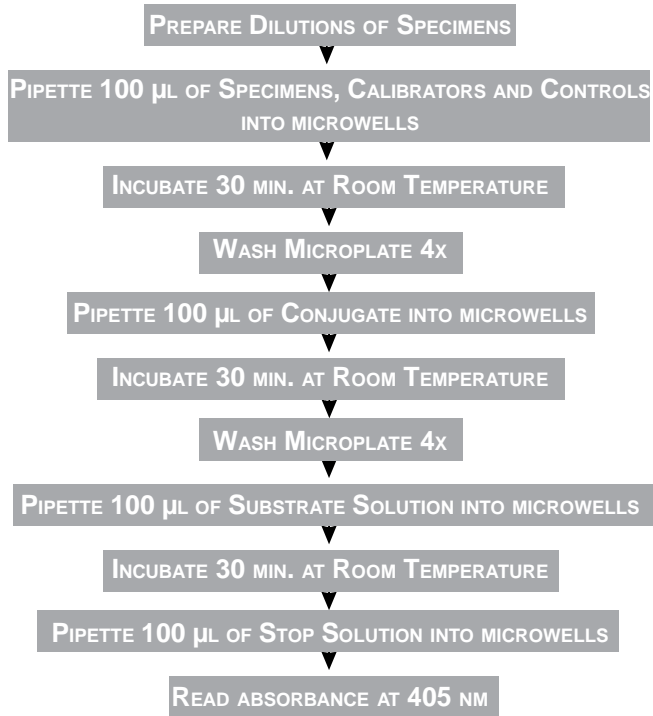


Immuliisa™ PROCEDURE AT A GLANCE



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Immuliisa™ Anti-Bactericidal/Permeability- Increasing Protein (BPI) Antibody ELISA

For Investigational Use Only

PRODUCT INSERT

Catalog No. 1155

96 Determinations

INTENDED USE

An enzyme linked immunosorbent assay (ELISA) for the detection and semi-quantitation of antibodies to bactericidal/ permeability-increasing protein (BPI) in human serum, to be used as an adjunct to clinical and other laboratory findings in the diagnosis of inflammatory bowel disease, primary sclerosing cholangitis and cystic fibrosis.

SUMMARY AND EXPLANATION

The presence of anti-neutrophil cytoplasmic antibodies (ANCA) in patients with vasculitis was first observed in 1982 by Davies *et al*. ANCA are a group of autoantibodies directed against proteins in the granules of neutrophils and in peroxidase-positive lysosomes of peripheral blood monocytes. The major antigens involved are proteinase 3 (PR3) and myeloperoxidase (MPO)². Autoantibodies against these antigens occur primarily in *Wegner's glomerulonephritis*, *microscopic polyangitis* and in many other autoimmune disorders characterized by vasculitis and inflammation^{3,4}. Recently, ANCA directed against another polymorphonuclear antigen have been described⁵⁻⁷. This antigen is an integral cell membrane, cationic, anti-microbial protein with a 55kD molecular weight and is identified to be BPI. Anti-BPI antibodies are closely associated with *ulcerative colitis*, *primary sclerosing cholangitis*, Crohn' disease, and cystic fibrosis⁵⁻⁷. On indirect immunofluorescence antibodies to BPI give a p-ANCA reaction pattern. The incidence of BPI-antibodies varies from 69% in primary sclerosing cholangitis, 49% in ulcerative colitis, 17% in Crohn's, and as high as 91% in Cystic Fibrosis⁵⁻¹⁰. Though, the pathophysiological nature of BPI antibodies remains to be established, it is generally accepted that BPI antibodies attenuate the endotoxin-neutralizing capacities of BPI, leading to over-expression of LPS as a bacterial stimuli. In conclusion, BPI represents one of the major ANCA specificity antigens and antibodies to which are recognized as a seromarker for a distinct disease entity.

PRINCIPLES OF PROCEDURES

The test is performed as a solid phase enzyme labeled immunosorbent assay (ELISA) in microwells coated with purified BPI antigen. Controls, calibrators and patient serum samples are incubated in the microwells allowing anti-BPI antibodies present in the serum to bind to the antigen. Unbound antibody and other serum proteins are removed by washing the microwells. Bound antibodies are incubated with an enzyme labeled anti-human IgG conjugate. Unbound conjugate is removed by washing the microwells. 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 the 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 expressed in Enzyme Units per milliliter (EU/ml).

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 (20-25°C) prior to use. When stored at 2-8°C, the reconstituted wash buffer is stable until the kit expiration date. Reconstitute the wash buffer to 1 liter with distilled or deionized water. 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 other than the same catalog number from IMMCO DIAGNOSTICS. Follow good laboratory practices to minimize microbial and cross contamination of reagents when handling. Do not use beyond expiration date on the label.

