REF 41429 ZENIT ra	ZENIT RA PR3	Distributed by A.MENARINI diagnostics
INSTRUCTIONS FOR USE	IVD +2°C +8°C Σ 50	CE

INTENDED USE

The *ZENIT RA PR3* test is a chemiluminescent immunoassay (CLIA) for use on the dedicated *ZENIT RA Analyzer* for quantitative determination of the specific IgG antibodies directed against proteinase 3 (PR3) in samples of human serum or plasma (EDTA, heparin).
This assay method is employed as a supplementary diagnostic technique in evaluation of ANCA-associated primitive systemic vasculitides and of Wegener's granulomatosis (WG) in particular.
CAUTION: Medical decisions cannot be based solely on the results of this test but must take into account all the available clinical and laboratory data.

CLINICAL SIGNIFICANCE

Antineutrophil cytoplasmic antibodies (ANCA) are autoantibodies directed against antigens contained in the cytoplasm of granulocytes and monocytes.[1] The ANCAs are serologic markers for several necrotizing vasculitides affecting small- and medium-sized blood vessels; in particular, Wegener's granulomatosis (WG), microscopic polyangiitis (MPA), pauci-immune necrotizing glomerulonephritis (PING, a form of MPA affecting only the kidney) with extracapillary proliferation, and, to a lesser degree, Churg-Strauss syndrome (CSS), which are often collectively defined as ANCA-associated vasculitides (AAVs).[2]
Much clinical and experimental evidence also suggests that the ANCAs may play a pathogenetic role in AAVs; this is particularly true of ANCA-MPO.[3]
Two principal fluoroscopic classes, c-ANCA and p-ANCA, are susceptible to recognition via the standard indirect immunofluorescence (IIF) method applied to normal human neutrophils fixed with absolute ethanol. Less frequently, two other fluoroscopic patterns, atypical c-ANCA and ANCA-At, not generally associated with the presence of idiopathic vasculitis, may be recognized by this technique.[4]
In patients affected with AAV, the main antigen targets of the ANCAs are myeloperoxidase (MPO) and proteinase 3 (PR3).[5,6]
MPO is an enzyme with significant bactericide properties that act by catalyzing peroxidation reactions that form toxic products such as HOCl, H_2O_2, and oxygen radicals. Additionally, hypochlorous acid and its metabolites can inactivate the protease inhibitors and therefore play an important role in tissue degradation and in maintenance of an "inflammatory" microenvironment.[3] MPO accounts for about 5% of the total protein content of the neurophils; it is a strongly cationic dimeric ester molecule with a molecular weight ~ 140 kDa.

PR3 is a weakly cationic serine protease contained in the primary granules (or azurophils) of granulocytes and monocytes, made up of 228 amino acids and having a molecular weight of 29-31 kDa, that exercises an anti-microbial action against bacteria and fungi. The majority of its biological functions depend on proteolytic activity. In an inflammatory context, it is released, together with other constituents of the granules and oxygen radicals, to the exterior of the cell where it can degrade collagen, the proteoglycans, and other constituents of connective tissue. Excessive, prolonged, or inappropriate proteolytic activity, however, is a cause of damage to the organism.[3] PR3-ANCAs interfere with enzymatic inhibition of PR3 by its physiological inhibitor, α1-antitrypsin.

In the majority of cases, a c-ANCA pattern is attributable to the presence of antibodies specific for PR3, while a p-ANCA pattern can be caused by antibodies directed against any of numerous proteins, among which MPO is the most frequent. To date, the clinical significance of ANCAs directed against other cytoplasmic components different from PR3 and MPO is not clear and for this reason they are not useful in laboratory diagnosis of the AAVs.[7,8]

Following publication of the results of the multi-center project for standardization of ANCA assay methods,[9] official guidelines for correct ANCA testing and reporting in patients with suspected vasculitides were drawn up; the reader is referred to said documents for more detailed information [4,10,11]. The above-mentioned study, the results of which were later amply confirmed, clearly showed that correct ANCA determination depends on a combination of the IIF method and identification of antigen specificity using systems specific for the two principal antigens, MPO and PR3 (ELISA, FEIA, CLIA methods).[12,13]

Combined use of the two methods permits obtaining a specificity > 98% even in the case of the control pathologies, a percentage much greater than that obtainable when the tests are used singly.

