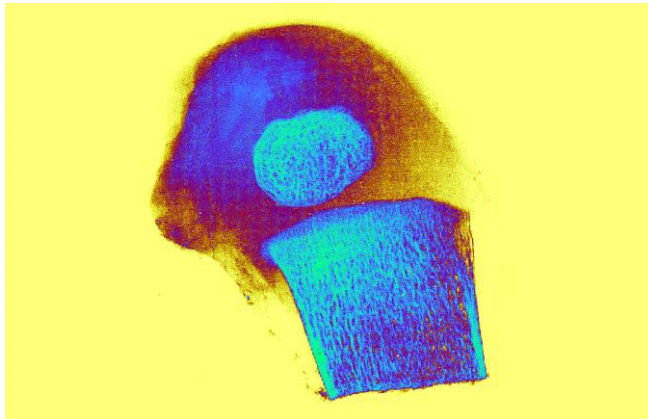


NEW
EDITION

Blyscan™

Sulfated Glycosaminoglycan Assay



biocolor
life science assays

**Internet Manual
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Blyscan Sulfated Glycosaminoglycan Assay

General Protocol

Detection Limit:
Time Required:

0.25µg
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. 1a, see outside back cover).

Centrifuge

Transfer the tubes to a microcentrifuge and spin at 12,000r.p.m. for 10 minutes (Fig. 1b).

ASSAY PROTOCOL CONTINUED ON INSIDE BACK COVER

PROTOCOL CONTINUED FROM INSIDE FRONT COVER

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.

Carefully invert and drain tubes (Fig. 1c).

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

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

Release and Recovery of s-Glycosaminoglycan Bound Dye

Add dissociation reagent (0.5ml) to each tube (Fig. 1d).

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, the samples are ready for measurement.

The colour is light stable, but should be read within 2 hours.

Keep the tubes capped until ready to measure absorbance.

Measurement

Transfer 200 μ l of each sample to individual wells of a 96 micro well plate (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 $\pm 5\%$ 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 5 μ g of the GAG molecules present.

SEE MANUAL FOR SAMPLE EXTRACTION AND PREPARATION DETAILS

Origin of Blyscan Assay Name

The name for the assay was found using a computer based dictionary. Looking for three or more letters in sequence from 'glycan' we found 'blyscan'. 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.

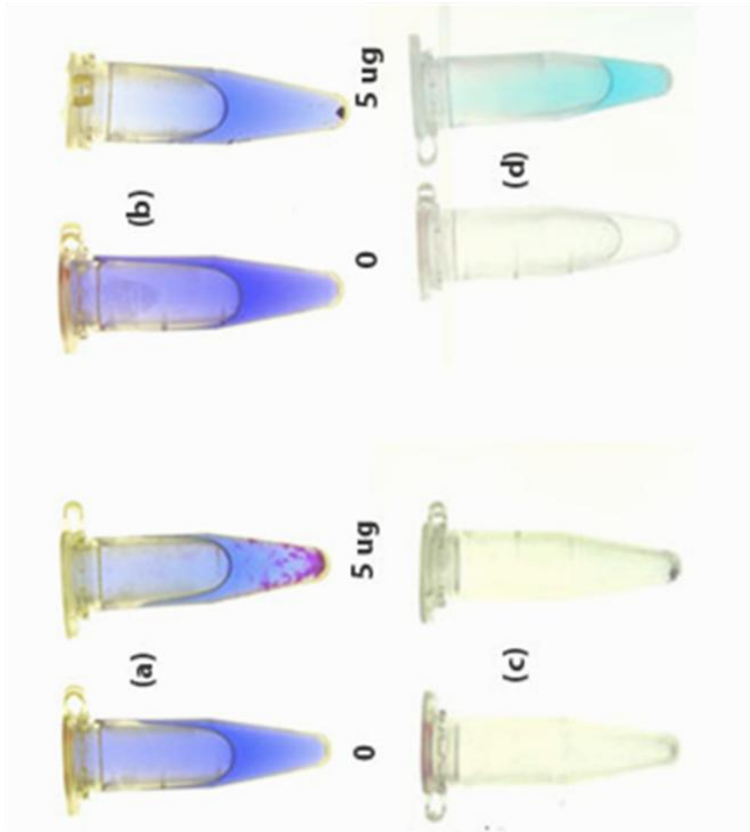


Fig. 1 Blyscan Assay; step-by-step

- (a)** 0 and 5µg 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.

