

REFERENCES

1. Toh B et al. Pernicious Anemia. N Eng J Med, 1997; 337: 1441-1448.
2. Toh B et al. Pernicious Anemia. Autoimmunity, 2004;37:357-361.
3. Mardh E et al. Diagnosis of gastritis by means of a combination of serological analyses. Clin Chim Acta 2002;320:17-27.
4. Carmel R et al. Prevalence of undiagnosed pernicious anemia in the elderly. Arch Intern Med; 1996;156:1097-1100.
5. Chuang JS et al. Diagnostic ELISA for parietal cell autoantibody using tomato lectin-purified gastric H+/K+-ATPase (proton pump). Autoimmunity 1992;2:1-7.
6. Goldkorn I et al. Gastric parietal cell antigens of 60-90, 92, and 100-120 kDa associated with autoimmune gastritis and pernicious anemia. Role of N-glycans in the structure and antigenicity of the 60-90-kDa component. J Biol Chem.1989;264:18768-74.
7. Basso N et al. Antigastric autoantibodies in Helicobacter pylori infection: role in gastric mucosal inflammation. Int J Clin Lab Res 2000;30:173-178.
8. Ching-Chu L et al. Implications of anti-parietal cell antibodies and anti-Helicobacter pylori antibodies in histological gastritis and patient outcome World J Gastroenterol 2005;11:4715-4720
9. Baxter AG et al. Genetic control of susceptibility to autoimmune gastritis. International Reviews of Immunology, 24: 55-62, 2005.
10. Uibo R. Contribution of epidemiological studies to gastritis immunology. Int Rev Immunol.2005;24:31-54.
11. Biosafety in Microbiological and Biomedical Laboratories. Centers for Disease Control, National Institutes of Health [HHS Pub. No. (CDC) 93-8395], 1993.

For technical assistance please contact:



IMMCO Diagnostics, Inc.
60 Pineview Drive
Buffalo, NY 14228-2120
Telephone: (716) 691-0091
Fax: (716) 691-0466
Toll Free USA/Canada: 1-800-537-TEST
E-Mail: info@immcodiagnostics.com

or your local product distributor

FEB2006
Document No. PI4165IUO



ImmuLisa™

Anti-Gastric Parietal Cell Antibody (AGPA) ELISA

For investigational use only

PRODUCT INSERT

Catalog No. 1165 96 Determinations

INTENDED USE

Enzyme linked immunosorbent assay (ELISA) for the qualitative and semi-quantitative detection of antibodies to the gastric parietal cell antigen H+K+ATPase (proton pump) in human serum. Detection of these antibodies aids in the diagnosis of chronic atrophic gastritis and pernicious anemia.

SUMMARY AND EXPLANATION

Atrophic gastritis is a chronic gastritis that affects the corporal mucosa. It is characterized histologically by chronic inflammation of the gastric mucosa with loss of glandular cells and replacement by intestinal-type epithelium and fibrous tissue. Clinically, it is characterized by hypo- or achlorhydria and loss of intrinsic factor resulting in pernicious anemia. The immunological hallmark of pernicious anemia is the presence of autoantibodies to gastric parietal cells (AGPA), parietal cell antigen H+K+ATPase and intrinsic factor.¹⁻⁶

AGPA are a marker for autoimmune gastritis whereas intrinsic factor antibodies are more closely associated with pernicious anemia. The progression of chronic atrophic gastritis to pernicious anemia may occur over a long period of time (20 to 30 years).¹ For this reason, the detection of both types of antibodies is important as together they impart greater confidence in the diagnosis and progression of autoimmune gastritis as well as the diagnosis of pernicious anemia.

AGPA are detected either by immunofluorescence on mouse stomach sections or by ELISA. The gastric parietal cell antigen H+K+ATPase is a membrane antigen with two alpha and beta subunits.¹⁻⁴ Autoantibodies to gastric parietal cell antigen react to both alpha and beta subunits of H+K+ATPase. AGPA occur in about 90% of patients with pernicious anemia, 30% of first degree relatives of patients with pernicious anemia and up to 50% of adults and 18% of children with *H. pylori* infection.⁷⁻⁹ About 65% of patients with gastritis and *H. pylori* infection are AGPA

positive. The presence of AGPA in such cases is associated with the duration of *H.pylori* infection and the degree of lymphocytic infiltrate and atrophy of the glandular epithelium^{7,8}. In addition there is high incidence of AGPA in patients with various autoimmune endocrinopathies.^{1,2} Normal subjects exhibit an age related increase in the incidence of AGPA from 2 to 8%.¹⁰ Also there is a general increase in the number of persons with atrophic gastritis with an increase in age.

