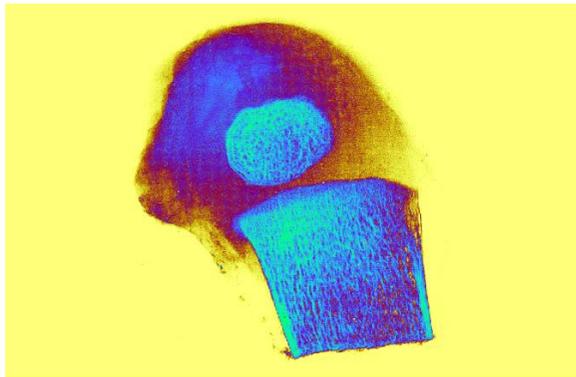


Blyscan™

Sulfated Glycosaminoglycan Assay



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Blyscan™ Sulfated Glycosaminoglycan Assay

The assay has been designed for research work only.
Handle the Blyscan Assay using Good Laboratory Practice.

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Published by

Biocolor Ltd.

**8 Meadowbank Road, Carrickfergus,
BT38 8YF, Northern Ireland, U.K.**

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Assay Manual

Intended Applications

The Blyscan Assay is a quantitative dye-binding method for the analysis of sulfated proteoglycans and glycosaminoglycans, (sGAG).

Test material can be assayed directly when present in a soluble form, or following papain extraction from biological materials. The assay can be used to measure the total sGAG content and can also be adopted to determine the *O*- and *N*-sulfated glycosaminoglycan ratio within test samples.

The dye label used in the assay is 1, 9-dimethylmethylene blue and the dye is employed under conditions that provide a specific label for the sulfated polysaccharide component of proteoglycans or the protein free sulfated glycosaminoglycan chains.

The assay is not suitable for small sulfated disaccharide fragments or for samples containing alginates, as these contain uronic acid.

Assay Sample Material

Soluble extracts from:

- fibrous and hyaline cartilages.
- arteries, heart, lung, skin and other material containing extracellular matrix, (connective tissue) and solid tumours.
- extracellular matrix components that may be released by live cells into the culture medium, some of which can be attached to cell culture plasticware.
- soluble sGAG from synovial fluid, urine and gel chromatography fraction aliquots.

Test Sample Composition

An extensive range of mammalian proteoglycans have been measured using this assay. The following sGAG, either still attached to the peptide/protein core, or as free chains can be assayed:

- chondroitin sulfates (4- & 6-sulfated)
- keratan sulfates (alkali sensitive & resistant forms)
- dermatan sulfate
- heparan sulfates (including heparins)

Hyaluronate is not detected by the Blyscan Assay. The presence of hyaluronate in test samples does not interfere with the measurement of sGAG.

Samples for analysis **must** be free of any particulate matter or turbidity (cell debris, insoluble extracellular matrix material). Soluble components such as proteins and neutral carbohydrates present in tissue extracts do not interfere with the assay.

Blyscan Pack Sizes and Storage Conditions

Standard Assay Kit Product Code: B1000 (110 assays)

Economy Pack Product Code: B3000 (440 assays)

All components are stable for one year, (from Invoice Date), when stored at 15-25°C. **Do not store below +4°C.**

Once opened the glass vial containing sGAG standard should be stored at +4°C.

Assay Kit Components

1. **Dye Reagent** contains 1, 9-dimethyl-methylene blue in an inorganic buffer, with surfactants.
2. **Dissociation Reagent** contains the sodium salt of an anionic surfactant. This reagent has been formulated to dissociate the sGAG-dye complex and enhance the spectrophotometric absorption profile of the free dye.
3. **Reference Standard** - a sterile solution of bovine tracheal chondroitin 4-sulfate. **Concentration: 100 µg/ml.**

N- and O-sGAG Differentiation Reagents

4. **Sodium Nitrite:** 5% (w/v) sodium nitrite, sterile solution
5. **Acetic Acid Solution:** 25% (v/v) acetic acid
6. **Ammonium Sulfamate:** 12.5% (w/v) sterile solution

Components Required for sGAG Extraction – not supplied

Papain Extraction Reagent; required for sample preparation prior to assay and is made up according to method on page 3.