The ANCA titer correlates (although with some exceptions) with disease activity; for this reason, quantitative methods for ANCA-MPO or ANCA-PR3 assay are recommended for use in patient monitoring.[14]

The specificity of the ANCAs (MPO or PR3) does not allow differentiating among the various AAVs (WG, MPA, PING, CSS); nevertheless, the presence of p-ANCA/MPO-ANCA is more suggestive of a diagnosis of MPA or PING, while c-ANCA/PR3-ANCA positivity is more frequently associated with WG.

In an active phase of WG and in MPA (including the kidney-restricted form), the prevalence of the ANCAs is 70-90%; in CSS, the ANCAs are instead present only in ≈ 40% of patients, with MPO as the prevalent antigen specificity.

Regarding the diagnostic utility of the laboratory datum, it is worthwhile noting that the positive and negative predictive values (PPV, NPV) depend, besides on the sensitivity and the specificity of the test, on the prevalence of the disease in the study population. An appropriate requirement (high pre-testing probability) permits obtaining a result with real clinical utility and significantly reduces the incidence of false positive results.[15]

The ANCA assay should therefore be conducted when there is a founded clinical suspicion; a positive result, while not per se sufficient for diagnosing AAV, should be evaluated in the light of the overall clinical and histologic picture.

Literature occasionally reports ANCA positivity in cases of pathologies different from ANCA-associated vasculitides, although these reports have not always been confirmed by demonstration of antigen specificity for MPO or PR3.

Differential diagnosis must consider numerous pathologies; among these, it is worthwhile to recall – due in part to their high frequency – the infectious diseases and subacute bacterial endocarditis in particular. Despite the fact that the NPV of a negative result is generally elevated, this fact does not completely exclude the possibility of an AAV, above all in patients showing strong clinical signs of primitive systemic vasculitis.[16]

PRINCIPLE OF THE METHOD

The *ZENIT RA PR3* kit for quantitative determination of the specific anti-PR3 IgG antibodies employs an indirect, two-step immunoassay method based on the principle of chemiluminescence.
The specific antigen is used to coat magnetic particles (solid phase) and a human anti-IgG antibody is labeled with an acridine ester derivative (conjugate).
During the first incubation, the specific antibodies present in the sample, in the calibrators, or in the controls bind with the solid phase.
During the second incubation, the conjugate reacts with the anti-PR3 IgG antibodies captured on the solid phase.
After each incubation, the material that has not bonded with the solid phase is removed by suction and repeated washing.
The quantity of marked conjugate bonded to the solid phase is evaluated by chemiluminescent reaction and measurement of the light signal. The generated signal, measured in RLU (Relative Light Units), is indicative of the concentration of the specific antibodies present in the sample, in the calibrators, and in the controls.

AUTOMATION

The *ZENIT RA Analyzer* automatically performs all the operations called for by the assay protocol: addition of the samples, calibrators, controls, magnetic particles, conjugate, and chemiluminescence activator solutions to the reaction vessel; magnetic separation and washing of the particles; measurement of the emitted light.
The system calculates the assay results for the samples and the controls using the stored calibration curves and prints a report containing all the assay- and patient-related information.

MATERIALS AND REAGENTS

Materials and Reagents Provided

REAG	1	MP	2.5 ml

Magnetic particles coated with PR3 (proteinase 3) antigen in phosphate buffer containing stabilizing proteins, detergent, and Pro-Clin 300 and sodium azide (< 0.1%) as preservatives.

