11. Assay Procedure Continued…

9. Add 100µl of Stop Solution **(Reagent 5)** to each well. To allow equal reaction times, the Stop Solution should be added to the wells in the same order as the TMB Substrate.

10. Read the optical density (OD) of each well at 450nm in a microplate reader within 10 minutes. A 620nm filter may be used as a reference wavelength.

12. Quality Control

Quality control data is supplied on the lot-specific QC certificate included in the kit.

The positive control is intended to monitor for substantial reagent failure.

Any well positive by spectrophotometer but without visible colour should be cleaned on the underside and re-read. If OD-values below zero are observed, the wavelengths used should be verified, the reader re-blanked to air and the measurements repeated.

13. Interpretation of Results

Quantitative Results

Plot the optical density of each standard against its concentration and draw the best-fit curve through the points. Read the unknowns off this curve. Values below 0.4 U/ml are normal. Values between 0.4 U/ml and 0.5 U/ml are indeterminate. Values above 0.5 U/ml are significantly elevated. Values above 30 should be repeated at a higher dilution e.g. 1:200

Qualitative Results

Normal values are less than 0.4 U/ml. Values above 0.4 U/ml should be repeated in the quantitative assay.

14. Limitations of the Procedure

Results must be interpreted in conjunction with other clinical findings. Positive anti-PR3 IgG results have been reported in infective disorders such as HIV infections, endocarditis, pneumonia and in cystic fibrosis patients. They may also be found in monoclonal gammopathy and malignancy without signs of secondary vasculitis.

15. Performance Characteristics

Recovery of Added Antibody

Ab Added (U/ml)	Result (U/ml)	Recovery %
0	14.6	-
15.0	31.2	110.0
50.0	63.5	97.0

Clinical Studies

Samples from 134 patients with and without clinically-defined Wegener's granulomatosis were tested by ELISA for anti-PR3 antibodies. The results are shown in the table below.

	Anti-PR3 -	Anti-PR3 +
WG -	97	1
WG +	0	36

Based on these data, the sensitivity and specificity of the PR3 ELISA is 100% and 99% respectively.

16. Reproducibility

Within Assay Precision

Mean U/ml	SD	CV%
10.5	0.67	6.3
45.2	3.10	6.8

Between Assay Precision

Mean U/ml	SD	CV%
11.5	0.84	7.3
43.7	4.23	9.6

17. Method Summary

- Dilute sera 1:50 with sample diluent **(Reagent 1)**
- Dispense standards as required, the positive control and the diluted sample into the microplate wells
- Incubate for **30 minutes** at room temperature.
- *Wash the wells three times*
- Dispense 100µl of conjugate **(Reagent 3)** into each well
- Incubate at room temperature for **30 minutes**
- *Wash the wells four times*
- Add 100µl of TMB substrate **(Reagent 4)** to each well
- Incubate at room temperature for **10 minutes**
- Add 100µl stop solution **(Reagent 5)** to each well
- Read the optical density at 450nm (single wavelength) or 450/620nm (dual wavelength).

18. Further Reading

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G·E·N·E·S·I·S Diagnostics

PR3 Antibodies ELISA Kit

Quantitative/qualitative assay for Proteinase 3 (IgG) antibodies

Product Code: GD003

For *in vitro* Diagnostic Use

CE

1. Materials Included in the Kit

- **Microplate:** 96 wells in 12 X 8 break-apart strips, pre-coated with PR3, with holder in a foil bag with desiccant
- **Reagent 1**: **Sample Diluent** 10mM Tris-buffered saline, pH 7.2 with antimicrobial agent, 50ml, ready to use (blue)
- **Reagent 2: Wash Buffer** 100mM Tris-buffered saline with detergent, pH 7.2, 100 ml, **concentrate** (x10)
- **Reagent 3: Conjugate** goat anti-human IgG conjugated to horseradish peroxidase in protein stabilising solution and antimicrobial agent, 12 ml, (red), ready to use
- **Reagent 4: TMB Substrate** aqueous solution of TMB and hydrogen peroxide, 12 ml, ready to use
- **Reagent 5: Stop Solution** 0.25M sulphuric acid, 12 ml, ready to use
- **Standards:** 0.4, 1.0, 3.0, 10, 30 U/ml, 1ml of 10mM Tris-buffered saline containing human serum IgG antibodies to PR3, ready to use
- **Positive control:** 1ml of 10mM Tris-buffered saline containing human serum antibodies to PR3, ready to use
- **Instructions for use**

2. Other Equipment Required

Test tubes for dilution • graduated cylinder for preparing wash buffer • precision pipettes and disposable tips to deliver 20µl, 100µl, 1ml • EIA microplate washer or multi-channel pipette or wash bottle • distilled or de-ionised water • absorbent paper • EIA microplate reader with 450nm and optional 620nm reference filter. Alternatively, a suitable, self-validated automated system may be used.

Instrumentation, whether manual or automated, should meet the following criteria: pipettes with better than 3% imprecision with no carry over between pipetting steps; microplate washers should remove 99% of fluid; automated machines should minimise time between washing and adding the next reagent.

3. Intended Use

The anti-PR3 kit is a rapid ELISA test for the detection of IgG antibodies to serine protease - 3 (PR3). It is intended for use in clinical laboratories as an aid to the diagnosis of Wegener's granulomatosis and associated diseases. The components of the kit are for *in vitro* diagnostic use only.