TISSUE SAMPLE PREPARATION PRIOR TO ASSAY

See sample processing flowchart on p12

Preparation of Test Material for Extraction

sGAG content within the test sample may be expressed as:

(i) μg sGAG /mg wet tissue (If the test material contains substantial fatty deposits prior treatment with chloroform / methanol ratio 1:3 should be considered) or

(ii) μg sGAG /mg dry tissue. (Place a weighed sample into a desiccator over a drying agent, or into a drying oven ($< 60^\circ\text{C}$) and dry to constant weight ($\sim 30\%$ of wet weight)).

Papain Extraction – prior to measurement of sGAG from tissue samples or cell culture samples the sGAG are extracted using a papain extraction reagent. This reagent is prepared as follows.

Papain Extraction Reagent. (shelf life 7 days @ 4°C).

To 50ml of a 0.2M sodium phosphate buffer, ($\text{Na}_2\text{HPO}_4 - \text{NaH}_2\text{PO}_4$), pH 6.4

add; 400 mg sodium acetate
 200 mg EDTA, disodium salt
 40 mg cysteine HCl

When the above components have dissolved in the buffer introduce 250 μl of a papain suspension, containing about 5 mg of the enzyme.

A Papain crystallized suspension produced by Sigma-Aldrich, (*Product Code P3125*) is recommended. Avoid using crude papain preparations that often contain other proteases which hasten digestion of the papain.

Tissue Samples (*in vivo*) - Papain Extraction of Total sGAG

1. Place test sample (20-50mg) and Papain Extraction Reagent (1ml) in 1.5 ml labelled microcentrifuge tubes.
2. Put the microcentrifuge tubes in a thermally regulated metal heating block or water bath at 65°C . Remove occasionally for mixing. A digestion time of 3 hours @ 65°C is sufficient to break up soft tissue aliquots of 20 to 50 mg, (wet weight). However, hard tissues, such as cartilage and bone, require overnight incubation @ 65°C .
3. Centrifuge tubes at $10000 \times g$ for 10 minutes. Decant off supernatant for use with Blyscan sGAG Assay Protocol (inside manual cover).

A three hour extraction protocol permits sGAG assays to be carried out on the same day as extraction (Fig. 2).

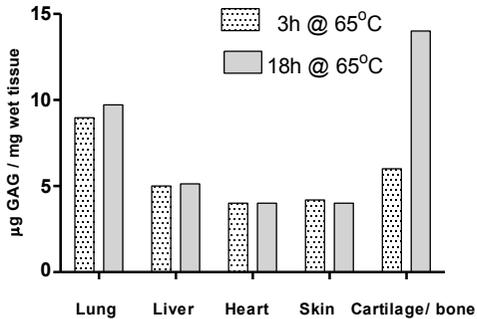


Fig. 2: Recovery of sGAG from mouse tissues, with 3 and 18 hours of hot papain.

Cell Culture Samples (*in vitro*) - Papain Extraction of Total sGAG

1. Remove medium from T-flask or microwell plate and retain for sGAG assay.
2. Wash cells with PBS and drain. Add Papain Extraction Reagent, (2ml for a T-25 flask, 1ml for 12 & 24 well plates and 250µl for 96 well plates). Incubate for 3 hours at 65°C with occasional mixing.
3. Remove digested extract from flasks or microwells and centrifuge at 10,000g for 10minutes. Retain supernatant for use with Blyscan sGAG assay protocol (inside manual cover), suggested sample volume 50µl (Fig. 3).
4. Total sGAG is calculated from the sum of cell medium sGAG levels and sGAG levels from the cell digest.