REAG	2	CONJ	15 ml

Goat anti-human IgG polyclonal antibody labeled with an acridine ester derivative (conjugate), in phosphate buffer containing stabilizer proteins and sodium azide (< 0.1%) as preservative.

REAG	3	DIL	25 ml

Sample Dilution Solution: phosphate buffer containing bovine serum albumin, detergent, inert blue dye, and Pro-Clin 300 and Gentamicin SO_4 as preservatives.

REAG	4	CAL A	1.6 ml

Human serum with low concentration of anti-MPO IgG antibodies in phosphate buffer containing bovine serum albumin, detergent, inert blue dye, and Pro-Clin 300 and Gentamicin SO_4 as preservatives.

REAG	5	CAL B	1.6 ml

Human serum with high concentration of anti-MPO IgG antibodies in phosphate buffer containing bovine serum albumin, detergent, inert blue dye, and Pro-Clin 300 and Gentamicin SO_4 as preservatives.

All the reagents are ready to use.
Reagents 1, 2, and 3 are assembled into a single reagents cartridge unit.
The concentrations of the calibrators are expressed in AU/ml (Arbitrary Unit) and calibrated against an internal reference standard. The concentration values are specific by product lot and are recorded on the DATA DISK included in the kit.

DATA DISK

A mini-DVD containing information about all the ZENIT RA products (Reagents, Calibrators, Control Sera) updated to the latest production lot and excluding products expired at the date of writing of each new DATA DISK.
The only DATA DISK needed to ensure that the information needed for correct system operation is always updated is that with the highest lot number.

Materials and Reagents Required but not Provided in the Kit

- ZENIT RA Analyzer — Code No. 41400
- ZENIT RA Cuvette Cube * — Code No. 41402
  Box of 960 cuvettes.
- ZENIT RA System Liquid * — Code No. 41409

1 – 0.5-liter bottle of 10x solution.

- ZENIT RA Wash Solution * Code No. 41407
  1 – 0.5-liter bottle of 20x solution.

- ZENIT RA Trigger Set * Code No. 41403
  1 – 250-ml vial of Trigger A (pre-activation solution)
  1 – 250-ml vial of Trigger B (activation solution)

- ZENIT RA D-SORB Solution Code No. 41436
  Box containing 2 – 1-liter bottles of ready-to-use solution.

- ZENIT RA Cartridge Checking System * Code No. 41401

- ZENIT RA Top Cap Set Code No. 41566
  300 top caps for capping the calibrator containers after first use.

(*)The ZENIT RA Analyzer and the accessories tagged with an asterisk are manufactured by Immunodiagnostic Systems S.A., Rue E. Solvay, 101, B-4000 Liège, Belgium and distributed by A. Menarini Diagnostics Srl.

Other Recommended Reagents

ZENIT RA ANCA/GBM CONTROL SET Code No. 41449

3 – 1.5 ml vials of human serum negative for anti-PR3 antibodies and 3 – 1.5 ml vials of human serum positive for anti-PR3 antibodies.

WARNINGS AND PRECAUTIONS

The reagents provided in the *ZENIT RA PR3* kit are intended for *in vitro* diagnostic use only and not for *in vivo* use in humans or animals.
This product must be used by professional users only and in strict accordance with the instructions set out in this document.

Menarini may not be held responsible for any loss or damage in any way resulting from or related to use of the product in manners not compliant with the instructions provided.

Safety Precautions

This product contains material of animal origin and therefore must be handled as though it contained infectious agents.

