BÜHLMANN
anti-SGPG Antibody ELISA

anti-Sulfate-3-Glucuronyl Paragloboside Antibodies

This product is for research use only
It is not intended for use in diagnostic procedures

EK-SGPG-U 96 tests

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BÜHLMANN LABORATORIES AG
Baselstrasse 55
CH - 4124 Schönenbuch, Switzerland
Tel.: +41 61 487 1212
Fax: +41 61 487 1234
info@buhlmannlabs.ch

ENGLISH

INTENDED USE

The BÜHLMANN anti-SGPG Antibody ELISA is intended for the semi-quantitative determination of human IgM-autoantibodies directed against sulfate-3-glucuronyl paragloboside [SGPG] and sulfate-3-glucuronyl-lactosaminyl-paragloboside [SGLPG] in serum (1-3, 5).
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PRINCIPLE OF THE ASSAY

The BÜHLMANN anti-SGPG Antibody ELISA employs the quantitative enzymatically amplified sandwich-type immunoassay technique. Highly purified SGPG and SGLPG from bovine *cauda equina* have been precoated onto a microtiter plate. The Calibrator, Controls and sera are incubated for two hours in the microtiter wells and anti-SGPG autoantibodies present are bound by the immobilized bovine SGPG. After washing away any unbound substances, horseradish peroxidase (HRP) labeled antibodies against human IgM are added to the wells and incubated for another 2 hours. After a washing, the substrate solution containing tetramethylbenzidin (TMB) is added to the wells and incubated for 30 minutes. A blue coloration develops in proportion to the amount of anti-SGPG autoantibodies bound in the initial step. The color development is stopped by addition of the acidic stop solution (H_2SO_4) which turns the blue solution to yellow. The intensity of the color absorbance is measured in a microtiter plate reader at a wavelength of 450 nm. The absorbance measured is directly proportional to the concentration of anti-human SGPG autoantibodies.

REAGENTS SUPPLIED AND PREPARATION

Reagents	Quantity	Code	Reconstitution
Microtiter Plate 96 wells precoated with bovine SGPG	12 x 8-well strips with holder	B-SGPG-MP	Ready to use
Plate Sealer	3 pieces		
Wash Buffer Concentrate (10x) with preservatives	1 bottle 100 ml	B-MAG-WB	Dilute with 900 ml of deionized water
Incubation Buffer with preservatives	1 bottle 100 ml	B-MAG-IB	Ready to use
Calibrator[1)] Human serum with preservatives	1 vial	B-SGPG-CA	Add 1 ml of Incubation Buffer
Control Low, Medium and High[2] Human serum with preservatives	3 vials	B-SGPG-CONSET	Add 1 ml of Incubation Buffer
Enzyme Label Anti-IgM-HRP in a protein-based buffer; preservatives	1 vial 11 ml	B-SGPG-ELM	Ready to use Blue solution
TMB Substrate TMB in Citrate buffer with Hydrogen Peroxide	1 vial 11 ml	B-TMB	Ready to use
Stop Solution 0.25 M Sulfuric acid	1 vial 11 ml	B- STS	Ready to use **Corrosive agent**

Table 1

[1)] The Calibrator consists of a diluted positive serum which has been standardized to an internal established reference (see chapter standardization and cut-off).

[2)] Low, Medium and High Control contain lot-specific amounts of anti-SGPG antibodies. Refer to the QC data sheet provided with the kit for the appropriate ratios.

STORAGE AND SHELF LIFE OF REAGENTS

Unopened Reagents	
Store at 2-8°C. Do not use past kit expiration date printed on the label.	
Opened / Reconstituted Reagents	
Microtiter Plate	Return unused strips immediately to the foil pouch containing the desiccant packs and reseal along the entire edge of zip-seal. Store for up to 2 months at 2-8°C.
Wash Buffer diluted	Store for up to 2 months at 2-8°C.
Calibrator	Store for up to 2 months at -20°C.
Controls	
Incubation Buffer	Store at 2-8°C until expiration date.
Enzyme Label	
TMB Substrate (protect from light)	
Stop Solution	Store at 18-28°C until expiration date.