The Immulisa™ AGPA ELISA offers certain advantages over immunofluorescence methods. Detection of AGPA by immunofluorescence requires experience with fluorescence microscopy techniques and the results obtained can be subjective. In addition, if not performed on a suitable substrate, one may interpret false positive results due to heterophile antibodies. IMMCO provides a sensitive and specific ELISA method for detecting AGPA. Coupled with the Intrinsic Factor Antibody ELISA, the AGPA ELISA offers a complete laboratory solution for the diagnosis of autoimmune gastritis and pernicious anemia.

PRINCIPLES OF PROCEDURE

The test is performed as a solid phase immunoassay. Microwells are coated with native purified porcine H+K+ATPase antigen followed by blocking the unreacted sites to reduce non-specific binding. Controls and patient serum samples are incubated in the antigen coated wells which allows specific antibodies present in the serum to bind to the H+K+ATPase antigen. Unbound antibody and other serum proteins are removed by washing the microwells. Antibodies bound to the antigen on the microwells are detected by adding an enzyme labeled anti-human IgG conjugate. Unbound conjugate is removed by washing. Specific enzyme substrate (pNPP) is then added to the wells and the presence of antibodies is detected by a color change produced by the conversion of pNPP substrate to a colored reaction product. The reaction is stopped and the intensity of the color change, which is proportional to the concentration of antibody, is read by a spectrophotometer at 405 nm. Results are reported as positive, indeterminate (borderline) or negative with units (EU/ml).

Specimen (EU/ml)	Inter-assay CV
1 (28.7 EU/ml)	4.4%
2 (54.7 EU/ml)	5.1%
3 (63.8 EU/ml)	6.4%

Based on 16 replicates of three specimens, the intra-assay Coefficient of Variation (CV) of the AGPA Antibody ELISA test was calculated.

Specimen (EU/ml)	Intra-assay CV
1 (28.7 EU/ml)	2.6%
2 (53.8 EU/ml)	6.0%
3 (62.2 EU/ml)	6.8%

Linearity

To determine acceptable linearity, plates were assayed with calibrators of known values. The r-squared values of the resulting standard curves were determined for multiple assays. The average r² for standard calibration curve was 0.9970 with the lowest of 0.9911. R-squared values greater than 0.95 are deemed acceptable.

Recovery

Samples with known AGPA levels were mixed with appropriate dilutions of other positive samples with known amounts of AGPA. The antibody levels of the mixed samples were determined and used to calculate percent recovery. The results follow:

	Ab. conc. Expected (EU/ml)	Ab. conc. Obtained (EU/ml)	% Recovery
Sample 1	25	29	116
Sample 2	42	47	112
Sample 3	67	64	96
Sample 4	49	54	110
Sample 5	62	60	97
Sample 6	44	45	102
Sample 7	53	51	96
Sample 8	69	64	93
Sample 9	65	63	97

specimens were confirmed, indicating a close correlation of the IMMCO AGPA ELISA results with the presence of AGPA.

- B. **IMMCO ELISA vs. IFA:** The same set of specimens was also tested for AGPA by immunofluorescence on mouse kidney/stomach sections (IMMCO cat No. 1107). Results demonstrating correlation are summarized below.

		AGPA by immunofluorescence		
		Positive	Negative	Total
IMMCO	Positive	34	19	53
AGPA	Negative	3	79	82
ELISA	Total	37	98	135
	Relative Agreement:	83.7%		
	Relative Sensitivity:	91.9%		
	Relative Specificity:	80.6%		

- C. **Cross Reactivity:** A total of 106 potentially cross-reactive specimens, including patients with other autoimmune disorders and infectious diseases were tested for AGPA and intrinsic factor antibodies using the Immulisa™ systems. Of these, none of the samples from celiac disease patients were positive for AGPA. All celiac disease patients were children. However a significant number of patients with *H.pylori* and other disease controls were positive for AGPA. It is reported that approximately 60% of the adult population in the US has the *H. pylori* infection and that AGPA is associated with this infection. This explains the elevated incidence of positive results of AGPA in adults as contrasted with children.