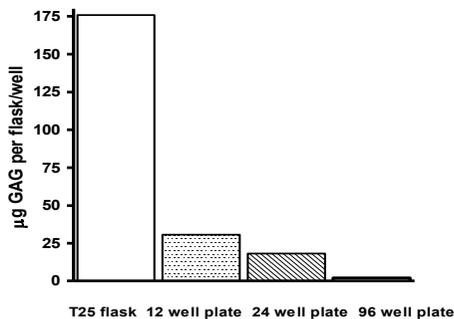


Fig. 3: A CHO cell line cultured for 72 hours, without a culture medium change, (cells confluent). The 12/24/96 well plates and T25 flasks were treated with papain to recover sGAG.

Papain Extraction of External and Interior Cell sGAG Fractions

There is little sGAG released into the culture medium until cell confluency occurs. This has led to the development of a sGAG assay option to determine the sGAG levels within different fractions of the cell and the extracellular matrix.

Volumes given in example are for a T25 Flask. If microwell plates are used adjust volumes accordingly.

Spent Cell Culture Medium sGAG Contents

Centrifuge culture medium at 10000 x g for 10mins. to remove cell debris and assay sGAG in supernatant.

Cellular sGAG Content

- (a) Wash cells with PBS and drain. To release anchored cells use Papain Extraction Reagent (1ml) for exactly **60 seconds @ 37°C**.
- (b) Tap flask or wells and transfer the cell suspension to 1.5 ml microcentrifuge tube(s). Immediately place in **ice-water bath**. Ensure cell suspension is well mixed when subsequently sampling aliquots for (1), (2) & (3).

(1) sGAG attached to the external surface of the cell (Fig. 4).

Take an aliquot (100µl) of cell suspension and proceed to Blyscan sGAG Assay Protocol (inside manual cover).

(2) sGAG from the extracellular matrix after cell removal (Fig. 4).

Centrifuge an aliquot of cell suspension for 10 mins @ 10000 x g, to remove cells from the supernatant. Take 100µl of the supernatant and proceed to Blyscan sGAG Assay Protocol.

(3) sGAG external & internal cell contents (Fig. 4).

To a 1.5ml microcentrifuge tube add an aliquot of cell suspension (we suggest trying 200µl) and 500µl of Papain Extraction Reagent. Incubate contents at 65°C for 3 hours, and then cool to room temperature.

Take 100µl of the papain digested cells and proceed to Blyscan sGAG Assay Protocol.

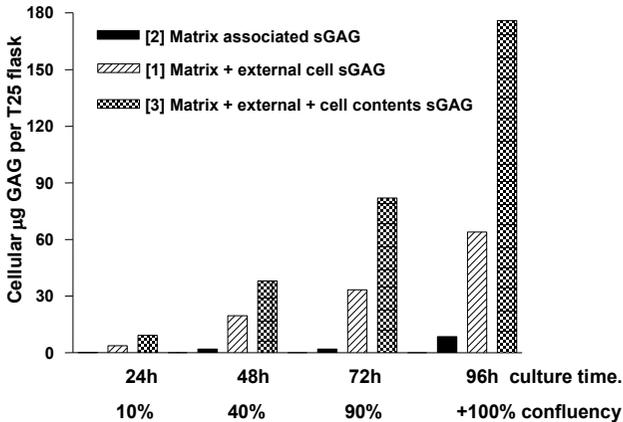


Fig. 4: CHO cells cultured in a T-25 flask.

Other Samples e.g. Synovial Fluid, Urine, Chromatography Fractions

Direct analysis of water based samples is possible provided they are transparent. Any turbidity or suspensions of particles need to be removed. If samples contain high salt concentrations and/or surfactants run a Standard sGAG curve using the sample as the dilutant.

Synovial Fluid: May require dilution before analysis. Viscous samples can require dilution with PBS to aid accurate pipetting. Cloudy samples will require clarification prior to analysis. Hyaluronate does not interfere with the recovery of sGAG.

Urine: Random specimens, (but preferably an early morning sample), stored at 4°C for same day analysis, or frozen for subsequent batch analysis. Analyze at two dilutions; (a) 25µl urine plus 75µl water and (b) 100µl urine sample; in 1.5ml microcentrifuge tubes. Add 1ml Blyscan Dye and shake gently for 30 mins. Complete the assay as described on the inside cover of this manual. Results are usually expressed as sGAG mg/100ml or as sGAG mg/mM of creatinine.

