Instruction

WIESLAB® sGAG quantitative kit

Alcian blue-binding assay for the detection of
sulphated glycosaminoglycans

Store the kit at +4° C

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For Research Use Only. Not for use in diagnostic procedures.

REF **GAG 201 RUO**

PURPOSE WITH RESEARCH PRODUCT

The Wieslab® sGAG test kit is a quantitative dye-binding assay for the *in vitro* analysis of sulphated glycosaminoglycans (sGAG). The assay is used to detect sGAGs in biological samples such as synovial fluid, blood, and tissue extracts.
The result shall not be used for clinical diagnosis or patient management.

SUMMARY AND EXPLANATION

The most abundant heteropolysaccharides in the body are the glycosaminoglycans (GAGs). They are located primarily on the surface of cells or in the extracellular matrix. As an example, cartilage is composed to a large extent of GAGs, which are the dominant part of the proteoglycan Aggrecan. GAGs are negatively charged long unbranched polymeric polysaccharides composed of repeating units of disaccharides containing one uronic acid or galactose and one amino sugar, either N-acetyl-glucosamine or N-acetylgalactosamine. The variation in charge may be very large since each disaccharide is more or less sulphated.

Based on GAG's high negative charge, a number of dye-binding procedures for their measurement have been developed. In most cases, however, they are not applicable to biological material without different forms of pre-treatment such as protease digestion. The present assay makes use of the dye Alcian blue (C.I.# 74240) which has a long history as histological tissue staining reagent. Alcian blue is a tetravalent cation with a hydrophobic core. The four charges allow the dye to bind to negatively charged polymers such as GAGs at high ionic strength, in contrast to other cationic dyes which are all monovalent. The molecular structure of Alcian blue, i.e. the plane tetragonal hydrophobic core with positive charges at its corners, may facilitate formation of aggregates of several molecules side by side rather than micelle formation. The ionic strength, pH and presence of detergents will affect the size of these aggregates in solution.

The ionic bonding between cationic dyes (such as Alcian blue) and the negatively charged GAGs are generally thought to be proportional to the number of negative charges present on the GAG chain, i.e. both sulphate and carboxyl groups.

Principle of the Wieslab® sGAG assay

The principle is based on the specific interaction between sulphated polymers and the tetravalent cationic dye Alcian blue. The assay is performed at a pH low enough to neutralize all carboxylic and phosphoric acid groups and at an ionic strength large enough to eliminate ionic interactions other than those between Alcian blue and sulphated GAGs (2). The Alcian Blue reagent may be obtained from a number of commercial sources. However, the quality of the reagent and its usability in proteoglycan assays differ dramatically from brand to brand. The Alcian Blue reagent in Wieslab's sGAG assay has been carefully selected and optimised for this particular use.
Hyaluronan, a non-sulphated GAG, does not react in this assay. There is no interference from proteins or nucleic acids in this method, in contrast to the DMMB-method or other dye binding methods (2).

WARNINGS AND PRECAUTIONS

- ***For Research Use Only. Not for use in diagnostic procedures***.

The Centers for Disease Control and Prevention and National Institutes of Health recommended that potentially infectious agents be handled at the Biosafety Level 2.
The kit contain a number of solutions contain corrosive/irritating chemical compounds such as sulphuric acid, guanidine hydrochloride and DMSO. They should all be handled by knowledgeable persons with proper care and according to routine precautions/regulations for handling hazardous chemicals.
- Avoid swallowing and contact with the skin or mucous membranes. If contact with skin or eyes occurs irrigate thoroughly with water and seek medical attention immediately.
- Never pipette by mouth or allow reagents to come into contact with skin.

- Material safety data sheet for all hazardous components contained in this kit are available on request from Euro Diagnostica.

Warning

DIL	GuHCL
CONTROL	

Contains: Guanidine hydrochloride

H302:	Harmful if swallowed.
H315:	Causes skin irritation.
H319:	Causes serious eye irritation.
P264:	Wash hands thoroughly after handling.
P280:	Wear protective gloves/protective clothing/eye protection/face protection.
P302+352:	IF ON SKIN: Wash with plenty of soap and water.
P305+351 +338:	IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing.
P332+313:	If skin irritation occurs: Get medical advice/attention.

Warning

Flammable

SOLN	Gu- Prop

Contains: Guanidine hydrochloride. 1-Propanol.

