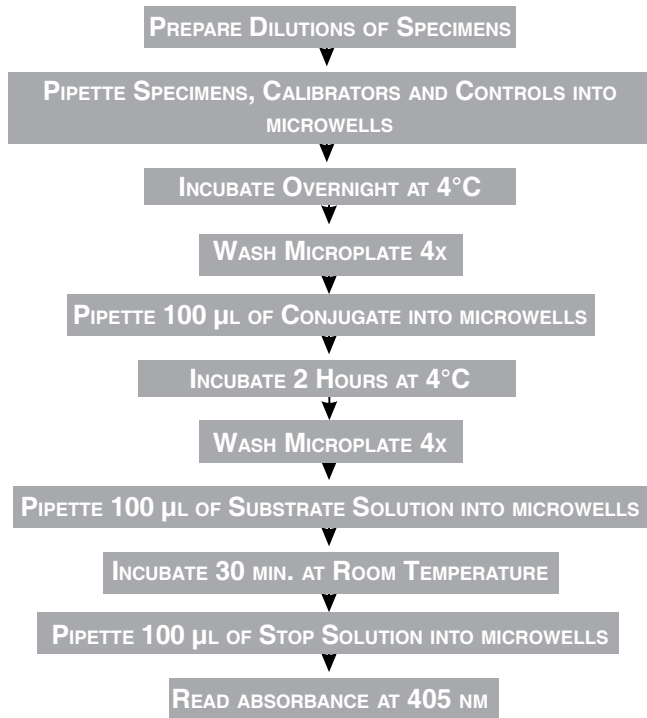


ImmuLisa™ PROCEDURE AT A GLANCE



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ImmuLisa™ Ganglioside Antibody ELISA

For Research Use Only

PRODUCT INSERT

Product Code 1180M / 1180G	anti-GM ₁ IgM / IgG	96 Determinations
Product Code 1181M / 1181G	anti-Asialo GM ₁ IgM / IgG	96 Determinations
Product Code 1183M / 1183G	anti-GD _{1b} IgM / IgG	96 Determinations
Product Code 1184M / 1184G	anti-GQ _{1b} IgM / IgG	96 Determinations
Product Code 1185M / 1185G	anti-Galactocerebroside IgM / IgG	96 Determinations
Product Code 1186M / 1186G	anti-GD _{1a} IgM / IgG	96 Determinations

INTENDED USE

These enzyme-linked immunosorbent assays (ELISA) are intended for the semi-quantitative detection of antibodies to gangliosides in human serum to be used as adjunct to clinical indications of patients with motor-neuron disorders / neuropathies.

SUMMARY AND EXPLANATION

Gangliosides are acidic glycosphingolipids localized in the outer layer of plasma membranes and occur in a variety of motor-neuron disorders / neuropathies. Neuropathies can be generally debilitating, and sometimes fatal if they affect vital organ function. Neuropathies can be hereditary conditions, or result from an infection or an autoimmune disease. Various neuropathies often present with similar symptoms and hence are difficult to diagnose. Accurate diagnosis is important since proper treatment modality and prognosis may differ depending on the particular neuropathy¹.

Some neuropathies, Guillain-Barré Syndrome (GBS) in particular, are better characterized than others. GBS refers to an autoimmune disorder characterized by rapid limb weakness and loss of tendon reflex and sensory dysfunctions. GBS is an acute inflammatory neuropathy. Patients with GBS elicit antibodies to GM₁ gangliosides^{2,3}. These antibodies are reported in patients with severe axonal damage and are directed against the galactose-galactosamine structure of the carbohydrate portion of the molecule⁴. GBS is a typical post-infectious autoimmune disorder. More than two thirds of GBS patients have an antecedent infection, usually mediated by a virus or bacterium. *Cytomegalovirus*, associated with upper-respiratory tract infections, and *Campylobacter jejunii*, associated with gastroenteritis, initiate a cross reactive immune response against gangliosides, resulting in immune complex deposition and demyelination of nerves leading to slow nerve conduction or blockage⁵.