Assay Protocol

The General Protocol can be found on the separate sheet supplied with each kit. The following manual sections contain supplementary details that should be read through, alongside the General protocol, before the assay is carried out.

MEASUREMENT OF TOTAL SULFATED GLYCOSAMINOGLYCANS (sGAG)

sGAG Concentration in Test Samples

The absorbance values of the reagent blank, reference standards and test samples are measured against water. The reagent blank value should be less than 0.10 absorbance units. The reagent blank's absorbance value is subtracted from the standards and test samples absorbance readings. It can be more convenient to set the microplate reader to zero using the reagent blank when low reagent blank values are consistently being obtained.

Variations in absorbance values between duplicate samples should be monitored. Initially a few wide variations may occur. If this is not due to inaccurate pipetting the most likely source of error is in the drainage step. Practice with draining and drying the top of the microcentrifuge tubes will lead to a consistent mode of practice. Duplicate samples should read within $\pm 5\%$ of their mean value.

Using a computer spreadsheet programmed with graphical output the sGAG reference standard absorbance means should be plotted against their known concentrations. Joining the points should produce a straight line graph that can be extended downwards to pass close to or through zero (Absorbance v Concentration). An example calibration curve is provided in Fig. 7.

Test sample concentration values can be read off the graph or calculated from the degree of the slope. Absorbance readings less than 0.1 and greater than 1.5 are unreliable. Samples in these regions should be re-assayed after the test material has been concentrated or diluted as appropriate. Values above 1.50 should not be further diluted with the Dissociation Reagent as 1ml of Blyscan Dye Reagent cannot dye saturate these increased sGAG levels.

The spectrum chart in Fig.6 of the Blyscan Dye in the Dissociation Reagent has a peak maximum of 656 nm. The absorbance peak is broad and most microplate readers will have a colour filter between 625 and 675 nm. This should provide an absorbance slope similar to, but not necessarily matching, that of the 656nm as in Fig.6. The sGAG Reference Standard curve was obtained using a microplate reader and is presented in Fig.7 to offer a guide for other microplate readers.

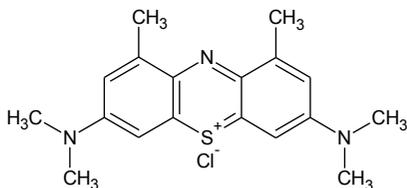


Fig. 5: Molecular Structure of Blyscan Dye.

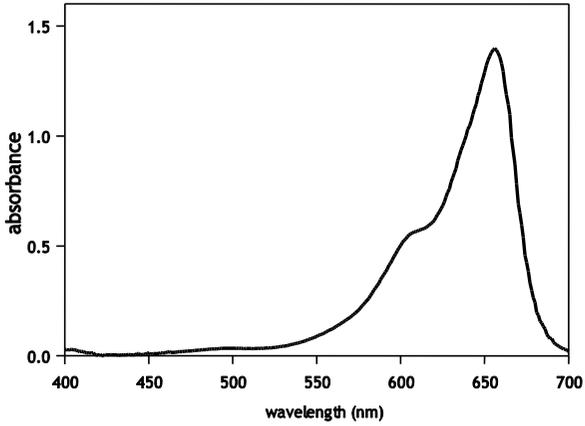


Fig. 6: Absorption Spectrum of Blyscan Dye.