Condition	# Tested	% Positive	
		AGPA	IF
Rheumatoid Arthritis	10	30	0
Celiac Disease	10	0	0
Hashimoto's Thyroiditis	10	30	0
Graves' Disease	10	30	0
<i>H. Pylori</i> positive	7	43	0
Hepatitis C positive	19	47	10
Blood donors	40	15	0

Precision

Based on 40 replicates of three specimens, the inter-assay Coefficient of Variation (CV) of the AGPA Antibody ELISA test was calculated.

REAGENTS

Storage and Preparation

Store all reagents at 2-8°C. **Do not freeze.**

Do not use if reagent is not clear or if a precipitate is present. All reagents must be brought to room temperature (22-30°C) prior to use.

Reconstitute the wash buffer to 1 liter with distilled or deionized water. When stored at 2-8°C, the reconstituted wash buffer is stable until the kit expiration date. Coated microwell strips are for one time use only.

Precautions

All human derived components used have been tested for HBsAg, HCV, HIV-1 and 2 and HTLV-I and found negative by FDA required tests. However, human blood derivatives and patient specimens should be considered potentially infectious. Follow good laboratory practices in storing, dispensing and disposing of these materials¹¹.

WARNING - Sodium azide (NaN₃) may react with lead and copper plumbing to form highly explosive metal azides. Upon disposal of liquids, flush with large volumes of water to prevent azide buildup. Sodium azide may be toxic if ingested. If ingested, report incident immediately to laboratory director or poison control center.

Instructions should be followed exactly as they appear in this kit insert to ensure valid results. Do not interchange kit components with those from other sources. Follow good laboratory practices to minimize microbial and cross contamination of reagents when handling. Do not use kit components beyond expiration date on the labels.

Materials provided

Immulisa™ Anti-Gastric Parietal Cell Antibody ELISA Catalog No. 1165

Kit contains sufficient reagents to perform 96 determinations.

12 x 8 Microplate with individual breakaway microwells. Coated with purified porcine H+K+ATPase antigen. Ready for use.

1 x 1.5 ml *Ready to use **Positive Control** (*red cap*). Contains human serum positive for gastric parietal cell antibodies. The expected concentration range in EU/ml is printed on the label.

1 x 1.5 ml *Ready to use **Negative Control** (*white cap*). Contains human serum.

- 4 x 1.5 ml** *Ready to use **set of 4 Calibrators**; Calibrator A (*green cap*), Calibrator B (*violet cap*), Calibrator C (*blue cap*) and Calibrator D (*yellow cap*). Human serum containing gastric parietal cell antibodies. Concentrations in EU/ml are printed on the labels.
- 1 x 12 ml** *Alk. Phos. goat anti-human IgG Conjugate. Ready for use. Color coded pink.
- 1 x 60 ml** *Serum Diluent. Ready for use. Color coded green.
- 1 x 12 ml** *Enzyme substrate. Contains pNPP. Ready for use.
Protect from light.
- 1 x 12 ml** Stop Solution. Ready for use.
- 2 x vials** Powder Wash Buffer. **Reconstitute to one liter each.**
- 1 x Extra** Frame Holder
- 2 x** Protocol Sheets
- *CAUTION - Contains <0.1% NaN₃

Materials Required But Not Provided

- Deionized or distilled water
- Squeeze bottle to hold diluted wash buffer
- Pipettes capable of delivering 5 µl to 1000 µl
- Disposable pipette tips
- Clean test tubes 12 x 75 mm and test tube rack
- Timer
- Absorbent paper towels
- Microplate reader capable of reading absorbance values at 405 nm. If dual wavelength microplate reader is available, the reference filter should be set at 600-650 nm
- Automatic microplate washer capable of dispensing 200 µl

SPECIMEN COLLECTION AND HANDLING

Only serum specimens should be used in this procedure. Grossly hemolyzed, lipemic or microbially contaminated specimens may interfere with the performance of the test and should not be used. Store specimens at 2°- 8°C for no longer than one week. For longer storage, serum specimens should be frozen. Avoid repeated freezing and thawing of samples.