This product contains components of human origin. All the serum or plasma units used in the manufacture of the reagents in this kit have been tested by FDA-approved methods and have been found to be non-reactive for HBsAg and anti-HCV, anti-HIV1, and anti-HIV2 antibodies.
Nevertheless, since no analysis method can offer complete assurance of the total absence of pathogenic agents, all material of human origin should be considered potentially infected/infectious and handled as such.
If the packaging is damaged in such a way as to cause leakage of the reagents, decontaminate the affected area with a dilute sodium hypochlorite (bleach) solution while wearing appropriate personal protective equipment (lab coat, gloves, goggles).
Dispose of used cleaning materials and the packaging materials affected by the leakage in accordance with national-level regulations for disposal of potentially infected/infectious waste.
Some of the reagents contain sodium azide as preservative. Since sodium azide may react with lead, copper, or brass in plumbing to form explosive azide compounds, do not flush reagents or waste to sewer. Dispose of such waste in accordance with national-level regulations for disposal of potentially hazardous substances.

Operating Precautions
In order to obtain reliable results, follow these instructions for use and the instructions provided in the analyzer operator's manual carefully.
The reagents supplied in the kit are intended for use only with the *ZENIT RA Analyzer* system.
The reagents cartridge components cannot be removed from the cartridge and reassembled.
Do not use the kit after the expiry date.

REAGENT PREPARATION

The reagents supplied in the kit are ready to use.

REAGENT STORAGE AND STABILITY

Store the reagents supplied in the kit in an upright position, at 2-8 °C, in a dark place.
In these conditions, the reagents cartridges and the unopened calibrators reagents are stable until the expiry date.
After opening, the reagents cartridges may be used for 60 days if stored refrigerated at 2-8 °C or onboard the machine.
After opening, the calibrators may be used for 60 days if stored refrigerated at 2-8 °C or if the on-board use time does not exceed 6 hours per session.
Do not freeze the reagents and/or the calibrators.

SAMPLE PREPARATION AND STORAGE

The assay must be performed on samples of human serum or plasma (EDTA - heparin).
Use of lipemic, hemolyzed, or turbid samples is not recommended.
If the assay is performed more than 8 hours after the blood sample is drawn, after separating the serum from the coagulate or the plasma from the red blood cells, transfer the surnatant from the gel separation tubes to secondary tubes.
Prior to analysis, the samples may be stored refrigerated at 2-8 °C for a maximum of 7 days.
If the samples must be stored for more than 7 days before testing, store frozen at < -20 °C.
Avoid repeated freezing and thawing.

ASSAY PROCEDURE

In order to obtain reliable analysis results, follow the instructions provided in the analyzer operator's manual carefully.

Loading the Reagents

All the reagents supplied in the kit are ready to use.
Before installing the reagents cartridge on the system, agitate the magnetic particles container by rotating horizontally, in order to ensure correct particle suspension. Do not allow the suspension to foam during agitation.
Position the reagents cartridge in the reagents area of the analyzer, using the guide for that purpose, and allow to agitate for at least 30 minutes prior to use.
The identification bar code is automatically read when the reagents cartridge is positioned on the analyzer. If the cartridge label is damaged or if for any other reason reading is not performed, the cartridge identification data may be entered manually.
The analyzer automatically performs continual agitation of the magnetic particles.
If the reagents cartridge is removed from the analyzer, store in an upright position, at 2-8 °C, in a dark place.

Loading the Calibrators and Controls

The ZENIT RA calibrators and controls are ready to use. Allow the calibrators and controls to stand at room temperature for 10 minutes before use. Agitate the contents gently, by hand or vortex; do not allow to foam. Do not upend the container and do not remove the seal cap with perforator (yellow cap for calibrators; green or blue caps for controls).
When using a calibrator or control for the first time, press the perforator cap down until it stops. This operation perforates the container seal membrane to permit accessing the liquid contents. If the perforator

cap is used correctly, red strip at the top of the label will be covered (See Fig. 1 – Sealed Container and Perforated Container).

Previously-used calibrator and/or control containers will be capped with a top cap (white cap) and the red label strip will be covered.

Load only perforated containers from which the top cap (white cap) has been removed and on which the red strip is covered (Fig. 1 – Perforated Container) onto the analyzer).