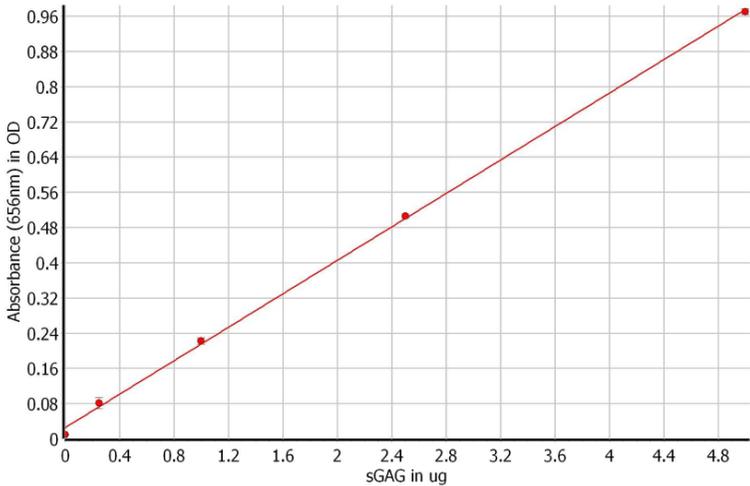


Fig.7: A typical straight line assay calibration curve using chondroitin 4-sulfate (bovine trachea).

Limits of Detection Using a 100 μ l sample aliquot, a 0.25 μ g quantity of chondroitin 4-sulfate (equivalent to 2.5 μ g/ml) gives a 656nm absorbance value of ~0.10. The maximum 656nm absorbance value for 5 μ g (equivalent to 50 μ g/ml) sGAG is ~1.0. Higher absorbance values will depart from a linear correlation between absorbance and concentration.

N-SULFATED AND O-SULFATED GLYCOSAMINOGLYCAN DIFFERENTIATION

Heparin and heparan sulfate contain *N*-sulfated hexosamines whereas some other proteoglycans contain *O*-sulfated hexosamines. Nitrous acid reacts with the *N*-sulfated *D*-glucosamine in preparations of heparin and heparan sulfate, by cleaving after the amino sugar to form a 2,5-anhydromannose residue. A mechanism for this reaction has been presented below (Fig. 8). After this reaction *O*-sulfated glycosaminoglycan levels can be measured using the Blyscan protocol as normal, and results subtracted from total sGAG levels (determined earlier) to give *N*-sulfated glycosaminoglycan levels.

Nitrous Acid Cleavage Method

sGAG samples (100 μ l), in microcentrifuge tubes, are mixed with 100 μ l of sodium nitrite reagent. Following addition of 25% acetic acid solution (100 μ l) the tube contents are vortexed. The reaction is allowed to proceed for 60 minutes at room temperature, with occasional mixing. During this period the tube contents may turn pale yellow and form gas bubbles.

After the reaction is completed ammonium sulphamate reagent (100 μ l) is added to each tube; vigorous bubbling can occur as the nitrous acid is removed. The contents are further mixed over a period of 10 minutes. A sample of the neutralized reaction mixture (100 μ l) is then assayed, with appropriate blanks and controls, according to the normal dye-binding assay procedure.

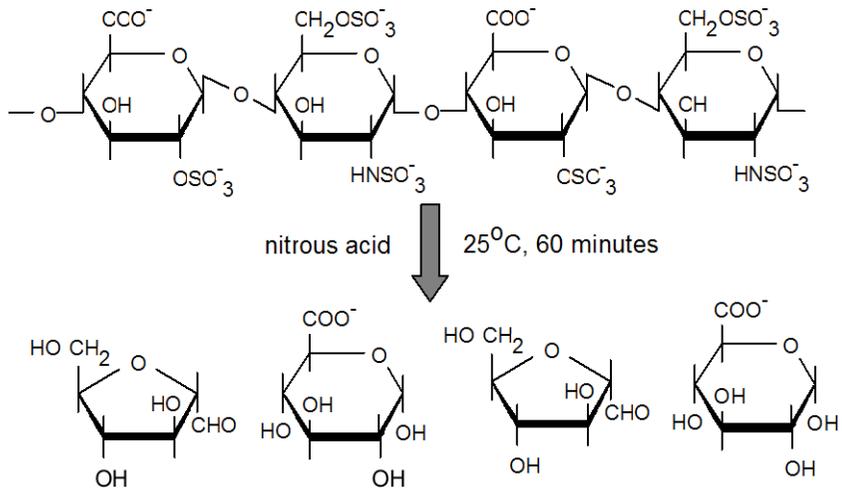


Fig.8: Proposed mechanism for the nitrous acid cleavage of *N*-sulfated glycosaminoglycans (heparan sulfate and heparin) (Carney, S.L., 1986).