Table 2

PRECAUTIONS

SAFETY PRECAUTIONS

- Both, Calibrator (B-SGPG-CA) and Controls (B-SGPG-CONSET) of this kit contain components of human origin. Although tested and found negative for HBV surface antigen, HCV and HIV1/2 antibodies, the reagents should be handled as if capable of transmitting infections and should be handled in accordance with good laboratory practices using appropriate precautions.
- **Substrate and Stop Solution:** The Substrate Solution (B-TMB) contains Tetramethylbenzidine (TMB), hydrogen peroxide and dimethylformamide. The Stop Solution (B-STS) contains sulfuric acid. Each of those reagents is irritant to eyes, skin and mucous membranes. Avoid contact with eyes, skin and clothing. After contact with eyes or skin, wash immediately with plenty of water.
- Unused solution should be disposed of according to local State and Federal regulations.

TECHNICAL PRECAUTIONS

- **Kit components:** Read carefully the instructions prior to carrying out the test. Test performance will be adversely affected, if reagents are incorrectly diluted, modified or stored under conditions other than those as detailed in this instruction for use:
- **Residues in the microtiter plate** wells result from the production process. They are removed in the washing step (Assay procedure step 3) and do not affect the results.
- **Steps 3-9:** Use cold (2-8°C) reagents for all these steps and keep them cold while pipetting.
  Recommendation: Prepare wash buffer in particular on the eve of using it in the assay and to place it in the fridge overnight.
- **Steps 3, 6, 9:** Make sure that the wells are completely empty after the last washing cycle.
- **Step 9:** Adjust TMB Substrate to room temperature (18-28°C) before using it.
  Recommendation: Take it out from fridge when starting the assay.
- **Step 11:** Shake microtiter plates during the incubation with substrate. Depending on the plate shaker, we recommend 400-600 rpm. The solution should be moved in the wells but must not spill over.

- If an **automated washer** is used, "plate mode" should be chosen so that dispensing is performed sequentially on all strips before aspirating.
- Components must not be used after the expiry date printed on the labels.
- Do not mix different lots of reagents.
- Every effort should be made to ensure that no cross contamination occurs between reagents, samples or between wells.
- Microwells cannot be re-used.

MATERIALS REQUIRED BUT NOT PROVIDED

- Precision pipettes with disposable tips: 2 µl, 100 µl and 1000 µl pipettes.
- Disposable polystyrene or polypropylene tubes for the preparation of sample dilutions.
- 1000 ml cylinder for the dilution of the Wash Buffer Concentrate.
- Microtiter plate washer or squeeze bottle for Wash Buffer.
- Microtiter plate rotator.
- Microtiter plate reader for measurement of absorbance at 450 nm.

SPECIMEN COLLECTION AND STORAGE

The procedure calls for <0.1 ml of blood or <50 µl of serum. Lipemic, hemolytic and icteric samples should not be used in this assay. Lipemic samples can be avoided by asking patients to fast for at least 12 hours prior to the sample being taken. Collect blood into plain tubes, avoid hemolysis, leave to clot for one hour at room temperature (RT) (18-28°C), centrifuge for 10 minutes at approximately 1000 x *g* at RT and collect the serum.

Store serum samples at ≤-20°C. Samples are stable for ≥1 year if stored at ≤-20°C. Avoid repeated freeze-thaw cycles. Frozen samples should be thawed and mixed thoroughly by gentle swirling or inversion prior to use.

ASSAY PROCEDURE

Note: use refrigerated reagents in steps 3. to 9.

1. Dilute all samples 1:1000 with cold Incubation Buffer (e.g. 2 µl of serum + 2 ml of Incubation Buffer). Mix thoroughly by vortexing and leave diluted samples for 60 minutes at 2 – 8 °C prior to pipetting.
2. Use a plate with enough 8-well strips to test the desired number of blank, calibrator, controls and samples. Remove excess strips from the holder and re-seal them together with the two desiccant bags without delay. Store refrigerated.
3. Wash the coated strips four times using at least 300 µl of wash buffer per well. Empty the wells and strike plate firmly onto blotting paper.