NOTES

REFERENCES

1. Davies DJ, Moran JE, Niall JF et al. Segmental necrotizing glomerulonephritis with antineutrophil antibody: possible arbovirus etiology? *Br Med J*; 1982, 285:606.
2. Hagen EC, Ballieux BE, van Es LA et al. Antineutrophil cytoplasmic autoantibodies: a review of the antigens involved, the assays, and the clinical and possible pathogenetic consequences. *Blood*; 1993, 81:1996-2002.
3. van der Woude FJ, Rasmussen N, Lobatto S et al. Autoantibodies against neutrophils and monocytes: tool for diagnosis and marker of disease activity in Wegener's granulomatosis. *Lancet*; 1985, 1:425-429.
4. Falk RJ, Jennette JC. Anti-neutrophil cytoplasmic autoantibodies with specificity for myeloperoxidase in patients with systemic vasculitis and idiopathic necrotizing and crescentic glomerulonephritis. *N Eng J Med*; 1988, 318:1651-1657.
5. Stoffel MP, Csernok C, Herzberg T et al. Anti-neutrophil cytoplasmic antibodies (ANCA) directed against bactericidal/permeability increasing protein (BPI): a new seromarker for inflammatory bowel disease and associated disorders. *Clin. Exp. Immunol*; 1996, 104:54-59
6. Zhao MH, Jayne DRW, Ardiles LG et al. Autoantibodies against bacterial/permeability-increasing protein in patients with cystic fibrosis. *Q.J. Med*; 1996, 89: 259-265.
7. Zhao MH, Jones SJ and Lockwood CM. Bacterial/permeability-increasing protein (BPI) is an important antigen for anti-neutrophil cytoplasmic autoantibodies (ANCA) in vasculitis. *Clin Exp Immunol*; 1995, 99:49-56.
8. Cambridge G, Rampton DS, Stevens TRJ et al. Anti-neutrophil antibodies in inflammatory bowel disease: prevalence and diagnostic role. *Gut*; 1992, 33:668-674.
9. Hertevig E, Wieslander J, Johansson C, et al. Anti-neutrophil cytoplasmic antibodies in chronic inflammatory bowel disease. Prevalence and diagnostic role. *Scand J Gastroenterol*; 1995, 30:693-698.
10. Vecchi M, Bianchi MB, Sinico RA et al. Antibodies to neutrophil cytoplasm in Italian patients with ulcerative colitis: sensitivity, specificity and recognition of putative antigens. *Digestion*; 1994, 55:34-39.
11. Biosafety in Microbiological and Biomedical Laboratories. Centers for Disease Control, National Institutes of Health, (HHS Pub No [CDC] 93-8395), 1993.

Materials provided

Catalog No. 1155

ImmuLisa™ Anti-Bactericidal/ Permeability-Increasing Protein (BPI) Antibody ELISA. Kit contains sufficient reagents to perform 96 determinations.

- 12 x 8** Ready to use **Microplate** with individual breakaway microwells coated with BPI antigen. .
- 1 x 1.5 ml** *Ready to use **Positive Control** (*red cap*). Contains human serum positive for anti-BPI 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 antibodies to BPI antigen. Concentrations in EU/ml are printed on the labels.
- 1 x 12 ml** *Ready to use **anti-human IgG Alk. Phos. Conjugate**. Color coded pink.
- 2 x 60 ml** *Ready to use **Serum Diluent**. Color coded blue.
- 1 x 12 ml** *Ready to use **Enzyme Substrate**. Contains pNPP. **Protect from light**.
- 1 x 12 ml** Ready to use **Stop Solution**.
- 2 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 630 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

- Before starting with the assay read the product insert carefully .
- Let serum specimens and test reagents equilibrate at 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 wells simultaneously. This speeds the process and provides for a more uniform incubation time.
- 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.
- Remove required microwell strips from the pouch and carefully reseal the pouch to prevent condensation in the unused wells. Return pouch immediately to refrigerator.

