table 1. Percent Agreement of the Immunoscan CCPlus® kit compared to an alternative CCP ELISA. A total of 628 frozen retrospective sera were assayed. 368 samples were RA samples and 260 samples were from apparently healthy blood donors. The following table summarises the results.

		Alternative ELISA		
		Positive	**Negative**	**Total**
Immunoscan CCPlus® Kit	**Positive**	275	5	280
	Negative	2	346	348
	Total	277	351	628

Positive Percent Agreement: 275/277 = 99.3%   95% CI = 97.4 – 99.9%
Negative Percent Agreement: 346/351 = 98.6%   95% CI = 96.7 – 99.5%
Overall Percent Agreement: 621/628 = 98.9%   95% CI = 97.7 – 99.6%

The 95% confidence interval (CI) was calculated using the exact method.

Table 2. Sensitivity and specificity. A total of 1180 frozen retrospective sera with clinical characterisation were assayed. The following table summarises the results

Control and Disease groups	Total number	Negative < 25 U/mL	Positive ≥ 25 U/mL
Blood donors	260	257	3
RA	399	90	309
WG	20	18	2
MP	20	20	0
SLE	66	64	2
Sjögren's syndrome	13	13	0
IBD	98	95	3

Osteoarthritis	21	21	0
Thyroiditis	20	20	0
Epstein Barr Virus	5	5	0
Parvovirus	5	5	0
Mycoplasma	9	9	0
Toxoplasma	6	6	0
Tuberculosis	5	5	0
Yersinia	8	8	0
Salmonella	3	3	0
Chlamydia	5	4	1
Malaria	4	4	0
Borrelia	9	9	0
Syphilis	5	5	0
Infectious endocarditis	3	3	0
Legionella	4	4	0
AST	3	3	0
Schistomiasis	4	4	0
Rubella	5	5	0
Chaga's syndrome	3	3	0
Scleroderma	17	16	1
Multiple Sclerosis	20	20	0
IDDM	20	20	0
PM/DM	20	20	0
MCTD	20	19	1
Routine samples	80	78	2

RA   = rheumatoid arthritis
WG   = Wegener's granulomatosis
MP   = microscopic polyangiitis
SLE   = systemic lupus erythematosus
PM/DM = Polymyositis/Dermatomyositis
IBD   = inflammatory bowel disease
AST   = anti-Streptolysine test
IDDM   = insulin dependent diabetes mellitus
MCTD   = mixed connective tissue disease

Sensitivity
RA = 309/399 = 77.4 %   95% CI = 73.3 - 81.5%

Specificity
Blood donors   = 257/260 = 98.8%   95% CI = 96.7 - 99.8%
WG   = 18/20 = 90.0%   95% CI = 68.3 – 98.8%
MP   = 20/20 = 100%   95% CI = 83.2 - 100%
SLE   = 64/66 = 97.0 %   95% CI = 89.5 - 99.6%
Sjogren's   = 13/13 = 100 %   95% CI = 75.3 - 100%
IBD   = 95/98 = 96.9%   95% CI = 91.3 - 99.4%
Osteoarthritis   = 21/21 = 100%   95% CI = 83.9 - 100%
Thyroiditis   = 20/20 = 100%   95% CI = 83.2 - 100%
Infectious Disease = 85/86 = 98.8%   95% CI = 93.7 - 100%
Scleroderma   = 16/17 = 94.1%   95% CI = 71.3 - 99.8%
Multiple Sclerosis = 20/20 = 100%   95% CI = 83.2 - 100%
IDDM   = 20/20 = 100%   95% CI = 83.2 - 100%
PM/DM   = 20/20 = 100%   95% CI = 83.2 - 100%

MCTD   = 19/20 = 95.0%   95% CI = 75.1 - 99.9%
Routine samples   = 78/80 = 97.5 %   95% CI = 91.3 - 99.7 %

The 95% confidence interval (CI) was calculated using the Exact method.

Table 3. Intra-assay precision was determined by testing six different samples eight times each.