Prevalence of Gastric Parietal Cell Antibodies^{8,10}

Disease Group	No. of Patients	Percent Positive
Normals	11,601	4
Duodenal ulcer	52	44
Gastric ulcer	14	79
Gastric carcinoma	44	70
Diabetes Type 1	229	30
Graves Disease	14	57
Chronic lymphocytic thyroiditis	115	27
<i>H. pylori</i>		
Children	54	28
Adults	26	84

Performance Characteristics

The Immulisa™ Anti-Gastric Parietal Cell Antibody ELISA was evaluated by testing AGPA positive and negative samples, disease controls and “normal” human sera and comparing with results obtained using commercially available ELISA and immunofluorescence assays.

Normal Range: 98 normal human sera specimens were tested on this system and the mean value was less than 12 EU/ml with a range of 1.8-72 EU/ml.

Comparative Sensitivity and Specificity

- A. **ImmLISA™ AGPA ELISA vs. Other Gastric Parietal Cell Antibody ELISA:** 137 specimens were tested with both systems. These studies show close correlation of the two systems.

		Other AGPA ELISA		
		Positive	Negative	Total
IMMCO	Positive	42	22	64
AGPA	Negative	0	73	73
ELISA	Total	42	95	137
Relative Agreement:		83.9%		
Relative Sensitivity:		100%		
Relative Specificity:		76.8%		

Samples testing positive on the IMMCO ELISA and negative on the other ELISA were confirmed by dot blot. Eleven of these twelve

Interpretation

Interpretation values were determined by testing 98 adult normal blood donors. The values depicted below are the mean of the normal subjects plus 3SD.

anti-AGPA Value	Interpretation
<20 EU/ml	Negative
20-25 EU/ml	Indeterminate (Borderline)
>25 EU/ml	Positive

LIMITATIONS OF PROCEDURE

Only serum specimens should be used in this procedure. Grossly hemolyzed, lipemic or microbially contaminated specimens may interfere with the performance of the test and should not be used. Store specimens at 2-8°C for no longer than one week. For longer storage, serum specimens should be frozen. Avoid repeated freezing and thawing of samples.

AGPA are present in 30% of first degree relatives of patients with pernicious anemia and in up to 50% of adults and 18% of children with *H. pylori* infections. In addition, there is 10% incidence of AGPA in patients with various autoimmune endocrinopathies. In normal subjects there seems to be an age related increase in the incidence of AGPA from 2 to 8%. Test results obtained by this assay should be considered in conjunction with antibodies to intrinsic factor, clinical and other laboratory findings.

EXPECTED VALUES

Test results in a normal population are expected to be negative. However, 4% of apparently healthy, asymptomatic individuals may test positive for gastric parietal cell antibodies.

The following table depicts the incidence of gastric parietal cell antibodies in normal individuals and patients with various diseases, as reported in the literature.

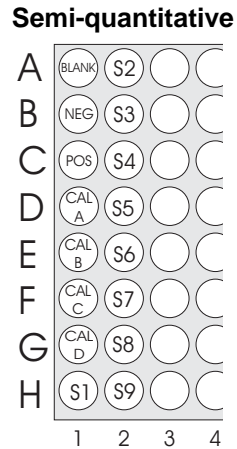
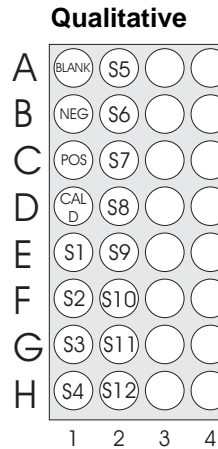
PROCEDURE

Procedural Notes

- Carefully read the product insert before starting the assay.
- Remove required microwell strips from the pouch and carefully reseal the pouch to prevent condensation in the unused wells. Return pouch immediately to refrigerator.
- Let patient specimens and test reagents equilibrate to room temperature before starting with the test procedure. Return all unused specimens and reagents to refrigerator immediately after use.
- All dilutions of the patient samples should be prepared prior to starting with the assay.
- **Good washing technique is critical.** If washing is performed manually, adequate washing is accomplished by directing a forceful stream of wash buffer with a wide tip wash bottle across the entire microplate. **An automated microplate washer is recommended.**
- Use a multichannel pipette capable of delivering 8 or 12 wells simultaneously. This speeds the process and provides more uniform incubation times.
- For all steps, careful control of timing is important. The start of all incubation periods begins with the completion of reagent addition.
- Addition of all samples and reagents should be performed at the same rate and in the same sequence.