GM₁, GD_{1a}, GD_{1b} and Asialo GM₁ share the same terminal Gal (β1-3) GalNAc residues. Increased titers of IgM antibodies against GM₁, GD_{1a}, GD_{1b} and Asialo GM₁ are found to co-exist in several types of motor-neuropathies⁶. Among these, the presence of anti-GM₁ antibodies are preferentially associated with multi-focal motor neuropathies and are never observed in lower motor neuron diseases⁷. This observation distinguishes them from anti-GD_{1b} and anti-Asialo GM₁ antibodies, which are preferentially observed in cases with lower motor neuropathies.

Serological profiles for anti-ganglioside antibodies in treated and untreated GBS patients have been reported⁸. Anti-GM₁ IgG and anti-GD_{1a} IgM titers peaked around forty (40) and ninety (90) days respectively following treatment. Anti-GM₁ IgG titers decreased following intravenous immunoglobulin (IVIg) treatment. The clinical utility of these antibodies is demonstrated in the correlation of antibody peaks with higher disability scores and worse clinical outcome⁸. Anti-GD_{1a} IgG antibodies are also found in 23% of patients with Multiple Sclerosis (MS) and 18% with Optic Neuritis (ON)⁹. The augmented GD_{1a} IgG responses can be used as a discriminatory feature between MS and GBS⁹. Immunopathological studies suggest that the target of immune attack is different in the various subtypes of GBS. In Acute Motor Axonal Neuropathy, the attack is directed against the axolemma and nodes of Ranvier. Antibodies to GD_{1a} selectively bind to motor nerve nodes rather than sensory nerve nodes of Ranvier, suggesting their pathogenic role¹⁰.

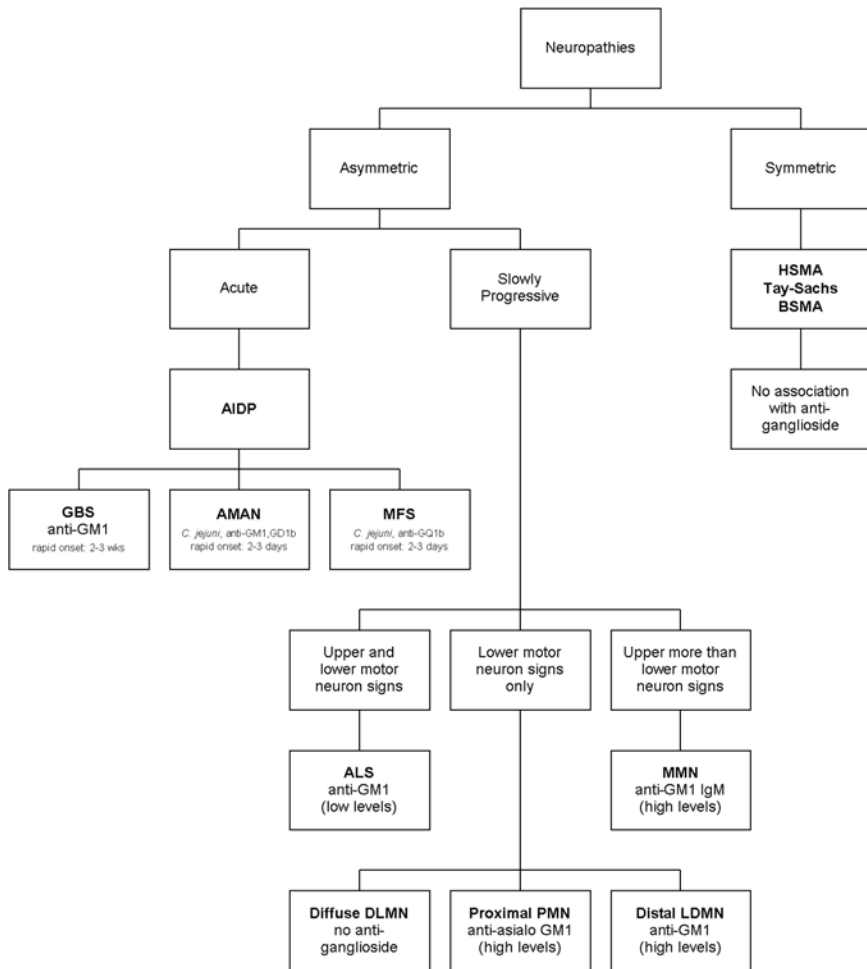
Anti-GM₁ and anti-GD_{1b} antibodies have also been reported in patients with multiple sclerosis (MS), systemic lupus erythematosus (SLE), Alzheimer's disease and certain normal individuals. It is important to note that anti-GM₁ and related antibody levels with a titer greater than 1:800 are strongly and specifically associated with lower motor neuron disease, sensory-motor neuropathy and motoneuropathy¹¹.