Following nitrous acid treatment, the colour produced by the assay represents the *O*-sulfated glycosaminoglycan content.

The *N*-sulfated content can be subsequently calculated from the difference between the total sGAG value and the *O*-sulfated glycosaminoglycan content in each sample.

A calibration curve is constructed by preparing known mixtures of *N*-sulfated and *O*-sulfated glycosaminoglycan from purified standards.

The dye binding (absorbance @ 656 nm), with / without nitrous acid treatment, can be expressed as a ratio and plotted against the relative content of the standard mixtures (Fig. 9).

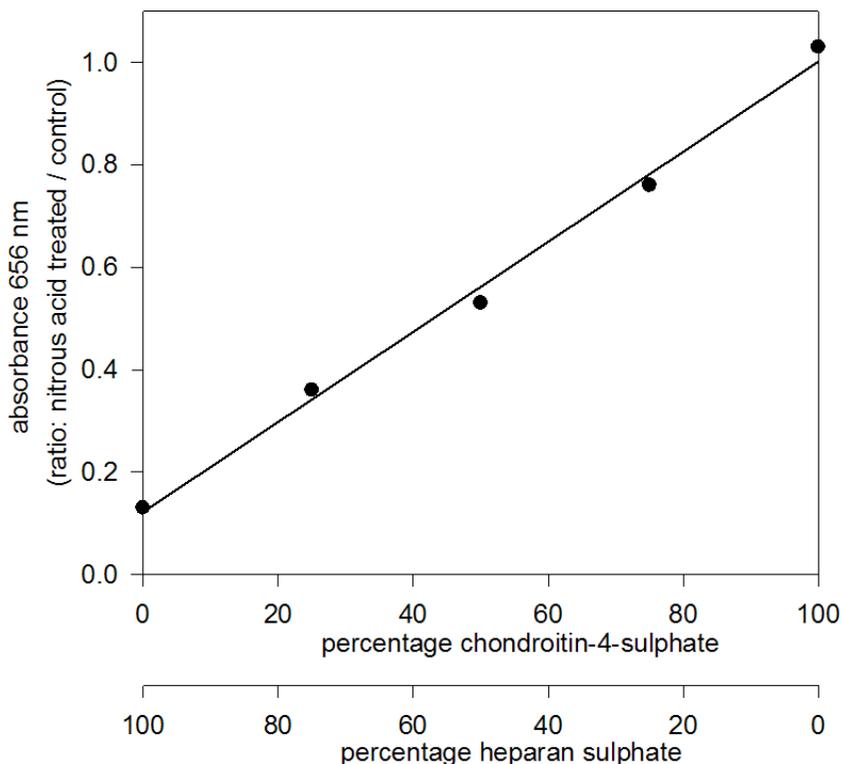


Fig. 9: A typical calibration curve suitable for the determination of the *O*-sulfated and *N*-sulfated glycosaminoglycan content of a test sample.

Origin of Blyscan Assay Name

Blyscan is an Old English word meaning 'to shine' and from which the word 'blush', (blushing), may have been derived. This was an appropriate choice as the Blyscan Assay contains a blue dye which turns bright pink when it binds to sulphated glycosaminoglycans!