Table 2

Incidence of Anti-BPI Antibodies in Various Disorders^{5,6}

Diagnosis	No. of Patients Tested	No. of Patients Positive	% Positive
Ulcerative Colitis	54	20	37
Crohn's Disease	44	10	23
Cystic Fibrosis	66	60	91
Primary sclerosing cholangitis	36	13	15
Wegener's Granulomatosis	86	13	15
Microscopic Polyangiitis	63	9	14
Henoch-Schönlein Purpura	22	1	5
IgA Nephropathy	10	0	0
Normal Donors	206	1	0

PERFORMANCE CHARACTERISTICS

ImmULisa™ Anti-BPI

	Positive	Negative	Total
Other ELISA	29	1	30
Positive	29	1	30
Negative	0	63	63
Total	29	64	93

Relative Agreement: 98.9%
 Relative Sensitivity: 96.6%
 Relative Specificity: 100.0%

Interpretation

The following serves only as a guide in the interpretation of the laboratory results. Each laboratory must determine its own normal values. These may vary with the population examined.

anti-BPI value	Interpretation
<15 EU/ml	Negative
15-20 EU/ml	Indeterminate (Borderline)
>20 EU/ml	Positive

LIMITATIONS OF THE PROCEDURE

The ImmuLisa™ Anti-BPI test should not be performed on grossly hemolyzed, microbially contaminated or lipemic samples. This method should be used for testing human serum samples only. A diagnosis should not be made solely on the basis of ELISA test results alone.

EXPECTED VALUES

Antibodies to polymorphonuclear antigens as detected by indirect immunofluorescence are present in various autoimmune disorders (Table 1). Three distinct staining patterns are usually observed on indirect immunofluorescence *i.e.* c-cytoplasmic, p-perinuclear and x or a- atypical. Anti-BPI antibodies are usually associated with p-ANCA reaction pattern. Anti-BPI antibodies are of IgG and IgA isotypes. IgA isotype anti-BPI antibodies are almost always associated with IgG isotype anti-BPI antibodies. The significance of anti-BPI antibodies in various diseases is summarized in Table 2.

Table 1

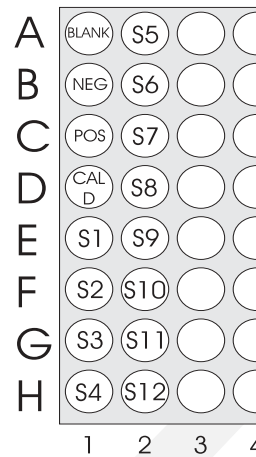
Incidence of ANCA by Indirect Immunofluorescence^{5, 8-10} (on ethanol fixed human polymorphonuclear leukocytes in gastrointestinal and liver disorders)

Diagnosis	No. of Patients Tested	No. of Patients Positive	% Positive
Ulcerative Colitis	307	151	49
Crohn's Disease	226	38	17
Celiac Disease	91	4	<1
Primary Sclerosing Cholangitis	36	25	69
Disease Controls	14	0	0
Normals	120	1	<1

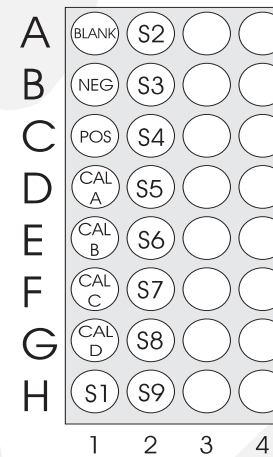
Test Method

- Step 1** Let all reagents and specimens equilibrate at 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 only the Ready to Use Low Calibrator D (*vial with yellow cap*).
- or** For a **semi-quantitative determination** use the Ready to Use Calibrators A through D as depicted in the sample layout below.

QUALITATIVE DETERMINATION



SEMI-QUANTITATIVE DETERMINATION



- Step 4** Prepare a **1:201** dilution of the patient samples by mixing **5 µl** of the patient sera with **1.0 ml** 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 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 from adding Stop Solution.
- Step 15** Read absorbance of each microwell at **405 nm** using a single or 405/630nm 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 the Calibrator D must be greater than that of the negative control and lesser 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:

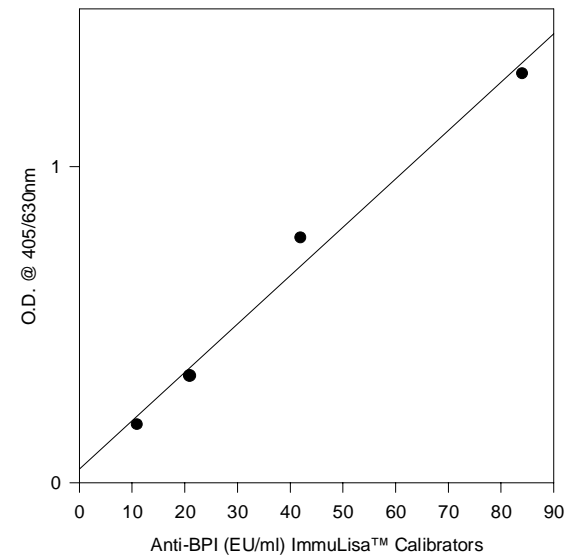
1. QUALITATIVE DETERMINATION

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

2. SEMI-QUANTITATIVE DETERMINATION

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

Anti-BPI Immulisa™ Standard Curve



Calibrator

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