4a. Pipet 100 µl of Incubation Buffer (blank) in duplicate into wells A1+A2.

4b. Pipet 100 µl of Calibrator in duplicate into wells B1+B2

4c. Pipet 100 µl of Low Control in duplicate into wells C1+C2

4d. Pipet 100 µl of Medium Control in duplicate into wells D1+D2

4e. Pipet 100 µl of High Control in duplicate into wells E1+E2

4f. Pipet 100 µl of each diluted sample in duplicate into the subsequent wells.

5. Cover the plate with a plate sealer and incubate for 2 hours (± 5 min) at 2-8°C.
6. Remove and discard plate sealer. Wash plate four times using at least 300 µl of wash buffer per well. Empty wells and strike plate firmly onto blotting paper.
7. Add 100 µl of Enzyme Label (blue solution) to all wells.
8. Cover plate with a new plate sealer, and incubate for 2 hours (± 5 min) at 2-8°C.
9. Remove and discard plate sealer. Wash plate four times using at least 300 µl of wash buffer per well. Empty wells and strike plate firmly onto blotting paper.

Important: Allow TMB substrate solution to come to 18-28°C prior to using it in step 10.

10. Add 100 µl of TMB substrate solution to all wells.
11. Cover plate and place plate on a plate rotator set at 800-1000 rpm, protect plate from direct light and incubate for 30 minutes (± 5 min) at 18-28°C.
12. Add 100 µl of stop solution to all wells. Remove air bubbles with a pipette tip. Proceed to step 13 within 30 minutes.
13. Read absorbance at 450 nm in a microtiter plate reader.

STANDARDIZATION

The Calibrator included in this kit has been calibrated against internal reference material (diluted positive serum). The dilution was chosen in the range between the OD of normal blood donors and positive sera.

RESULTS AND CALCULATION

Calibrator: Record absorbance at 450 nm (OD_{450}) and subtract the averaged blank value. Average the duplicate values. The Ratio of the calibrator is set to a value of 1 (divided by itself).

Samples and Controls: Record absorbance at 450 nm (OD_{450}) for each sample and control well and subtract the averaged blank value. Average the duplicate values. Calculate Ratio from the averaged sample absorbance to the averaged absorbance of the Calibrator.

$$\text{Ratio} = \frac{\text{mean net OD450 of sample}}{\text{mean net OD450 of calibrator}}$$

Note: Results presented in Table 3 are examples. Calibrator and Controls must be used in each individual assay.

QUALITY CONTROL

A thorough understanding of this instruction for use (IFU) is necessary for the successful use of the product. Reliable results will be obtained only by using precise laboratory techniques (current GLP guidelines) and accurately following this IFU.

BÜHLMANN strongly recommends testing Blank, Calibrator, Control and samples in duplicate.

Since there is no control serum for anti-SGPG antibodies commercially available, we recommend using a positive serum pool for internal quality controls.

All controls must fall within established confidence limits. The confidence limits for the Controls are lot-specific and printed on the additional data sheet.

The reproducibility of calibrator and control values should be within established limits of laboratory acceptability.

If the precision of the assay does not correlate with the established limits and repetition excludes errors in technique, check the following issues: i) pipetting, temperature controlling and timing devices ii) ELISA reader settings iii) expiration dates of reagents iv) storage and incubation conditions v) TMB Substrate Solution should be colorless vi) purity of water.

PERFORMANCE CHARACTERISTICS

Intra-Assay Precision (Within-Run): 4.9%. The intra-assay precision was calculated from the results of 20 pairs of values from four human sera obtained in a single run (cf. Table 4).

Inter-Assay Precision (Run-to-Run): 10.0%. The inter-assay precision was calculated from the results of 20 pairs of values from 6 human sera obtained in 20 different runs (cf. Table 5)

Dilution Linearity/Parallelism: 151%. 13 human serum samples containing high titer of anti-SGPG antibodies were diluted with Incubation Buffer 1:1000 to 1:128'000, left for one hour at 18-28°C and subsequently assayed according to the assay procedure. The O/E ratio (observed/expected) was calculated step by step (*cf.* Table 6). It is suggested that the relatively high deviation particularly at high antibody titers in the samples is due to antibody aggregations. In general, pathological sera show strongly elevated antibody titers therefore it has no influence to the positive/negative discrimination.