Test Method

- Step 1** Let all reagents and specimens equilibrate to room temperature.
- Step 2** Label protocol sheet to indicate sample placement in the wells. It is good laboratory practice to run samples in duplicate.
- Step 3** For a **qualitative determination** use the Ready to Use Low Calibrator D (*vial with yellow cap*) only.
- or**
- For a **semi-quantitative determination** use the Ready to Use Calibrators A through D as depicted in the sample layout below.



- Step 4** Prepare a **1:101** dilution of the patient samples by mixing **5 µl** of the patient sera with **500ul** of Serum Diluent.
- Step 5** Remove the required microwells from pouch and return unused strips in the sealed pouch to refrigerator. Securely place the microwells into the extra provided holder.
- Step 6** Pipette **100 µl** of Ready to use Calibrators, Positive and Negative controls and diluted patient samples (**1:101**) to the appropriate microwells as per protocol sheet.
- Note:** Include one well which contains **100 µl** of the Serum Diluent as a reagent blank. Zero the ELISA reader against the reagent blank.
- Step 7** Incubate **30 minutes** (± 5 min) at room temperature.
- Step 8** Wash **4x** with wash buffer. For manual washing, fill each microwell with reconstituted wash buffer. Discard the fluid by inverting and tapping out the contents of each well or by aspirating the liquid from each well. To blot at the end of the last wash, invert strips and tap the wells vigorously on absorbent paper towels. For automatic washers, program the washer as per manufacturer's instructions.
- Step 9** Pipette **100 µl** of Conjugate into microwells.
- Step 10** Incubate **30 minutes** (± 5 min) at room temperature.
- Step 11** Wash all microwells as in Step 8.
- Step 12** Pipette **100 µl** of Enzyme Substrate into each microwell in the same order and timing as for the Conjugate.
- Step 13** Incubate **30 minutes** (± 5 min) at room temperature.
- Step 14** Pipette **100 µl** of Stop Solution into each microwell using the same order and timing as for the addition of the Enzyme

Substrate. Read absorbance values within 1 hour of adding Stop Solution.

- Step 15** Read absorbance of each microwell at **405 nm** using a single or at 405/630nm using a dual wavelength microplate reader against the reagent blank set at zero absorbance.

Quality Control

Calibrators, Positive and Negative Controls and a reagent blank must be run with each assay to verify the integrity and accuracy of the test. The absorbance reading of the reagent blank should be <0.3. The Calibrator A should have an absorbance reading of not less than 1.0, otherwise the test must be repeated. The negative control must be <20 EU/ml. If the test is run in duplicate, the mean of the two readings should be taken for determining EU/ml. While performing Qualitative determinations, the optical density of Calibrator D must be greater than that of the negative control and less than the absorbance of the positive control. For semi-quantitative determinations the positive control must give values in the range stated on the vial.

RESULTS

Calculations

The concentrations of the patient samples can be determined by either of two methods:

1. QUALITATIVE DETERMINATION

$$\frac{\text{Abs. of Test Sample}}{\text{Abs. of Calibrator D}} \times \text{EU/ml of Calibrator D} = \text{EU/ml Test Sample}$$

2. SEMI-QUANTITATIVE DETERMINATION

Plot the absorbance of Calibrator A through D against their respective concentrations on linear-linear graph paper. Plot the concentrations in EU/ml on the X-axis against the absorbances on the Y-axis and draw the best fit curve. Determine the concentrations of the patient samples from the curve according to corresponding absorbance values.

Calibrator

The Ready to Use Calibrators are included to provide semi-quantitation and must be used with each run. Patient samples containing high antibody levels may give absorbance values greater than that of Calibrator A. For determining accurate semi-quantitative values, such specimens should be further diluted (usually 1:400) so they fall within the range of the calibrator curve when retested. For determining EU/ml values, multiply the units obtained by the dilution factor.