Autoimmune responses in patients suffering from other neuropathies can be directed against GQ_{1b} and sulfatides gangliosides⁷. GQ_{1b} antibodies are associated with Miller Fisher syndrome, an unusual variant of GBS, afflicting patients with *ophthalmoplegia*, *ataxia* and *areflexia* and often follow an infectious episode. Anti-galactocerebroside antibodies appear to be closely related to anti-sulfatides antibodies and have been observed in patients with *leprous neuritis*, *African trypanosomiasis* and *muco-cutaneous leishmaniasis*. Ganglioside antibodies of clinical significance are usually of IgM and/or IgG isotypes. It has been demonstrated that plasma exchange is an effective therapeutic measure, provided it is initiated not later than two weeks after the onset of symptoms¹². Hence, laboratory tests based on early detection of antibodies to various gangliosides can effectively complement such therapies.

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Figure 1. Disease Association of Anti-Ganglioside Antibodies



Legend

- GBS = Guillain-Barré Syndrome
- AMAN = Acute Motor Axonal Neuropathy
- MFS = Miller-Fisher Syndrome
- ALS = Amyotrophic Lateral Sclerosis
- MMN = Multifocal Motor Neuropathy
- HSMA = Hereditary Spinal Muscular Atrophy
- LDMN = Lower Distal Motor Neuron Syndrome
- PMN = Proximal Motor Neuron Syndrome
- DLMN = Diffuse Lower Motor Neuron Syndrome
- AIDP = Acute Inflammatory Demyelinating Polyneuropathy

PRINCIPLES OF PROCEDURES

The ImmuLisa™ anti-ganglioside antibody assays are performed as solid phase enzyme-linked immunosorbent assays (ELISA). Purified antigen-coated microwells are used to incubate Controls, Calibrators and patient serum samples to allow specific antibodies present in serum to bind to the respective antigen. Unbound antibodies and other serum proteins are removed by washing the microwells. After addition and incubation with an enzyme labeled anti-human conjugate, unbound conjugate is removed by washing the microwells. The addition of pNPP enzyme substrate to the microwells results in a color change produced by the conversion of the substrate to a yellow reaction product. The reaction is stopped and the intensity of the color change, which is proportional to the concentration of antibody, is read on a spectrophotometer at 405 nm. Antibody concentration range is expressed in ELISA Units per milliliter (EU/ml) on the Calibrator and Positive Control. These values are used to determine compliance with assay quality controls and to determine qualitative results. Positive specimen results should be expressed in titer.

REAGENTS

Storage and Preparation

Store all reagents at 2-8°C. **Do not freeze.** Do not use if reagents exhibit turbidity or if a precipitate is present. **All reagents must be kept at 2-8°C throughout their use,** except Enzyme Substrate, which must be brought to room temperature (20-25°C) prior to use. When stored at 2-8°C, reconstituted Wash Buffer is stable until kit expiration date.

