anti-SGPG Autoantibodies ELISA

SGPG: Sulfate-3-Glucuronyl Paragloboside

For research use only.
Not for use in diagnostic procedures.

EK-SGPG-U 96 tests

Release Date: 2018-11-16
Version A1

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

ENGLISH

INTENDED USE

The BÜHLMANN anti-SGPG Autoantibodies ELISA is intended for the semi-quantitative determination of human IgM-auto-antibodies directed against sulfate-3-glucuronyl paragloboside [SGPG] and sulfate-3-glucuronyl-lactosaminyl-paragloboside [SGLPG].

For research use only. Not intended for use in diagnostic procedures.

PRINCIPLE OF THE ASSAY

The anti-SGPG Autoantibodies ELISA employs the enzymatically amplified sandwich-type immunoassay technique. The microtiter plates of the test are precoated with highly purified SGPG and SGLPG from bovine *cauda equina*. Calibrator, controls, and sera are incubated in the microtiter wells and anti-SGPG auto-antibodies present in the samples bind to the immobilized SGPG/ SGLPG. After washing off of unbound substances, the anti-SGPG auto-antibodies are detected with horseradish-peroxidase (HRP) labelled antibodies against human IgM. Following a second washing step, in which unbound enzyme label is removed, a substrate solution containing tetramethylbenzidine (TMB) is added. A blue colour develops in proportion to the amount of anti-SGPG auto-antibodies bound to the immobilized SGPG and SGLPG. Colour development is stopped by adding an acidic stop solution (diluted sulphuric acid) which turns the blue solution into yellow. The intensity of the colour is measured at 450 nm.
The measured absorbance is proportional to the titre of auto-antibodies present in a given sample. The titers of anti-human SGPG auto-antibodies are expressed as ratios of the calibrator and can be assigned to titer categories.

REAGENTS SUPPLIED AND PREPARATION

Reagents	Quantity	Code	Reconstitution
Microtiter Plate precoated with bovine SGPG	12 x 8-wells	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 IgM Anti-human IgM Ab conjugated to HRP in a protein-based buffer with preservatives	1 vial 11 mL	B-SGPG-ELM	Ready to use Blue solution
TMB Substrate TMB in citrate buffer	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).

[2)] Controls Low, Medium and High 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

Sealed / Unopened Reagents	
All sealed/unopened kit components are stable at 2-8 °C until the expiration date printed on the labels.	
Opened / Reconstituted Reagents	
Microtiter Plate	Return unused strips immediately to the aluminium pouch containing the desiccant packs and reseal along the entire edge of zip-seal. Store for up to 2 months at 2-8 °C.
Diluted Wash Buffer	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 printed on the labels.
Enzyme Label	
TMB Substrate	
Stop Solution	Store at 18-28 °C until expiration date printed on the labels.

Table 2

MATERIALS REQUIRED BUT NOT PROVIDED

- Precision pipettes with disposable tips capable of pipetting the following volumes: 2 µL, 100 µL and 1000 µL.
- Disposable polystyrene or polypropylene tubes for the preparation of sample dilutions.
- 1000 mL cylinder for the dilution of the wash buffer.
- Squeeze bottle for wash buffer or automatic microtiter plate washer.
- Blotting paper.
- Orbital shaker for microtiter plates.
- Microtiter plate reader for measurement of absorbance at 450 nm.

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.

- Stop solution: The stop solution (B-STS) contains sulfuric acid (0.25 M). The reagent is an irritant to eyes, skin and mucous membranes. Avoid contact with eyes, skin and clothes. After contact with eyes or skin, wash immediately with plenty of water.
- Reagents: Avoid contact of reagents with the skin, eyes, or mucous membranes. If contact does occur, immediately wash with generous amounts of water; otherwise, irritation / burns can occur.
- Unused solution should be disposed of according to local state and federal regulations.

Technical precautions

- 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.
- Prepare reagents before starting the assay procedure. Reagents used in steps 3-9 must be cold (2-8 ˚C) and kept cold while pipetting and washing. Put the TMB substrate at room temperature (18-28 ˚C).
- Steps 3-9: Use cold (2-8 °C) reagents for all these steps and keep them cold while pipetting. Recommendation: Prepare the wash buffer the evening before performing the assay and place it into the fridge overnight.
- Wash steps 3, 6 and 9: The wash steps are crucial for removing residues in the microtiter plate wells resulting from the production process (step 3) as well as any unbound auto-antibodies (steps 6 and 9).
  → Always perform the wash steps with cold (2-8 ˚C) wash buffer.
  → Make sure that all wells are completely empty after the last washing cycle.
- Step 10: Adjust TMB substrate to room temperature (18-28 °C) before using it.
- Step 11: Shake the microtiter plates during the incubation with substrate. Depending on the plate shaker, we recommend 400-600 rpm. The solution should move 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.

SPECIMEN COLLECTION AND STORAGE

- The procedure requires <0.1 mL of blood and <50 µL of serum, respectively.
- Lipemic, hemolytic and icteric samples should not be used in this assay. Lipemic samples can be avoided by asking subjects to fast for at least 12 hours prior to the sample being taken.
- Collect blood into plain tubes (no anti-coagulant), avoid haemolysis, leave to clot for one hour at room temperature (18-28 °C), centrifuge for 10-30 minutes at 1000-2000 x *g*, 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.
- We recommend freezing aliquots of samples in order to avoid repeated freezing/thawing.

