



ImmuLisa™

Anti-Intrinsic Factor (IF) Antibody ELISA

For investigational use only

PRODUCT INSERT

REF 1164 96 Determinations

INTENDED USE

Enzyme linked immunosorbent assay (ELISA) for the qualitative and semi-quantitative detection of antibodies to intrinsic factor in human serum.

SUMMARY AND EXPLANATION

Pernicious anemia is one of the most common causes of Vitamin B₁₂ (cobalamin) deficiency. Vitamin B₁₂ deficiency can result in hematological, neurological, and psychiatric complications. Histologically, pernicious anemia is characterized by gastric mucosal atrophy, selective loss of parietal and chief cells from the gastric mucosa and sub mucosal lymphocytic infiltrate. Immunologically, the hallmark of pernicious anemia is the presence of autoantibodies against gastric parietal cells, proton pump (H+K+ATPase), and against the cobalamin absorbing protein, intrinsic factor.¹⁻³

Intrinsic factor is a 60 kD glycoprotein produced by the parietal cells of the stomach lining and enables the absorption of vitamin B₁₂. In acquired pernicious anemia there is a significant decrease in intrinsic factor expression due to the loss of intrinsic factor producing gastric parietal cells, which results in the body's inability to absorb vitamin B₁₂ in the stomach. Approximately 2% of the population aged >60 have undiagnosed pernicious anemia⁴⁻⁶.

Intrinsic factor antibodies are of the IgG isotype and occur in about 70% of patients with pernicious anemia. Anti-gastric parietal cell antibodies (AGPA) are usually detected by immunofluorescence on mouse stomach substrate or by ELISA using gastric H+K+ATPase as the antigen. These antibodies are diagnostic of autoimmune gastritis and not of pernicious anemia. For the diagnosis of pernicious anemia, intrinsic factor antibodies are more specific and very closely associated with pernicious anemia⁷⁻⁹. Antibodies to intrinsic factor are detected by

radioimmunoassay (RIA) or by enzyme linked immunosorbent assay (ELISA).¹⁰⁻¹²

ELISA offers advantages over RIA and other methods that rely on the ability of autoantibodies to inhibit the binding of labeled vitamin B₁₂ to intrinsic factor. First, ELISA detects antibodies of both Type I and II whereas RIA or other inhibiting assays detect only Type I antibodies. Second, RIA and other B₁₂ inhibiting antibody methods give false positive results as these assays are subject to interference by high B₁₂ levels. Patients with a suspected diagnosis of pernicious anemia may already be on vitamin B₁₂ therapy causing elevated levels of B₁₂ in circulation.

The IMMCO Intrinsic Factor Antibody ELISA provides an advantage over other similar ELISA assays. It incorporates a very pure recombinant glycosylated holoenzyme as the antigen which yields significantly improved sensitivity and specificity. Other ELISA systems primarily utilize porcine intrinsic factor which may have limitations related to purity and antigenicity of the preparation. In addition, porcine antigen may have vitamin B₁₂ already bound to it, which may interfere in detection of intrinsic factor antibodies.

PRINCIPLES OF PROCEDURE

The test is performed as a solid phase immunoassay. Microwells are coated with a recombinant glycosylated human intrinsic factor antigen followed by a blocking step to reduce non-specific protein binding during the assay run. Controls and patient sera are incubated in the antigen coated wells to allow specific antibodies present in the serum to bind to the intrinsic factor antigen. Unbound antibodies and other serum proteins are removed by washing the microwells. Bound antibodies are detected by adding an enzyme labeled anti-human IgG conjugate to the microwells. 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 or negative.

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 materials¹³.

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-Intrinsic Factor Antibody ELISA REF 1164

Kit contains sufficient reagents to perform 96 determinations.

12 x 8 MICROPLATE IF Microplate with individual breakaway microwells. Coated with purified recombinant glycosylated human intrinsic factor antigen. Ready for use.

1 x 1.5 ml CONTROL + IF* Ready to use **Positive Control** (*red cap*). Contains human serum positive for anti-intrinsic factor antibodies. The expected concentration range in EU/ml is printed on the label.

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

4 x 1.5 ml CALIBRATOR A IF*
CALIBRATOR B IF* Ready to use **set of 4 Calibrators**; Calibrator A (*green cap*), Calibrator

CALIBRATOR C IF*
CALIBRATOR D IF*

B (*violet cap*), Calibrator C (*blue cap*) and Calibrator D (*yellow cap*). Human serum containing antibodies to intrinsic factor antigen. Concentrations in EU/ml are printed on the labels.

- 1 x 12 ml IgG-CONJ ALKPHOS* Alk. Phos. goat anti-human IgG Conjugate. Ready for use. Color coded pink.
- 2 x 60 ml DIL* Serum Diluent. Ready for use. Color coded green.
- 1 x 12 ml SUBSTRATE* Enzyme substrate. Contains pNPP. Ready for use. **Protect from light.**
- 1 x 12 ml STOP Stop Solution. Ready for use.
- 2 x vials BUF WASH 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.

