ImmuLisa™ Procedure at a Glance

Prepare Dilutions of Specimens

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Pipette 100 µL of Specimens, Calibrators and Controls into microwells

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Incubate 30 min. at Room Temperature

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Wash Microplate 4x

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Pipette 100 µL of Conjugate into microwells

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Incubate 30 min. at Room Temperature

↓

Wash Microplate 4x

↓

Pipette 100 µL of Substrate Solution into microwells

↓

Incubate 30 min. at Room Temperature

↓

Pipette 100 µL of Stop Solution into microwells

↓

Read absorbance at 405 nm

For technical assistance please contact:

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ImmuLisa™

Anti-Glomerular Basement Membrane (GBM) Antibody ELISA

IVD

PRODUCT INSERT

Catalog No. 1154 96 Determinations

INTENDED USE

An enzyme linked immunosorbent assay (ELISA) for the detection and semi-quantitation of anti-glomerular basement membrane (GBM) antibodies in human serum. The presence of GBM antibodies can be used as an adjunct to clinical and other laboratory findings in the diagnosis of autoimmune renal disorders such as *Goodpasture's Syndrome*.

SUMMARY AND EXPLANATION

Rapidly progressive glomerulonephritis (RPGN) is a clinical syndrome developing over days or weeks characterized by crescentic glomerulonephritis on histopathology of the kidney. The prognosis is poor if not recognized early and if an appropriate treatment is not instituted. To optimize patient management, RPGN may be classified based on a) clinical assessment, b) direct immunofluorescence and electron microscopic studies of renal biopsy and c) serum antibody studies.

Using the above criteria, RPGN may be classified into a) immune complex mediated disease characterized by the presence of anti-DNA antibodies or anti-streptococcal antibodies, b) anti-glomerular basement membrane (GBM) mediated glomerulonephritis and Goodpasture's Syndrome and c) anti-neutrophil cytoplasmic antibody (ANCA) associated glomerulonephritis. In a study by Jayne et al[1], of 889 RPGN suspected patients, 47 (5%) had anti-GBM, 246 (28%) had ANCA and 576 (65%) had neither antibodies. Two percent had both ANCA and anti-GBM antibodies. Anti-GBM antibodies can be detected by indirect immunofluorescence or by ELISA[2-10]. The antigen associated with anti-GBM antibodies is a non-collagenous domain of collagen IV.

PRINCIPLES OF PROCEDURE

The ELISA test is performed in microwells coated with purified GBM antigen. Controls, calibrators and patient serum samples are incubated in the microwells allowing anti-GBM 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 materials[11]. WARNING - Sodium azide (NaN_3) 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.

Materials provided

ImmuLisa™ Anti-Glomerular-Basement Membrane (GBM) Antibodies *Catalog No. 1154*

Kit contains sufficient reagents to perform 96 determinations.

12 x 8 Ready to use **Microplate** with individual breakaway microwells coated with GBM antigen.

1 x 1.5 ml *Ready to use **Positive Contro**l *(red cap*). Contains human serum positive for anti-GBM 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 GBM 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% NaN_3

REFERENCES

1. Jayne DRW, Marshall PD, Jones SJ and Lockwood CM. Autoantibodies to GBM and neutrophil cytoplasm in rapidly progressive glomerulonephritis. Kidney Int; 1990, 37:965-970.
2. Saxena R, Bygren P, Arvastson B, Wieslander J. Circulating autoantibodies as serological markers in the differential diagnosis of pulmonary renal syndrome. J Intern Med. 1995. 238:143-152.
3. Wieslander J, Bygren P, Heinegård D. Anti-basement membrane antibody: Immunoenzymatic assay and specificity of antibodies. Scand J Clin Lab Invest. 1981. 41:763-772.
4. Butkowski R, Langeveld J, Wieslander J, Hamilton J, Hudson BG. Localization of the Goodpasture epitope to a novel chain of basement membrane collagen. J Biol Chem, 1987; 262:7874-7877.
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7. Wieslander J, Bygren P, Heinegård D. Isolation of the specific glomerular basement membrane antigen involved in Goodpasture Syndrome. Proc Natl Acad Sci USA. 1984. 81:1544-1548.
8. Wieslander J, Barr JF, Butkowski RJ, Edwards SJ, Bygren P, Heinegård D, and Hudson BG. Goodpasture antigen of the glomerular basement membrane: localization to noncollagenous regions of type IV collagen. Proc Natl Acad Sci USA. 1994. 81:3838-3842.
9. Hudson BG, Wieslander J, Wisdom B, Noelken ME. Biology of disease. Goodpasture Syndrome: Molecular architecture and function of the basement membrane antigen. Lab Invest. 1989. 61:256-269.
10. Hudson BG, Reeders ST, Tryggvason K. Type IV collagen: Structure, gene organization, and role in human disease. J Biol Chem. 1993. 268:26033-26036.
11. Biosafety in Microbiological and Biomedical Laboratories. Centers for Disease Control, National Institutes of Health, 1993 [HHS Pub. No. (CDC) 93-8395].

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.

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-GBM value	Interpretation
<20 EU/ml	Negative
20-25 EU/ml	Indeterminate (Borderline)
>25 EU/ml	Positive

LIMITATIONS OF THE PROCEDURE

The results obtained serve only as an aid in the diagnosis of GBM antibody mediated nephritis and *Goodpasteur's Syndrome* and should not be interpreted as diagnostic in themselves. Serum of several patients with RPGN may be negative for anti-GBM antibodies

EXPECTED VALUES

Prevalance of anti-GBM Antibodies in different disease groups

Disease	No.	Negative	Positive	% Positive
Normals	120	120	0	0
Anti-GBM	65	1	64	98.4
Nephritis				
Systemic Vasculitis	40	38	1	2.5
Systemic Lupus				
Erythematosus	40	40	0	0
Rheumatoid Arthritis				
or Scleroderma	40	39	1	2.5

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 carefully the product insert.
- 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.

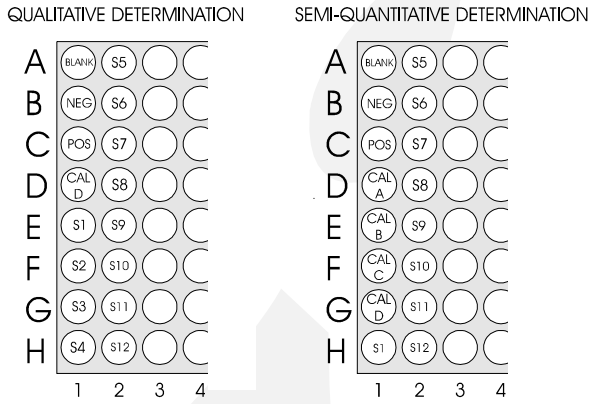
Test Method

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.



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.