	High		**High**		**High**	
	U/mL	OD	U/mL	OD	U/mL	OD
Mean.	2672	1.421	2685	1.432	1150	1.664
S.D.	138	0.01	205	0.01	55.3	0.02
% C.V.	5.2	0.4	7.6	0.4	4.8	0.9
	Medium		**Low**		**Low**	
	U/mL	OD	U/mL	OD	U/mL	OD
Mean	239	1.014	56	0.421	28	0.232
S.D.	2.3	0.01	2.1	0.01	0.5	0.01
%C.V.	1.0	0.4	3.8	2.8	3.6	1.3

Table 4. Inter-assay precision was determined by testing six different samples eight times each. Results were obtained for three different runs.

	High		**High**		**High**	
	U/mL	OD	U/mL	OD	U/mL	OD
Mean.	2696	1.426	2600	1.422	1168	1.706
S.D.	328	0.01	299	0.01	101.7	0.07
% C.V.	12.2	0.7	11.5	0.8	8.7	3.8
	Medium		**Low**		**Low**	
	U/mL	OD	U/mL	OD	U/mL	OD
Mean	242	1.031	59	0.428	28	0.232
S.D.	5.0	0.03	3.1	0.02	0.5	0.01
%C.V.	2.1	2.5	5.2	3.8	1.8	0.9

Table 5. Lot to lot variation was determined by testing six different samples eight times each. Results were obtained for three different lots.

	High		High		High	
	U/mL	OD	U/mL	OD	U/mL	OD
Mean.	2896	1.408	2870	1.408	1530	1.807
S.D.	405	0.02	335	0.02	260.4	0.03
% C.V.	14.0	1.4	11.7	1.5	17.0	1.6
	Medium		Low		Low	
	U/mL	OD	U/mL	OD	U/mL	OD
Mean	259	1.100	60	0.462	62	0.471
S.D.	21.8	0.04	4.2	0.02	6.6	0.04
%C.V.	8.4	3.9	6.9	4.4	10.8	8.2

Table 6. Dilution recovery was determined by testing five serial dilutions for three different samples.

Sample	Dilution	Mean Measured Concentration (U/mL)	Calculated Concentration (U/mL)	Dilution Corrected % Recovery
1	1/50	395	395	100
	1/100	195	198	98
	1/200	104	99	105
	1/400	53	50	106
	1/800	26	25	104
Sample	**Dilution**	**Mean Measured Concentration (U/mL)**	**Calculated Concentration (U/mL)**	**Dilution Corrected % Recovery**
2	1/50	921	921	100
	1/100	486	461	105
	1/200	257	230	112
	1/400	124	115	107
	1/800	63	58	109
Sample	**Dilution**	**Mean Measured Concentration (U/mL)**	**Calculated Concentration (U/mL)**	**Dilution Corrected % Recovery**
3	1/50	2962	2962	100
	1/100	1496	1481	101
	1/200	771	741	104
	1/400	349	370	94
	1/800	194	185	105

Two additional samples were diluted 1/50-1/1600 in the linear range. The mean concentrations were 164-6.0 U/mL and 321-11 U/mL respectively, with a dilution corrected recovery between 98-105%.

Detection Limit
The detection limit of the assay was determined by running the zero standard 14 times on three different lots. The detection limit of 1.6 U/mL was calculated by finding the mean plus two standard deviations.

Interference Study
Three low positive samples were spiked with bilirubin at 0.2 mg/mL, haemoglobin at 400 mg/dl, lipid at 15 mg/mL and rheumatoid factor at 200 IU/mL. The data indicates that the assayed concentrations do not interfere with the anti-CCP results.

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15. Vossenaar, E. K. Thesis
University of Nijmegen page 24 Table 1 Overview of CCP Sensitivity and Specificity (2004)

APPENDIX
Symbols used on labels

LOT	Batch number.
REF	Catalogue number.
⧖	Use-by date.
	Temperature limit.
☣	Biological risk.
📖i	Consult instruction for use.
▲▲	Manufacturer.
Σ∇ 96	Contains sufficient for 96 tests.

Ag			Antigen
DIL			Diluent.
BUF	WASH	20X	Wash buffer 20x concentrate.
H_2SO_4	0.5M		Sulphuric Acid, 0.5 molar (stop solution).
CONJ			Conjugate.
SUBS	TMB		Solution TMB (substrate solution).
CAL			Calibrator.
CONTROL	+		Positive control.
CONTROL	-		Negative control.
CONTROL	REF		Reference control.

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