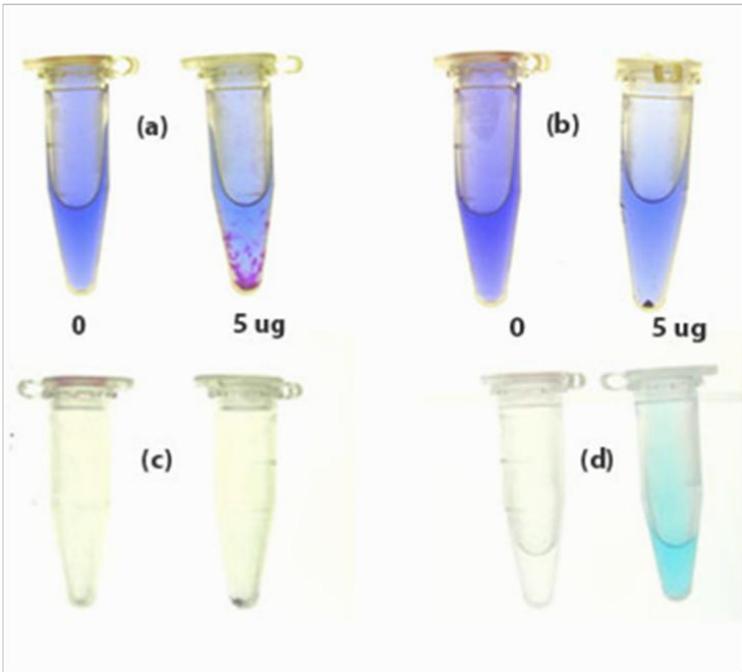
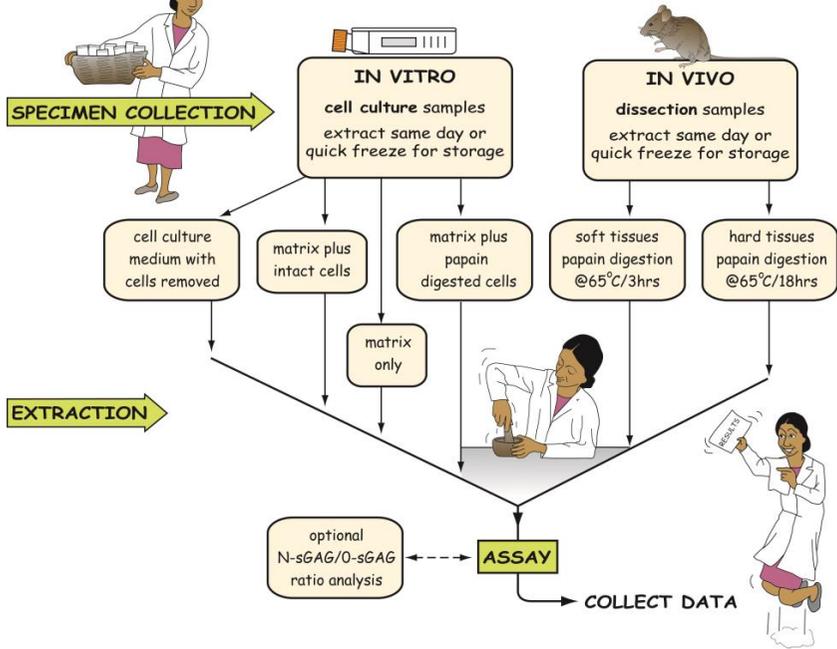


Fig. 10 Blyscan Assay; step-by-step

- (a) 0 and 5 μ g of sGAG and Blyscan Dye, (after 15 minutes mixing).
- (b) 30 min mixing and then centrifuged, (note the sGAG-Dye pellet).
- (c) The non-sGAG Dye was drained from tubes with pellet retained.
- (d) Dye released from sGAG using the Dye Dissociation Reagent.

BLYSCAN FLOWCHART



Useful Journal Reviews: S-Glycosaminoglycans & Proteoglycans

Proteoglycans: from structural compounds to signaling molecules.

Schaefer, L. & Schaefer, R.M. (2010) *Cell Tissue Res.* **339**, 237-246.

Proteoglycans and more – from molecules to biology.

Heinegard, D. (2009) *International J.Exper.Path.* **90**, 575-586.

The Structure of Glycosaminoglycans and their Interactions with Proteins.

Gandhi, N.S. & Mancera, R.L. (2008) *Chem.Biol.Drug.Des.* **72**, 455-482.

Glycosaminoglycans and their proteoglycans: host-associated molecular patterns for initiation and modulation of inflammation.

Taylor, K.R. & Gallo, R.L. (2006) *FASEB Journal* **20**, 9-22.

Monographs & Practical Reference Sources

Proteoglycans; Structure, Biology and Molecular Interactions [2000] Editor: R.V. Iozzo. Publisher: Marcel Dekker Inc., New York.