13th August 2010

Blyscan™

Sulfated Glycosaminoglycan Assay

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

TECHNICAL INFORMATION

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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 – (Extraction Protocols page 8)

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 (120 assays)

Economy Pack Product Code: B3000 (475 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 however be stored at +4°C.

Assay Kit components; supplied

1. **Dye Reagent** contains 1,9-dimethyl-methylene blue in an inorganic buffer, which also contains surfactants. [The Blyscan Dye in the current kit remains unaltered from the original composition and produces results that are fully compatible with any previous Blyscan based studies].
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:** 33% (v/v) acetic acid
6. **Ammonium Sulfamate :** 12.5% (w/v) sterile solution

Extraction components required for sample preparation; not supplied

7. **Papain.** Crystallized papain suspension, a Sigma-Aldrich product, (Code: P3125), was used during the development of this update. Avoid using low cost crude papain as it often contains other proteases that hasten digestion of the papain.
8. **L-Cysteine Hydrochloride,** (0.005 M).
9. **Ethylenediaminetetraacetic Acid, Disodium Salt,** (EDTA), (0.01 M).
10. **Potassium Phosphate Buffer,** pH 6.40, (0.2 M).
11. **Sodium Acetate** (0.1 M)
12. **Papain Buffered Reagent** (see page 8)

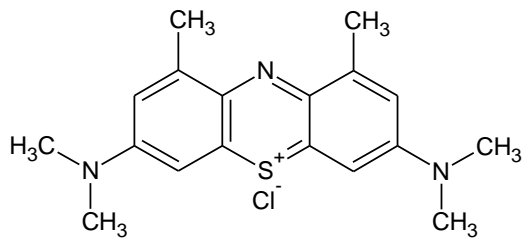


Fig. 2: Molecular Structure of Blyscan Dye.

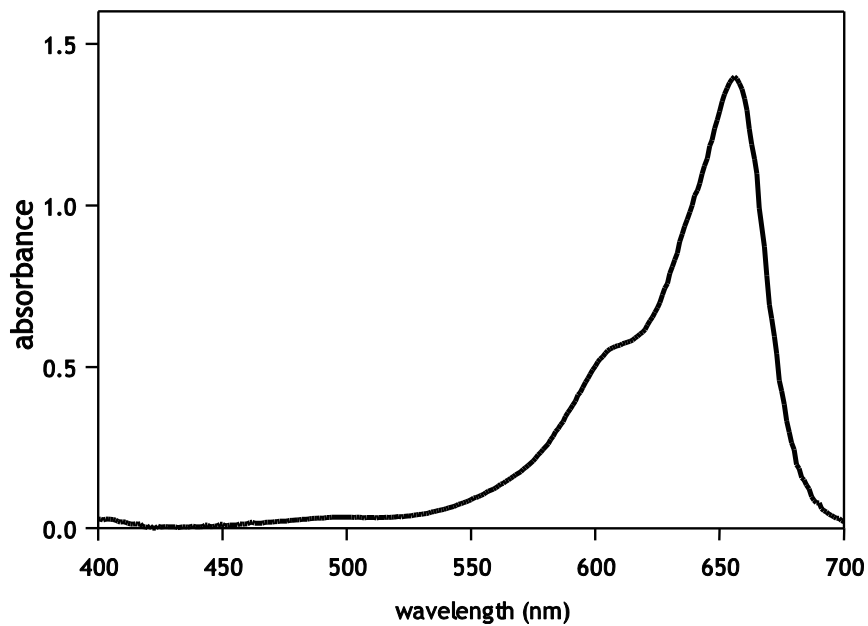


Fig. 3: Absorption Spectrum of Blyscan Dye.

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 three 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. 4.

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 and samples should be re-assayed after either concentration or dilution of the test material. Values above 1.50 should not be further diluted with the Dissociation Reagent as 1.00ml of Blyscan Dye Reagent cannot dye saturate these increased sGAG levels.

Notes on Microplate Readers

Many microplate readers use coloured glass filters unlike spectrophotometers that provide wavelength selection with a prism or ruled grating.