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

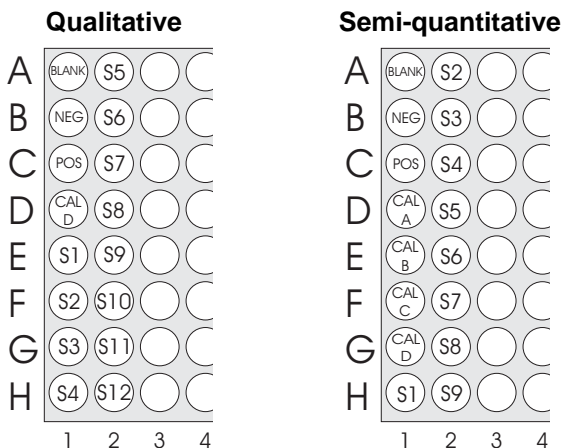
Step 1 Let all reagents and specimens equilibrate to room temperature.

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 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.

Interpretation

Interpretation values were determined by testing 40 adult normal blood donors. The values depicted below are the mean of the normal subjects plus 3SD.

anti-IF 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.

The presence of anti-intrinsic factor autoantibodies correlates well with cases of pernicious anemia and may be present in association with other autoimmune diseases, but rarely in normal subjects. Certain patients that are positive for Hepatitis C or H. pylori may also be positive for IF antibodies. Test results obtained by this assay should be considered in conjunction with clinical and other laboratory findings such as B₁₂ levels and the Schilling test.

EXPECTED VALUES

Test results in a normal population are expected to be negative. However, 0.1% - 0.2% of apparently healthy, asymptomatic individuals may test positive for anti-intrinsic factor antibodies.

The following table depicts the incidence of intrinsic factor antibodies in individuals diagnosed with pernicious anemia as reported in the literature.

Prevalence of Intrinsic Factor Antibodies in Pernicious Anemia

Study	No. of Patients	Intrinsic Factor Antibodies
Carmel 1 ¹⁴	34	78-88%
Carmel 2 ¹⁵	147	73%
Davidson ¹⁶	324	70%

Performance Characteristics

The ImmuLisa™ Intrinsic Factor Antibody ELISA for the detection of intrinsic factor antibodies was evaluated by testing well-characterized sera from patients with pernicious anemia alongside disease controls and “normal” human sera. These specimens were also tested on other commercially available kits and the results compared. Specimens were obtained from a reference laboratory utilizing a RIA method and from research groups studying cobalamin deficiency.

Normal Range: 40 normal human sera specimens were tested on the Intrinsic Factor ELISA and the mean value was less than 8 EU/ml.

Comparative Sensitivity and Specificity

A. Clinical Correlation: ImmuLisa™ Intrinsic Factor Antibody ELISA was tested with well characterized sera of patients with pernicious anemia: A total of 94 samples were tested on the IMMCO ELISA, including 10 pernicious anemia specimens, 14 healthy controls and 70 controls from patients with rheumatoid arthritis, celiac disease, Hashimoto’s thyroiditis, Graves’ disease, elevated *H. pylori* antibody levels, and hepatitis C.

		CLINICAL DIAGNOSIS		
		Positive	Negative	Total
IMMCO	Positive	10	5	15
IF ELISA	Negative	0	79	79
	Total	10	84	94
	Sensitivity:	100%		
	Specificity:	94%		

B. Immulisa™ anti-IF ELISA vs. a commercially available radioimmunoassay (RIA): A total of 47 specimens were tested on both systems, including 33 suspected pernicious anemia cases and 14 healthy controls. This study showed strong correlation in patients with low B12 levels, which is indicative of patients with pernicious anemia, but poor correlation in patients with normal or elevated B12 levels, which may be indicative of patients receiving B12 therapy. B12 therapy is known to cause false positive results on the RIA systems.¹²

C. Cross Reactivity: A total of 106 potentially cross-reactive specimens from individuals with other autoimmune disorders or infectious diseases were tested for IF antibodies using the Immulisa™ system. Of these, two Hepatitis C specimens were found positive for IF antibodies. The literature shows a higher incidence of IF antibodies in patients that are Hepatitis C positive.

Condition	#Tested	% Positive
Rheumatoid Arthritis	10	0%
Celiac Disease	10	0%
Hashimoto's Thyroiditis	10	0%
Graves' Disease	10	0%
<i>H. Pylori</i> positive	7	0%
Hepatitis C positive	19	11%
Normal Human Sera	40	0%

Precision

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

Specimen (EU/ml)	Inter-assay CV
1 (24.9 EU/ml)	4.6%
2 (50.3 EU/ml)	4.7%
3 (89.7 EU/ml)	1.2%

Based on 16 replicates (specimens 1 and 2) or 8 replicates (specimen 3) of three specimens, the intra-assay Coefficient of Variation (CV) of the IF Antibody ELISA test was calculated.

Specimen (EU/ml)	Intra-assay CV
1 (26.3 EU/ml)	4.6%
2 (46.7 EU/ml)	3.0%
3 (83.1 EU/ml)	2.6%

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. An r-squared value greater than 0.95 is deemed to be acceptable. The average r-squared value for this assay was greater than 0.99.

Recovery

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

Anti-IF	Ab. conc. expected (EU/ml)	Ab. conc. obtained (EU/ml)	% Recovery
Sample 1	30	27	90
Sample 2	45	42	93
Sample 3	40	41	103
Sample 4	75	60	80
Sample 5	47	43	92
Sample 6	42	42	100
Sample 7	57	59	104
Sample 8	76	73	96

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