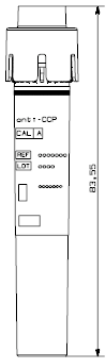
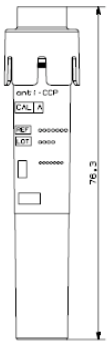
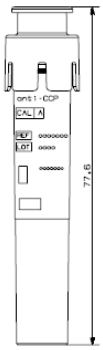
Read the barcode and insert the calibrators or controls into the samples area of the analyzer. The barcode data may be entered manually if the label is damaged or if for any other reason reading is not performed.

The concentration values of the anti-PR3 IgG antibodies contained in the calibrators and the controls are stored on the DATA DISK and are automatically transferred to the analyzer. The data may be entered manually if for any reason data transfer is not successful.

At the end of each session, reseal the calibrator and control containers with the appropriate top caps (white caps) and transfer to storage at 2-8 °C until next use (See Fig. 1 – Capped Container).

The calibrators are sufficient for up to four uses.

Figure 1: Container Layout

Sealed Container	Perforated Container	Capped Container
		

Loading the Samples

Identify the samples via the barcode reader and insert them in the appropriate container on the analyzer. If a sample barcode is missing or illegible or for any other reason not read, the sample identification data may be entered manually.

Select the analysis parameters for each sample.

Calibration

The *ZENIT RA Analyzer* uses a calibration curve (master curve) that is generated by the manufacturer for each lot of reagents cartridges.

The master curve parameters, as well as the calibrator concentration values, are stored on the DATA DISK and transferred to the analyzer database.
Calibrators A and B are used for recalibrating the master curve for the instrument used and for the reagents installed onboard.
To recalibrate, analyze three replicates of the two calibrators (A and B) and one replicate of each control. The concentration values obtained with the controls permit validating the new calibration.
Once a master curve recalibration has been accepted and stored in memory, all the successive samples can be analyzed with no further calibration being required, exception made for the cases listed below:

- when a reagents cartridge with a new lot number is installed on the analyzer;
- when the control values do not fall within the acceptability interval;
- after analyzer maintenance;
- after expiry of the period of validity of the recalibrated master curve.

A master curve recalibrated for the *ZENIT RA PR3* kit has a period of validity of 15 days.
Recalibration management is handled automatically by the analyzer.

Assay

Press the start button.

1. The system draws 80 µl of sample dilution solution, 40 µl of magnetic particles, 100 µl of sample dilution solution, and 6 µl of sample or control, in that order; for the calibrators, the positive serum is supplied prediluted with the sample dilution solution and the volume drawn is 106 µl. The solutions and suspension are dispensed into the reaction cuvette.
2. The reaction cuvette is incubated on the rotor at 37°C for 10 minutes.
3. At the end of this incubation phase, the magnetic particles are separated and washed.
4. 200 µl of conjugate are dispensed into the cuvette.
5. The reaction cuvette is incubated on the rotor at 37°C for 10 minutes.
6. At the end of this last incubation phase, the magnetic particles are separated and washed and the cuvette is transferred to the reading chamber.
7. The quantity of conjugate bound to the solid phase, expressed in RLU (Relative Light Units), is directly proportional to the concentration of anti-PR3 IgG in the sample.
8. The results are interpolated on the calibration curve and expressed in concentrations.

If the concentration value of a sample exceeds the upper limit of the measurable interval, the sample may be diluted and retested. The new value thus obtained is multiplied by the dilution factor to obtain the final result.

QUALITY CONTROL

In order to ensure the validity of the assay, control sera at different concentrations (at least one negative serum and one positive serum) must be tested every day on which samples are assayed.
If individual laboratory practice so dictates, more frequent or more numerous controls may be performed for verification of assay results. Follow local quality control procedures.