The spectrum chart in Fig.3 of the Blyscan Dye in the Dissociation Reagent with a peak maximum in the visible region of 656 nm was obtained with a dual beam spectrophotometer.

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 650nm filter as used in our microplate reader (Bio-Tek Instruments Inc.).

The sGAG Reference Standard curve was obtained using the above microplate reader and is presented in Fig.4 to offer a guide for filter selection in other microplate readers.

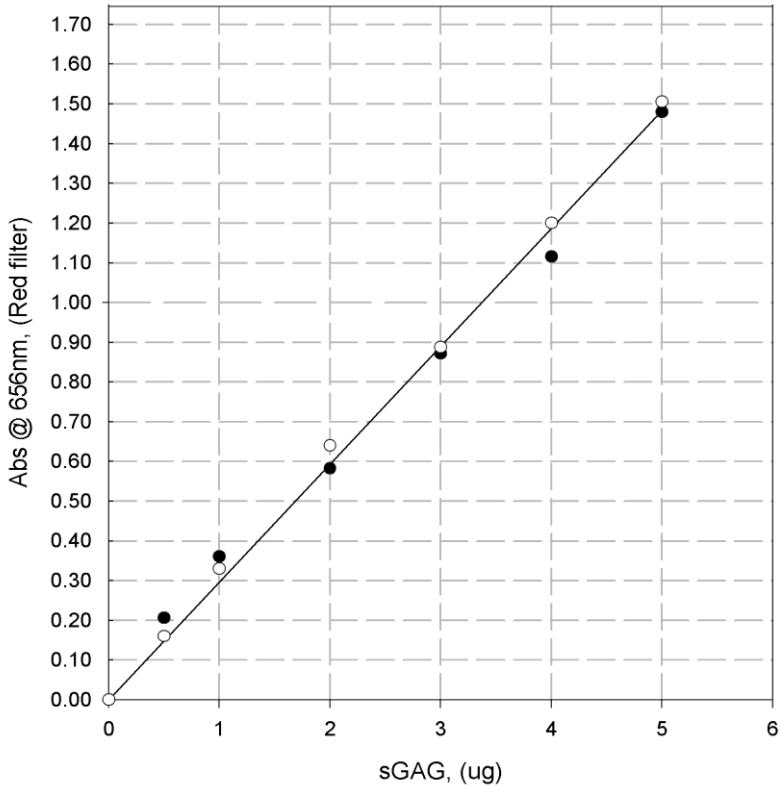


Fig.4: A typical straight line assay calibration curve. Test sample chondroitin 4-sulfate, (bovine trachea).

The Blyscan Assay provides the ability to measure sGAG over a concentration range from 2.5µg/ml to 500µg/ml, when using 100 to 10µl sample aliquots.

Limits of Detection

The assay detects a minimum acceptable absorbance value of 0.10 at 650nm for 0.25µg sGAG in a 100µl test sample aliquot, to a maximum absorbance value of 1.5 for 5µg sGAG in a 10µl sample aliquot. Higher absorbance values depart from a linear correlation between absorbance and concentration.

N-SULFATED AND O-SULFATED GLYCOSAMINOGLYCAN DIFFERENTIATION

Heparin and heparan sulfate differ from some other glycosaminoglycan types by containing *N*-sulfated hexosamines. Nitrous acid reacts with the *N*-sulfated-D-glucosamine in preparations of both these sGAG, by cleaving after the amino sugar to form a 2,5-anhydromannose residue. A mechanism for this reaction has been presented below (Fig. 5).

Nitrous acid cleavage method

100 μ l samples of sGAG, in microcentrifuge tubes, are mixed with 100 μ l of sodium nitrite reagent.

Following addition of the 33% 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 100 μ l ammonium sulphamate reagent 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.