Extracellular Matrix Protocols, (Methods in Molecular Biology Volume 139) [2000] Editors: C.H. Streuli & M.E. Grant. Publisher: Humana Press, New Jersey.

Extracellular Matrix, Vol. 1; Tissue Functions, Vol. 2. Molecular Components [1996] Editor: W.D. Comper. Publisher: Harwood Academic, The Netherlands.

The Extracellular Matrix Facts Book [1994] Editors: Shirley Ayad, R.Boot-Handford, M.J.Humphries, K.E.Kadler & A.Shuttleworth. Publisher: Academic Press, London.

Guidebook to the Extracellular Matrix and Adhesion Proteins [1993] Editors: T.Kreis & R.Vale. Publisher: Oxford University Press, Oxford.

Proteolytic enzymes [1989] Editors: R.J.Beynon & J.S.Bond. Publisher: IRL Press, Oxford.

Carbohydrate analysis [1989] Editors: M.F.Chaplin & J.F.Kennedy. Publisher: IRL Press, Oxford.

Blyscan Sulfated Glycosaminoglycan Assay

General Protocol

Detection Limit: 0.25µg/100µl (2.5µg/ml)

Time Required: 1 hour

Set Up Assay

Label a set of 1.5 ml microcentrifuge tubes. All samples, standards and blanks should be run in duplicate.

Prepare;

Reagent blanks - 100 µl of deionised water or the test sample buffer.

Glycosaminoglycan standards - use aliquots containing 1.0, 2.0, 3.0, 4.0 and 5.0 µg of the reference standard.

Make each standard up to 100µl using the same solvent as the Reagent blanks. The standards and the reagent blank (0µg) are used to produce a calibration curve.

Test samples - use volumes between 10 and 100 µl.

Adjust the contents of all tubes to 100 µl with deionised water or appropriate buffer. Where there is no previous knowledge of the glycosaminoglycan (GAG) content 50µl of the test material is suggested for a trial run. Samples should not contain excessive salt that could interfere with the GAG- dye interaction; and GAG content must not exceed 5µg or dye-GAG saturation will not occur.

Commence Assay

To each tube add Blyscan dye reagent (1.0ml).

Cap tubes; mix by inverting contents and place tubes in a gentle mechanical shaker for 30 minutes, (or manually mix at 5 minute intervals).

During this time period a sulphated glycosaminoglycan-dye complex will form and precipitate out from the soluble unbound dye (Fig.10a).

Centrifuge

Transfer the tubes to a microcentrifuge and spin at 13000 x g. for 10 minutes (Fig.10b).

Example images can be found on p12 of the main assay manual



Draining

Important: firmly packing the insoluble sGAG-dye complex at the bottom of the tubes is required to avoid any pellet loss during draining of unbound dye. *Longer centrifuge times can be used to improve packing of pellet*

Carefully invert and drain tubes (Fig.10c).

Any remaining droplets can be removed from the tubes by *gently* tapping the inverted tube on a paper tissue.

Release and Recovery of s-Glycosaminoglycan Bound Dye

Do not attempt to physically remove any fluid that is in close contact to the deposit.

Add dissociation reagent (0.5ml) to tubes (Fig.10d).

Re-cap the tubes and release the bound dye into solution. A vortex mixer is suitable.

When all of the bound dye has been dissolved, (usually within 10 minutes), centrifuge at 13000 x g for 5 mins to remove foam.

Keep the tubes capped until ready to measure absorbance.

Measurement

Transfer 200 µl of each sample to individual wells of a 96 micro well plate. Avoid rapid pipetting as foaming can cause abnormal absorbance readings. Keep a record map of the contents of each well; A1 to H12.

Set the microplate reader to 656nm or the closest matching red filter.

Measure absorbance against water for the reagent blanks, standards and test samples.

Obtain s-glycosaminoglycan concentrations from the Standard Curve. Duplicates should be close to ±5% of their mean value.

If the sample absorbance value is at the top end of the standard curve the test sample should be diluted five or ten-fold and the assay repeated. The dye concentration available will not be sufficient to fully label more than 5µg of the GAG molecules present.