Detection limit (LOB): <0.01 Ratio. 20 duplicates of Incubation Buffer were assayed in a single run. Mean and standard deviation (SD) were calculated for the absorbance values (OD). After subtraction of the blank absorbance value and calculation of the ratio a value of 0.006 was obtained.

Detection limit (LOQ): <0.15 Ratio. An anti-SGPG antibody positive serum was subsequently diluted (1:1000 – 1:100'000). 20 duplicates of each dilution were assayed in a single run. Mean, standard deviation (SD) and coefficient of variation (%CV) were calculated from the absorbance values. We found a ratio <0.145 with a CV of less than 10% (cf. Table 6).

Specificity: Two sets of experiments were performed to assess the specificity of the BÜHLMANN anti-SGPG Antibody ELISA:

1. Neutralization of anti-SGPG Autoantibodies:
Two sera with high anti-SGPG titers could be increasingly inhibited from binding to the microtiter plates coated with SGPG in concentration-dependent manner when preincubated for 16 hours by 4°C Incubation Buffer supplemented with increasing amount of SGPG (32 ng to 1.6 µg SGPG in Eq/galactose) prior to testing in the ELISA.

2. Specificity of anti-SGPG autoantibody binding:
Ten sera of medium and high anti-Ganglioside autoantibody titers (GA1, GM1, GM2, GD1a, GD1b and GQ1b) and five negative sera were tested in the BÜHLMANN anti-SGPG Antibody ELISA. 9/10 of these disease state sera and all negative sera resulted in a ratio lower than 0.6. The positively tested sample was further assayed by thin layer chromatography (TLC) for anti-SGPG and anti-GD1a autoantibodies. The existence of both kind of antibodies could be confirmed by thin layer chromatography (TLC).

Table 3: Examples of results

	OD_{450nm}	Mean OD_{450nm}	Mean Ratio
Blank	0.039		
Calibrator	0.378		
Calibrator	0.318	0.348	1.00
Control LOW	0.010		
Control LOW	0.012	0.011	0.03
Control MEDIUM	0.294		
Control MEDIUM	0.226	0.260	0.75
Control HIGH	0.834		
Control HIGH	0.731	0.783	2.25
Sample 1	0.953		
Sample 1	0.850	0.902	2.6
Sample 2	0.522		
Sample 2	0.537	0.530	1.5

Table 4 Intra-assay Precision

Sample Type	Mean Ratio	SD	CV(%)
Serum 1	3.8	0.3	7.6
Serum 2	2.8	0.1	4.5
Serum 3	2.1	0.1	3.3
Serum 2 dil.	1.2	0.05	4.1
Mean			**4.9**

Table 5 Inter-assay Precision

Sample Type	Mean Ratio	SD	CV(%)
Serum 1	4.2	0.5	11.3
Serum 5	4.1	0.3	6.9
Serum 3	2.6	0.3	12.0
Serum 2	2.1	0.1	6.2
Serum 2 dil.	1.2	0.1	10.2
Serum 2 dil.	0.5	0.1	13.4
Mean			**10.0**

Table 6 Dilution Linearity/Parallelism

Sample Type	Range [min – max]	Mean [Observed/Expected]
Serum 4HF	100-114%	112%
Serum 5K	169-200%	175%
Serum 1C	86-133%	108%
Serum 2G	108-164%	141%
Serum 3GL	80-121%	110%
Serum 5	133-175%	161%
Serum 6	140-196%	180%
Serum 7	114-200%	157%
Serum 8	166-192%	175%
Serum 1	133-186%	171%
Serum 2	133-183%	161%
Serum 3	120-192%	164%
Serum 4	165-200%	185%
Mean		**154%**