ASSAY PROCEDURE

1. Dilute all samples to be investigated 1:1000 with incubation buffer. Use e.g. 2 µL of serum + 2000 µL of incubation buffer. Mix thoroughly by vortexing and leave diluted samples for one hour at 18-28°C. Put samples as well as reconstituted calibrators and controls for 10 minutes on ice prior to pipetting in step 4.
2. Prepare a plate-frame with the required number of strips to test the calibrators, controls and samples. Reseal the remaining strips in the foil pouch together with the desiccant packs immediately. Store refrigerated.

Note: Use cold reagents in steps 3 to 9.

3. Wash coated wells four times using at least 300 µL of cold! wash buffer per well. Empty wells and tap plate firmly onto blotting paper to remove remaining liquid completely.

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 control low in duplicate into wells C1+C2

4d. Pipet 100 µL of control medium in duplicate into wells D1+D2

4e. Pipet 100 µL of control high 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 plate sealer. Empty the wells and wash four times using at least 300 µL of cold wash buffer (2-8 °C) per well. Empty the wells and strike the plate firmly onto blotting paper in order to remove washing buffer completely.
7. Add 100 µL of enzyme label IgM to all wells.

8. Cover plate with a plate sealer, and incubate for 2 hours (± 5 min) at 2-8 °C.
9. Remove plate sealer. Empty the wells and wash four times using at least 300 µL of cold wash buffer (2-8 °C) per well. Empty the wells and strike the plate firmly onto blotting paper in order to remove wash buffer completely.

Note: Adjust TMB substrate solution to room temperature (18-28 °C).

10. Add 100 µL of TMB substrate solution to each well.
11. Cover plate with a plate sealer, incubate plate on an orbital plate shaker at 400-600 rpm for 30 ± 2 minutes at 18-28 °C. Protect the plate from direct light.
12. Add 100 µL of stop solution to all wells. Proceed to step 13 within 30 minutes.
13. Read absorbance at 450 nm in a microtiter plate reader.

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 good understanding of this instruction for use is necessary to obtain reliable results. These will be obtained only by using precise laboratory techniques (current GLP guidelines) and accurately following the instruction for use.
BÜHLMANN strongly recommends testing blank, calibrator, control and samples in duplicate.
Since there is no control serum for anti-SGPG auto-antibodies commercially available, we recommend using a positive, and negative serum pool for internal quality control.
All controls must be within established confidence ranges. The confidence ranges of the controls are lot-specific and printed on the QC data sheet delivered with this kit.
Performance characteristics should be within established limits. If these characteristics are not in conformity with established limits and repetition excludes handling failures, check the following issues: i) Have all reagents, used in step 3-9, been kept at 2-8 °C? ii) accuracy of pipets, thermometers, and timers, iii) settings of ELISA washer and reader, iv) expiration date of the reagents v) storage and incubation conditions vi) colour of the TMB substrate solution (should be colourless) vii) purity of the water.

STANDARDIZATION

The calibrators included in this kit have been standardized against an internal reference material (diluted positive serum). The dilution was chosen in the range between the OD of normal blood donor samples and positive sera.

LIMITATIONS

The anti-SGPG Autoantibodies ELISA has not been validated for plasmapheresis samples.

PERFORMANCE CHARACTERISTICS

Intra-Assay Precision (Within-Run): 4.9%. The intra-assay precision was calculated from results of 20 pairs of values from four human sera obtained in a single run (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 (table 5).

Dilution Linearity/Parallelism: 154%. Thirteen (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 (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. Twenty (20) duplicates of incubation buffer were assayed in a single run. Mean and standard deviation (SD) were calculated for the absorbance values (OD). The mean OD value of the measurements was subtracted from the mean OD + 2 SD value. A final ratio of 0.006 to the calibrator was obtained.

Detection Limit (LoQ) : <0.15 Ratio. An anti-SGPG antibody positive serum was subsequently diluted (1:1000-1:100'000). Twenty (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 % (table 7).

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

1. Neutralization of anti-SGPG auto-antibodies: Two (2) 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 with 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 auto-antibody binding: Ten (10) sera of medium and high anti-Ganglioside auto-antibody titers (GA1, GM1, GM2, GD1a, GD1b and GQ1b) and five negative sera were tested in the anti-SGPG Autoantibodies ELISA. Nine (9) out of ten 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 auto-antibodies. The existence of both kind of antibodies could be confirmed by TLC.

APPENDIX I

TABLES

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 3

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 4

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 5

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 6

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				

Table 7

APPENDIX III

SHORT PROTOCOL

anti-SGPG Autoantibody 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 (± 2 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**

APPENDIX IV

SYMBOLS

Symbol	Explanation
	Use By
REF	Catalogue number
LOT	Batch code
	Contains sufficient for <n> tests
	Consult Instructions for Use
	Temperature Limitation
MP	Microtiter Plate
SOLN STOP	Stop Solution

Symbol	Explanation
BUF WASH 10X	Wash Buffer Concentrate (10x)
BUF INC	Incubation Buffer
CAL	Calibrator
CONTROL L	Low Control
CONTROL M	Medium Control
CONTROL H	High Control
EL IgM	Enzyme Label IgM
SUBS TMB	TMB Substrate