H225:	Highly flammable liquid and vapour.
H302:	Harmful if swallowed.
H315:	Causes skin irritation.
H319:	Causes serious eye irritation.
H336:	May cause drowsiness or dizziness.
P210:	Keep away from heat/sparks/open flames/hot surfaces. – No smoking.
P264:	Wash hands thoroughly after handling.
P280:	Wear protective gloves/protective clothing/eye protection/face protection.
P302+352:	IF ON SKIN: Wash with plenty of soap and water.
P305+351 +338:	IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing.
P332+313:	If skin irritation occurs: Get medical advice/attention.

KIT COMPONENTS AND STORAGE OF REAGENTS

- **10 mL Alcian Blue stock solution** containing 0.1% H_2SO_4 and 0.4 M GuHCl. To be diluted with solution "SAT".
- **10 mL GuHCL,** 8 M Guanidine-HCl. Used to dilute samples.
- **65 mL SAT solution** containing 0.3% H_2SO_4 and 0.75% Triton X-100. Used to dilute Alcian Blue stock solution and as addition to samples.
- **100 mL DMSO solution** containing 40% dimethylsulphoxide and 0.05 M $MgCl_2$. Used to wash pellets.
- **100 mL Gu-Prop solution** containing 4 M GuHCl, 33% 1-propanol and 0.25% Triton X-100. Used to dissolve pellets before reading the absorbance.
- **Six calibrators** containing 1.1 mL of chondroitin sulphate-6 (CS-6), **CAL1** (400 ug/mL), **CAL2** (200 ug/mL), **CAL3** (100 ug/mL), **CAL4** (50 ug/mL), **CAL5** (25 ug/mL), **CAL6** (12.5 ug/mL) diluted in water.
- **Control (Ctrl)** containing 1.1 mL of cartilage extract diluted in water.

All reagents should be stored at 4° C.

MATERIALS OR EQUIPMENT REQUIRED BUT NOT PROVIDED

- Microplate reader or a spectrophotometer with preferably a 620 nm filter (readings can be made at 600-620 nm).
- Precision pipettes with disposable tips.
- Disposable syringe with 18 G needle for convenient removal of supernatants
- Capped polypropylene vials (1.5 or 2 mL size) of Eppendorf type are recommended
- Centrifuge capable of giving a centrifugal force of at least 12 000 x g

SAMPLE PREPARATION

- GAGs are stable at 4° C in the absence of cells. The protein core of proteoglycans may be degraded by proteolytic activity in sample which, however, does not alter the GAG chains.
- Cell debris and insoluble material should be removed by centrifugation (12 000 x g, 15 minutes).
- Sample volumes given below are only suggestive. The method is equally applicable at any scale as long as the final concentration of GuHCl is 0.4 M at pH 1.5.
- The sGAG assay may be used with serum, plasma or synovial fluid. Handle as if capable of transmitting agents.
- Store samples between +2-8° C if testing will take place within five days. If specimens are to be kept for longer periods, store at -20° C or colder. Do not use a frost-free freezer because it may allow the specimens to go through freeze-thaw cycles and degrade antibody. Samples that are improperly stored or are subjected to multiple freeze-thaw cycles may yield spurious results.
- The NCCLS provides recommendations for storing blood specimens, (Approved Standard-Procedures for the Handling and Processing of Blood Specimens, H18A, 1990).

PROCEDURE

The procedure outlined here is valid for measuring sGAG in test tubes (2). For information on alternative applications such as electrophoresis (3) and dot blot assay (4) please contact Euro-Diagnostica.
All solutions should be used at room temperature (RT).

Preparation of dye working solution

Mix reagents with the following proportions to prepare Alcian Blue working solution:
50 mL SAT + 90 mL distilled water + 10 mL Alcian Blue stock solution.
This volume is sufficient for 200 tests.
The working solution is stable for 1 week at 4° C. Smaller volumes can be made using the same proportions.

Test tube procedure

1. Pipet 50 µL/vial in duplicate of blank (BL) use water, calibrators (C), control (Ctrl) and samples (S) in test tubes according to the following layout.