If the ZENIT RA control sera are used, the expected mean values and the acceptability limits are those reported in the DATA DISK supplied with the controls.
Should different control sera be used, the expected values must be defined with the ZENIT RA reagents and analysis system before the products are used.
Should the values obtained with the controls not fall within the specified acceptability range, the relative assay results cannot be considered valid and it will be necessary to retest the respective samples.
In this case, recalibrate before repeating the assay/s in question.

CALCULATION AND INTERPRETATION OF RESULTS

Calculation of Results

The system automatically calculates the concentration of the anti-PR3 IgG antibodies in the tested sample. The values may be displayed on video or may be printed.
The concentrations are expressed in AU/ml.
Calculation of the analyte concentration in a sample proceeds by reading of the result obtained for each sample on a calibration curve calculated in accordance with a 4-parameter logistic fitting model (4PL, weighted Y), which is corrected periodically on the basis of the calibrator assay results.

For detailed information on how the system calculates the results, refer to the system operator's manual.

Interpretation of Results

The measurability range for the *ZENIT RA PR3* assay is: 0.0 – 1067 AU/ml.
Values less than 0.0 AU/ml are extrapolated values and may be reported as "equal to 0.0 AU/ml."
Values in excess of 1067 AU/ml may be reported as "greater than 1067 AU/ml" or the sample may be retested following appropriate dilution.
The results for a sample may be interpreted as set forth below:

(AU/ml)	Interpretation
< 10	The sample should be considered Negative for the presence of anti-PR3 IgG
10÷20	The sample should be considered inconclusive for the presence of anti-PR3 IgG
> 20	The sample should be considered Positive for the presence of anti-PR3 IgG

The values reported above are suggested values only. Each laboratory will establish its own reference intervals.

LIMITS TO THE ASSAY METHOD

For diagnostic purposes, the results obtained with the *ZENIT RA PR3* kit and the *ZENIT RA Analyzer* system should always be used in conjunction with the other clinical and laboratory data available to the case physician.

Bacterial contamination of the samples and inactivation by heat may influence the results of the assay.

Heterophilic antibodies present in the human serum samples may react with immunoglobulin-based reagents, causing interference with in vitro immunoassays. Such samples may yield anomalous values when analyzed with the *ZENIT RA PR3* kit.

EXPECTED VALUES

Samples from 99 randomly-selected donors were analyzed to check for the presence of anti-PR3 IgG antibodies.

The results for all the samples analyzed were negative, with a mean value of 1.0 AU/ml and a standard deviation of 1.239 AU/ml.

The results thus obtained were used to calculate the “Limit of Blank” (LoB = the highest value that may be expected in a series of samples that do not contain the analyte). The Limit of Blank corresponding to the 95th percentile of the negative population was 3.5 AU/ml with reagents lot no. 1.

CLINICAL SENSITIVITY AND SPECIFICITY

A total of 333 samples were tested with the *ZENIT RA PR3* kit. Of these, 50 samples were from patients affected by ANCA-associated primitive systemic vasculitis diagnosed as Wegener’s granulomatosis (WG), 45 samples were from patients affected by ANCA-associated primitive systemic vasculitis diagnosed as microscopic polyangiitis (MPA), 109 samples were from patients affected by different pathologies 14 with chronic inflammatory intestinal disorders, 44 with systemic lupus erythematosus, 7 with systemic connective tissue disorders, 20 with non-ANCA-associated vasculitides, 10 with rheumatoid arthritis, 14 with infectious diseases), 30 samples were from normal subjects, and 100 samples were from donor subjects.

In the presumably negative study population (45 samples from patients diagnosed with MPA, 109 samples from patients affected by different non-ANCA associated pathologies, 30 samples from normal subjects, and 100 samples from donor subjects), 9 samples tested positive, 8 tested inconclusive, and 266 tested negative.

- **Diagnostic Specificity: 94.0 % (266/283)**

Of the 17 samples that tested “not negative,” 7 were from the patient group diagnosed with MPA, 9 were from the group of patients affected by different pathologies, and one was from the group of normal subjects.