Table 7 Functional Sensitivity

Sample Type	dilution	Ratio	SD	%CV
Serum 1	1:1	2.8	0.1	4.5
Serum 1	1:2	1.2	0	4.1
Serum 1	1:10	0.37	0.03	7.5
Serum 1	1:20	0.22	0.01	5.6
Serum 1	1:50	0.13	0.01	11.1
Serum 1	1:100	0.07	0.04	60.0
%CV =10% at a Ratio of 0.145				

1. Ariga T, *et al*.: Characterization of sulphated glycuronic acid containing glycolipids reacting with IgM M-proteins in patients with Neuropathy. J Biol Chem 262(2),848-853 (1987).
2. Burger et al.: Anti-myelin-associated glycoprotein antibodies in patients with a monoclonal IgM gammopathy and polyneuropathy, and a simplified method for the preparation of glycolipid antigens. J Immunol Meth 140; 31-36 (1991).
3. Baumann N.: Specificity of antiglycolipid antibodies. Clin Rev Allergy Immunol 19;31-40 (2000)
4. Campant RM et al.: Détection des anticorps anti-myéline: mise au point et evaluation dans 75 cas de neuropathies associées à une IgM monoclonale. Ann Biol Clin 57;69-75 (1999)
5. Hadden RDM et al. : Paraproteinemic demyelinating Neuropathies in European Handbook of Neurological Management : Volume 1, 2nd edition, edited by Gilhus NE et al (2011), Chapter 22.

BÜHLMANN anti-SGPG Antibody ELISA

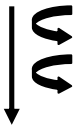
Precoated Microtiter Plate

wash 4 x

100 µL Calibrator, Controls or Serum Samples (1:1000)

incubate 2 hours (± 5 min) at 2-8°C
wash 4 x

add 100 µL Enzyme Label



incubate 2 hours (± 5 min)at 2-8°C
wash 4 x

add 100 µL TMB Substrate

incubate 30 minutes (± 5 min) at 18-28°C
on a plate rotator

add 100 µL Stop Solution

Read absorbance at 450 nm (within 30 minutes)

TIME TO RESULT: 4.5 HOURS

Symbol	Explanation
	Use By Verwendbar bis Utiliser jusqu'au Utilizzare entro Fecha de caducidad
REF	Catalogue number Bestellnummer Référence du catalogue Numero di catalogo Número de catálogo
LOT	Batch code Chargenbezeichnung Code du lot Codice del lotto Codigo de lote
	Contains sufficient for <n> tests Ausreichend für "n" Ansätze Contenu suffisant pour „n" tests Contenuto sufficiente per „n" saggi Contenudo sufficiente para <n> ensayos
	Consult Instructions for Use- Gebrauchsanweisung beachten Consulter le mode d'emploi Consultare le istruzioni per l'uso Consulte las instrucciones de uso
	Temperature limitation Zulässiger Temperaturbereich Limites de température Limiti di temperatura Limite de temperatura
MP	Microtiter Plate Mikrotiter-Platte Microplaque Micropiastra Microplaca

Symbol	Explanation
BUF WASH 10X	Wash Buffer Concentrate (10x) Wasch-Puffer Konzentrat (10x) Tampon de lavage concentré (10x) Tampone di lavaggio concentrato (10x) Tampón de lavado concentrado (10x)
BUF INC	Incubation Buffer Inkubations-Puffer Tampon d'incubation Tampone di incubazione Tampón de incubación
CAL	Calibrator Kalibrator Calibrateur Calibratore Calibrador
CONTROL L	Low Control Kontrolle tief Contrôle bas Controllo basso Control bajo
CONTROL M	Medium Control Kontrolle medium Contrôle médium Controllo medio Control medio
CONTROL H	High Control Kontrolle hoch Contrôle elevé Controllo alto Control alto
EL IgM	Enzyme Label IgM Enzymmarker IgM Marqueur enzymatique IgM Marcato enzimatico IgM marcada enzimáticamente IgM
SUBS TMB	TMB Substrate TMB Substrat Substrat TMB Substrato di TMB Substrato de TMB
SOLN STOP	Stop Solution Stopp-Lösung Solution stop Soluzione stoppante Solución de parada