BL	BL	Cal 6	Cal 6	Cal 5	Cal 5	Cal 4	Cal 4	Cal 3	Cal 3	Cal 2	Cal 2
Cal 1	Cal 1	Contr	Contr	S1	S1	S2	S2	etc	etc		

2. Add 50 µL of 8 M GuHCl to each vial, mix and incubate at RT for 15 minutes.
3. Add 50 µL of SAT solution to each vial, mix and incubate at RT for 15 minutes.
4. Add 750 µL of Alcian Blue working solution to each vial, mix and incubate at RT for at least 15 minutes. Alternatively, the samples can be incubated overnight at 4°C.
5. Centrifuge for 15 minutes at 12 000 x g.
6. Carefully remove supernatant by suction with a syringe and discard.
7. Add 500 µL of DMSO solution to the pellet. Mix thoroughly. Make sure that the pellet becomes suspended. Mix for 15 minutes on a shaker at RT.
8. Centrifuge for 15 minutes at 12 000 x g.
9. Remove supernatant as above and discard.
10. Add 500 µL Gu-Prop solution to the pellets. Mix for 15 minutes on a shaker. Check that the pellet is completely dissolved.
11. Dispense 2 x 240 µl into flat bottom microplate wells. Read the absorbance at 620 nm in a microplate reader. If needed, absorbance can be read between 600-620 nm, but 620 nm is the optimal wavelength. Alternatively, absorbance can be read in a spectrophotometer. However, carryover between samples can be substantial giving less accurate results.

CALCULATIONS

Plot absorbance values against amount of GAG in each calibrator (12.5 - 400 µg/mL). The plot should be a straight line with an absorbance of approximately 1.4 at 400 µg/mL. Fit a linear equation to data points and calculate the amount of GAG in each sample. Alternatively, if only a single calibrator concentration is used, a factor is calculated by dividing the calibrator concentration with the corresponding absorbance.
The absorbance of the control and unknown sample is then multiplied with the factor to obtain the amount in each sample.

QUALITY CONTROL

The value of the control in this kit batch is found on the kit lot Certificate.

Notes

1. All commercial sulphated GAG samples (Cs, CsC, Ds, Ks, Hs) should give a similar colour yield. Hyaluronan, DNA or RNA do not react with Alcian Blue under these conditions
2. The sample must not contain any particles that sediment during centrifugation or is insoluble in 0.4 M GuHCl at pH 1.5. If the sample does contain particles, it must be centrifuged before analysis and the supernatant used for analysis. If the sample contains material that precipitates in 0.4 M GuHCl at pH 1.5, it must be removed by preceding preparative step.
3. The final concentration of GuHCl in the reagent mixture must be kept at 0.4 M and the pH at 1.5–2.0.
4. Care should be taken that pellets are left intact when supernatants are removed by suction. The use of a manually operated syringe for suction and immediate removal of the supernatants after centrifugation is therefore recommended. The tube should be held at an angle and in such a way that the pellet and needle is visible during the entire operation. The needle should be gradually lowered with the meniscus during suction and never be allowed to touch the pellet.
5. Care should be taken that pellets are completely dissolved in steps where this is required.

BIBLIOGRAPHY

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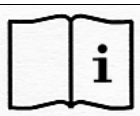
2. **Björnsson S.** Simultaneous preparation and quantitation of proteoglycans by precipitation with Alcian Blue. Anal Biochem 210: 282-291, 1993
3. **Björnsson S.** Size dependent separation of proteoglycans by electrophoresis in gels of pure agarose. Anal Biochem 210:292-298, 1993
4. **Björnsson S.** Quantitation of proteoglycans as glycosaminoglycans in biological fluids using an Alcian Blue dot blot analysis. Anal Biochem 256: 229-237, 1998
5. **Volck B. et al.** Studies on YKL-40 in knee joints of patients with rheumatoid arthritis and osteoarthritis. Involvement of YKL-40 in the joint pathology. Osteoarthritis and Cartilage 9: 203-214, 2001.

EXPLANATION OF SYMBOLS.

	Use-by date
	Biological risks
	Temperature limit
	Manufacturer.
	Batch code.
	Catalogue number.
	Consult instructions for use.
	Warning.
	Flammable.
 200	Contains sufficient for 200 tests.

SOLN **DMSO**	DMSO solution.
DIL **GuHCL**	Guanidine-HCl.
SOLN **SAT**	SAT solution.
SOLN **Gu-Prop**	Gu-Prop solution.
CONTROL	Control.
SOLN **Alcian blue**	Alcian Blue stock solution.
CAL **1 - 6**	Calibrators.

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