In the presumably positive study population (50 samples from patients diagnosed with WG), 11 samples tested negative and 39 tested positive.

- **Diagnostic Sensitivity: 78.0 % (39/50)**

Of the 11 samples that tested “not positive,” 7 samples tested positive for the indirect immunofluorescence “gold standard” test, with a p-ANCA pattern, and 4 samples tested negative for the indirect immunofluorescence “gold standard” test (one showing an “atypical” pattern).
Based on the diagnostic specificity and sensitivity results, **diagnostic concordance is 91.6 %** (305/333).

PERFORMANCE

Caution: The data presented are not representative of kit operating specifications but constitute experimental evidence of how kit performance is aligned with the manufacturer’s stated specifications.

Precision and Reproducibility

The precision and reproducibility of the *ZENIT RA PR3* kit assays were assessed using a protocol based on the guidelines provided by Clinical and Laboratory Standards Institute (CLSI) document EP5-A2.

Precision was calculated by analyzing the results for 20 replicates of five sera (one negative and four positive at different anti-PR3 IgG concentrations) run with two different reagent lots during the same experimental session.

The anti-PR3 IgG concentration found in the negative serum (N2) fell in the intervals from 0.0 to 3.0 AU/ml and from 2.1 to 4.4 AU/ml when tested with reagent lots no. 1 and no. 2, respectively

The results obtained with the 4 positive sera are reported in the table below.

Sample	Reagents Lot No.	Average Concentration (AU/ml)	SD	CV %
P1	1	23.0	0.80	3.5
	2	28.5	0.86	3.0
P2	1	70.0	1.83	2.6
	2	80.0	1.68	2.1
P3	1	110.2	3.74	3.4
	2	145.8	2.93	2.0
P4	1	386.7	6.64	1.7
	2	434.6	8.78	2.0

Reproducibility was calculated by analyzing the results for five sera (one negative and four positive at different anti-PR3 IgG concentrations) assayed in single replicates in 30 different sessions, with two different reagent lots.

The anti-PR3 IgG concentration found in the negative serum (N2) fell in the interval from 0.0 to 3.8 AU/ml.

The results obtained with the 4 positive sera are reported in the table below.

Sample	Average Concentration (AU/ml))	SD	CV %

P1	24.5	1.56	6.4
P2	71.3	3.09	4.3
P3	124.6	11.05	8.9
P4	391.8	22.87	5.8

Linearity of Dilution

The linearity of the *ZENIT RA PR3* kit dilutions was evaluated by following a protocol based on the guidelines provided by Clinical and Laboratory Standards Institute (CLSI) document EP6-A.

Scaled dilutions of 3 sera containing high concentrations of anti-PR3 IgG, diluted with the sample dilution solution, were assayed.

The results of this study are summarized in the table below.

Sample	Dilution Factor	Measured Concentration (AU/ml)	Expected Concentration (AU/ml)	Recovery %
1	1	377.4	-	(100)
	2	186.7	188.7	98.9
	4	84.7	94.4	89.7
	8	37.0	47.2	78.4
2	1	957.1	-	(100)
	2	493.1	478.6	103.0
	4	256.1	239.3	107.1
	8	131.8	119.6	110.2
	16	71.0	59.8	118.7
3	1	206.9	-	(100)
	2	111.6	103.5	107.8
	4	64.0	51.7	123.8
	8	33.4	25.9	129.0

It must in any case be noted that not all sera, when measured at different dilutions, can give results within the measurability interval, since the result is dependent not only on concentration but also on the affinity of the antibodies in the sample.