Precautions

All human derived components 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. If additional reagents are required, use only components with the same lot 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

Product	Catalog No.	Product	Catalog No.
ImmuLisa™ anti-GM ₁ IgM	1180M	ImmuLisa™ anti-GM ₁ IgG	1180G
ImmuLisa™ anti-Asialo GM ₁ IgM	1181M	ImmuLisa™ anti-Asialo GM ₁ IgG	1181G
ImmuLisa™ anti-GD _{1b} IgM	1183M	ImmuLisa™ anti-GD _{1b} IgG	1183G
ImmuLisa™ anti-GQ _{1b} IgM	1184M	ImmuLisa™ anti-GQ _{1b} IgG	1184G
ImmuLisa™ anti-Galactocerebroside IgM	1185M	ImmuLisa™ anti-Galactocerebroside IgG	1185G
ImmuLisa™ anti-GD _{1a} IgM	1186M	ImmuLisa™ anti-GD _{1a} IgG	1186G

Kits contain sufficient reagents to perform 96 determinations.

- 12 x 8** Ready to use **Microplate** with individual breakaway microwells coated with respective ganglioside antigen. Remove strips as needed and return in sealed pouch to refrigerator. Coated microwells are for one time use only.
- 1 x 1.5 ml** Ready to use **Positive Control*** (*red cap*). Contains human serum positive for respective ganglioside antibody. Expected concentration range in EU/ml is printed on the vial label.
- 1 x 1.5 ml** Ready to use **Negative Control*** (*white cap*). Contains ganglioside antibody negative human serum.
- 1 x 1.5 ml** Ready to use **Calibrator*** (*green cap*). Derived from human serum containing antibodies to respective gangliosides. Concentration in EU/ml is printed on the vial label.
- 1 x 12 ml** Ready to use **anti-human IgM or IgG Alk. Phos. Conjugate*†**. Color coded pink.
- 1 x 60 ml** Ready to use **Serum Diluent***. Color coded blue.
- 1 x 12 ml** Ready to use **Stop Solution**
- 2 vials** Powder **Wash Buffer**. Reconstitute each vial with 1 liter distilled or deionized water.

Above reagents must be used cold at 2-8°C.

- 1 x 12 ml** Ready to use **Enzyme Substrate***. Contains pNPP. **Protect from light.**

- 2 x** Protocol Sheets

* CAUTION - Contains <0.1% NaN₃
 † Conjugate isotype matches final character of code number (e.g. isotype of conjugate in 1180M is IgM).

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

LIMITATIONS OF THE PROCEDURE

Test results obtained by this assay alone are not diagnostic and should be considered in conjunction with the clinical presentation of the patient. Any test with borderline reactivity should be retested to confirm the result. It is also recommended that patients with borderline results be retested after a subsequent blood draw. Immunosuppressive therapy, plasmapheresis, initiation or alteration in the treatment of a patient with neuropathy should not be performed on the basis of positive reaction in this assay. All clinical observations and, most importantly, electrophysiological data such as motor nerve conduction block or other related symptoms should be taken into consideration along with the results of the ganglioside assay. Serum from some patients with motor neuropathies may be negative for certain gangliosides. Such patients should be tested for autoantibodies to other gangliosides.

EXPECTED VALUES

The following flow chart depicts the presence of anti-ganglioside antibodies in certain well characterized neuropathies^{1,7}. This chart is only to assist in the differential diagnosis and should not be used as conclusive information of all neuropathies listed. See limitations of the procedure.

RESULTS

Calculations

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

1. Qualitative

Results obtained by this method should be reported as positive or negative.

Abs. of Test Sample

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

Abs. of Calibrator




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

2. Quantitative

Quantitative values may be determined for samples testing positive using the qualitative method. Quantitative values for positive specimens should be expressed in titer of the specimen.¹⁴ To determine the titer of positive patients, make four-fold serial dilutions of the patient sample starting from 1:100 and perform the assay as indicated in Test Method. The last dilution that has an EU/ml greater than 25 is the endpoint titer of the patient. A table has been provided below to indicate proper preparation of the four-fold dilution series.

Tubes	1	2	3	4
Serum	10 µl			
	+			
Serum Diluent	1000 µl	800 µl	800 µl	800 µl
				
Transfer		200 µl	200 µl	200 µl
Final dilution	1:100	1:400	1:1600	1:6400

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 at -20°C. Avoid repeated freezing and thawing of samples.