4. Explanation of the Test

Anti-neutrophil cytoplasmic antibodies (ANCA) that recognise PR3 are strongly associated with Wegener's granulomatosis and are relevant to the immunopathogenesis of the associated vasculitis. Several studies have demonstrated good correlation between disease activity and anti-PR3 antibody titres. Thus, measurement of PR3 antibodies is useful in assessing the effects of therapy and in predicting relapse.

5. Principle of the Test

Diluted serum samples are incubated with PR3 immobilised on microtitre wells. After washing away unbound serum components, goat anti-human IgG conjugated to horseradish peroxidase is added to the wells, and this binds to surface-bound antibodies in the second incubation. Unbound conjugate is removed by washing, and a solution containing 3,3',5,5'-tetramethylbenzidine (TMB) and enzyme substrate is added to trace specific antibody binding. Addition of stop solution terminates the reaction and provides the appropriate pH for colour development. The optical densities of the standards, positive control and samples are measured using a microplate reader at 450nm.

6. Safety Precautions

1. All reagents in this kit are for *in vitro* diagnostic use only.
2. Only experienced laboratory personnel should use this test. The test protocol must be followed strictly.
3. CAUTION: the device contains material of human and animal origin and should be handled as a potential transmitter of diseases. All human source material used in the preparation of standards and control for this product have been tested and found negative by ELISA for antibodies to HIV, HbsAg and HCV. No test method, however, can offer complete assurance that infectious agents are absent. Therefore, all reagents containing human material should be handled as if potentially infectious. Operators should wear gloves and protective clothing when handling any patient sera or serum based products.
4. Reagents of this kit contain antimicrobial agents and the Substrate solution contains 3,3',5,5'-tetramethylbenzidine. Avoid contact with the skin and eyes. Rinse immediately with plenty of water if any contact occurs.
5. The Stop Solution contains 0.25M sulphuric acid. Avoid contact with skin and eyes. Rinse immediately with plenty of water if contact occurs.
6. Any liquid that has been brought into contact with potentially infectious material has to be discarded in a container with a disinfectant. Dispose of plates and specimens as clinical waste. Any unused reagents should be flushed away with copious amounts of water. Disposal must be performed in accordance with local legislation.

7. Technical Precautions

1. Strips and solutions should not be used if the foil bag is damaged or liquids have leaked.
2. Allow all reagents and the microplate to reach room temperature before use. Ensure that the microplate foil bag containing any unused strips is well sealed and contains the desiccant to avoid moisture. Store at 2 – 8ºC after use.
3. When automating, consider excess volumes required for setting up the instrument and dead volume of robot pipette
4. Include the positive control in every test run to monitor for reagent stability and correct assay performance.
5. Strictly observe the indicated incubation times and temperature.
6. Ensure that no cross-contamination occurs between wells. Keep all pipettes and other equipment used for enzyme conjugate completely separate from the substrate reagent.
7. When pipetting Conjugate or TMB Substrate, aliquots for the required numbers of wells should be taken to avoid multiple entry of pipette tips into the reagent bottles. Never pour unused reagents back into the original bottles.
8. Do not allow microwells to dry between incubation steps.
9. Strictly follow the described wash procedure. Insufficient washing may cause high background signal.
10. Avoid direct sunlight and exposure to heat sources during all incubation steps.
11. Replace colour-coded caps on their correct vials to avoid cross-contamination
12. It is important to dispense all samples and the positive control into the wells without delay. Therefore ensure that all samples are ready to dispense.

8. Shelf Life and Storage Conditions

On arrival, store the kit at 2 - 8°C. Once opened the kit is stable for 3 months (or until its expiry date if less than 3 months). Do not use kits beyond their expiry date. Do not freeze any kit component. The diluted Wash buffer has a shelf life of 3 months if stored in a closed bottle at 2 - 8ºC.

9. Specimen Collection and Storage

Serum or plasma samples may be used and should be stored at -20°C for long-term storage. Frozen samples must be mixed well after thawing and prior to testing. Repeated freezing and thawing can affect results. Addition of preservatives to the serum sample may adversely affect the results. Microbially contaminated, heat-treated or specimens containing particulate matter should not be used. Grossly haemolysed, icteric or lipaemic specimens should be avoided.

10. Preparation of Reagents

Dilute the Wash Buffer **(Reagent 2)** 1:9 in distilled water to make sufficient buffer for the assay run e.g. add 50ml wash buffer concentrate to 450ml water.

11. Assay Procedure

1. Dilute patient samples 1:50 in sample diluent (e.g. 20µl serum plus 1ml diluent).

2. Assemble the number of strips required for the assay.

3. For quantitative assays, dispense 100 µl of sample diluent as the 0 U/ml standard followed by 100 µl of each standard, the positive control and the diluted patient samples into appropriate wells. For qualitative assays, dispense only the 0.4 U/ml standard together with the positive control and samples.

4. Incubate for **30 minutes** at room temperature.

5. After 30 minutes, decant or aspirate the well contents and wash the wells 3 times using automated washing or the manual wash procedure (see below). Careful washing is the key to good results. **Do not allow the wells to dry out.**

   <u>Manual Wash Procedure</u>
   Empty the wells by inversion. Using a multi-channel pipette or wash bottle, fill the wells with wash buffer. Empty by inversion and blot the wells on absorbent paper. Repeat this wash process 2 more times.

6. Dispense 100µl of Conjugate **(Reagent 3)** into each well. Incubate the wells for **30 minutes** at room temperature.

7. After 30 minutes, discard the well contents and carefully wash the wells 4 times with wash buffer. Ensure that the wells are empty but do not allow to dry out.

8. Using a repeating dispenser, rapidly dispense 100µl of TMB Substrate **(Reagent 4)** into each well. Incubate the plate for **10 minutes**.