Analytical Sensitivity

The analytical sensitivity of the *ZENIT RA PR3* kit, expressed as the ***Limit of Detection*** (***LoD:*** the smallest quantity of analyte that can be measured by the method), was evaluated by following a protocol based on the guidelines provided by Clinical and Laboratory Standards Institute (CLSI) document EP17-A and the formula: $LoD = LoB + C_\beta SD_s$ (where LoB is the value of the Limit of Blank, SD_s is the estimated standard deviation of the low-concentration sample distribution, and C_β is derived from the 95th percentile of the standard normal [Gaussian] distribution)

Four (4) samples at low analyte concentration were assayed in single replicates, using two different reagents lots, in 30 different experiments.

The resulting Limit of Detection of the *ZENIT RA PR3* kit was 7.3 AU/ml.

The Limit of Detection values, clinical considerations, and the results of comparison with reference methods contributed to definition of the cutoff value.

Analytical Specificity: Interferences

A study based on the guidelines provided by CLSI document EP7-A2 demonstrated that assay performance is not influenced by inclusion in the sample of the potentially interfering substances listed below, at concentrations up to those tested.

Potentially Interfering Substances	Maximum Concentration Tested
Free Bilirubin	20 mg/dl
Conjugated Bilirubin	28 mg/dl
Hemoglobin	1000 mg/dl
Fatty acids	3000 mg/dl

Use of lipemic, hemolyzed, or turbid samples is not recommended.

Analytical Specificity: Cross-reactivity

A study of 24 samples, all with high levels of other autoantibodies and negative for anti-PR3 IgG, was conducted to evaluate potential cross-reactions with the antigen used for sensitizing the magnetic particles.
The samples were so divided: SS-A (2), SS-B (3), U1-snRNP (1), Jo-1 (2), Scl-70 (2), Cenp B (2), histone (1), nucleolar (1), β_2-GPI/CL IgG (2), and Gliadin/t-TG (3), CCP (1), RF (1), dsDNA (3).
The study revealed no significant cross-reactions between the solid-phase antigen and the other autoantibodies.

High-dose Hook Effect

Some methods for immunoassay of samples containing extremely high concentrations of analyte may provide apparent analyte levels that underestimate actual content (high-dose saturation or hook effect).
The dual-incubation method employed by the *ZENIT RA PR3* kit is not influenced by this effect.
A sample containing an extremely high concentration (above the top limit of the measurement interval) of anti-PR3 IgG has confirmed the absence of the hook effect up to a concentration of 20831 AU/ml.

Relative Sensitivity and Specificity

The presence of anti-PR3 IgG antibodies was determined, using the *ZENIT RA PR3* kit and a commercially-available ELISA method, in 226 samples: 50 samples from patients affected by ANCA-associated primitive systemic vasculitis diagnosed as Wegener's granulomatosis, 43 samples from patients affected by ANCA-associated primitive systemic vasculitis diagnosed as microscopic polyangiitis, 103 samples from patients affected by different pathologies (chronic inflammatory intestinal disorders, systemic lupus erythematosus, systemic connective tissue disorders, non-ANCA-associated vasculitides, rheumatoid arthritis, infectious diseases), and 30 normal subjects.
The ZENIT RA assay and the commercially-available ELISA assay gave discordant results for 4 samples.

Relative concordance was therefore 98.2 % (222/226).

Relative sensitivity was shown to be 97.8 % (45/46).

Relative specificity was shown to be 98.3 % (177/180).

The sample that tested negative with the *ZENIT RA PR3* kit and positive with the ELISA kit were from the group of patients affected with systemic lupus erythematosus.

The three samples that tested positive with the *ZENIT RA PR3* kit and negative with the ELISA kit were: one from the Wegener's granulomatosis sample group and one from the microscopic polyangiitis sample group.

Reference Sera

The quantity of anti-PR3 IgG antibodies in the "PR3-ANCA HUMAN REFERENCE SERUM # 16 (CDC, Cat. No. IS2721, Lot. No. 07-0002) sample tested with the *ZENIT RA PR3* kit, *after appropriate dilution,* was 2282 AU/ml.

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