PROCEDURE

Procedural Notes

- Read Product Insert carefully before starting the assay.
- Remove required microwell strips from pouch and, to prevent condensation, immediately return unused microwells in sealed pouch to refrigerator.
- **Let Enzyme Substrate equilibrate to room temperature prior to use. All other reagents, including wash buffer should be kept at 2-8°C and used cold.** Return unused samples and reagents to refrigerator immediately after use.
- Dilute patient samples immediately prior to starting the assay and store dilutions at 2-8°C until use.
- Prepare wash buffer and equilibrate its temperature to 2-8°C. Wash buffer cannot be prepared just prior to running the assay.
- **Good washing technique is critical and automated microplate washer is recommended.** If washing is performed manually, adequate washing can be accomplished by directing a forceful stream of **cold** wash buffer across all microwells with a wide tip wash bottle. However, manual washing is not recommended for this assay.
- Use multichannel pipette capable of pipetting simultaneously into 8 well strip. This speeds the process and provides for more uniform incubation times.
- Careful control of timing is important for all steps. Pipetting of all samples and reagents should be performed at the same rate and in the same sequence and start time of all incubations beginning with the completion of reagent addition. Each incubation period must be timed independently from previous one.

Test Method

- Step 1** Maintain 2-8°C temperature of all reagents (except Enzyme Substrate) and microtiter plate strips through Step 11 of the assay.
- Step 2** Label protocol sheet to indicate sample placement in the wells according to the following figure. It is good laboratory practice to run samples in duplicate.
- Step 3** Prepare a **1:101** dilution of the patient serum samples by mixing **5 µl** of serum samples with **500 µl** of **cold** Serum Diluent.
- Step 4** Remove the required microwells from the **cold** pouch and return unused strips in the sealed pouch to refrigerator. Securely place microwell strips into the extra provided holder.
- Note:** While performing Steps 1 through 5, microplate should be kept on ice packs to maintain 2-8°C temperature.
- Note:** Pipette **100 µl** of **cold** Serum Diluent into one microwell as a reagent blank. All Steps should be performed rapidly to avoid warming of the microwells and serum dilutions.
- Step 5** Pipette **100 µl** of Ready to Use **cold** Calibrator, Positive and Negative Controls into microwells. Pipette **100 µl** of **cold** diluted samples.

QUALITATIVE DETERMINATION

A	BLANK	S5		
B	NEG	S6		
C	POS	S7		
D	CAL	S8		
E	S1	S9		
F	S2	S10		
G	S3	S11		
H	S4	S12		
	1	2	3	4

- Step 6** Incubate 16-18 hours (overnight) at 2-8°C.
- Step 7** Wash **4x** with **cold** wash buffer. For manual washing, fill each microwell with reconstituted wash buffer. Aspirate contents of each well. Invert microplate and tap gently on absorbent paper towels. Keep inverted for ~1 minute to drain all liquid from microwells. **Note: Do not tap vigorously, tap lightly to remove excess liquid from wells. Keep all strips cold during this operation.**
- Step 8** Pipette **100 µl** of **cold** Conjugate into microwells.

- Step 9** Incubate 2 hours at 2-8°C.
- Step 10** Wash all microwells as in Step 7.
- Step 11** Pipette **100 µl** of **room temperature equilibrated Enzyme Substrate** into each microwell in the same order and timing as the conjugate.
- Step 12** Incubate **30 minutes** (\pm 5 min) at room temperature.
- Step 13** 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 one hour of adding Stop Solution.
- Step 14** Zero ELISA reader against reagent blank. Read absorbance of each microwell at **405 nm** using a single or 405/630nm dual wavelength microplate reader.

Quality Control

Reagent blank must be run with each assay to verify the integrity and accuracy of the test. The OD reading of the reagent blank should be <0.3 . The EU/ml of the Positive Control should be within the range indicated on the label. The Negative Control must be < 20 EU/ml. The OD of the Calibrator must be greater than that of the Negative Control and less than that of the Positive Control. For improved reproducibility samples may be run in duplicate and EU/ml calculated using the mean of the OD readings for the duplicate wells.