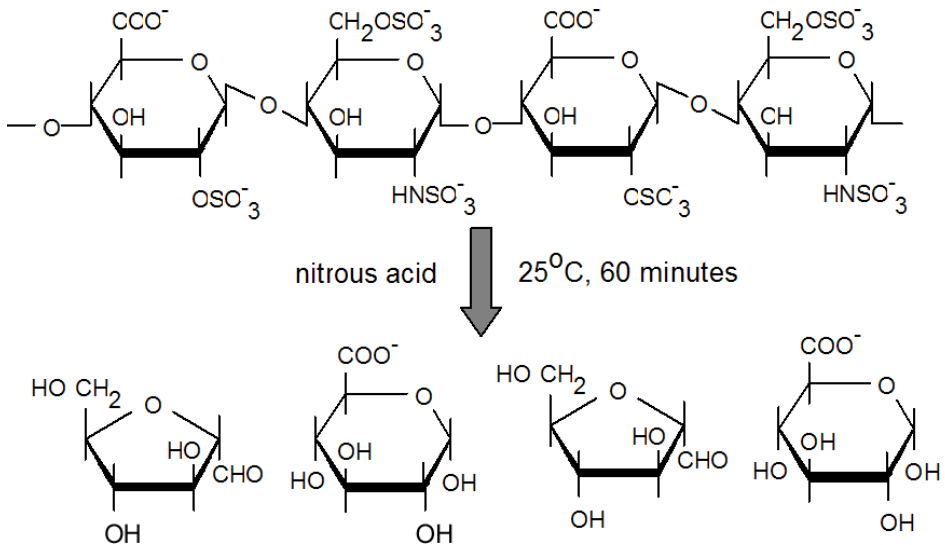


Fig.5: Proposed mechanism for the nitrous acid cleavage of N-sulfated glycosaminoglycans (heparin sulfate and heparin) (Carney,S.L.,1986).

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. 6).

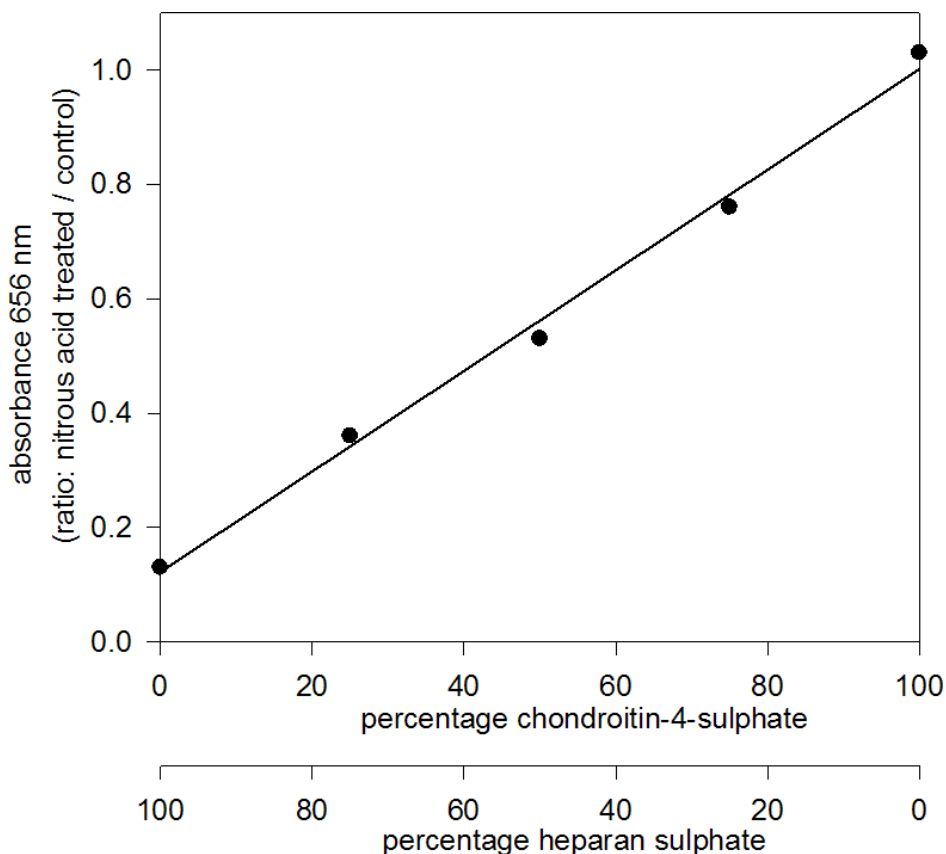


Fig. 6: A typical calibration curve suitable for the determination of the O-sulfated and N-sulfated glycosaminoglycan content of a test sample.

TISSUE SAMPLE PREPARATION PRIOR TO ASSAY

Preparation of Test Material for Extraction

Do you require the sGAG within test sample to 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) could be considered.

(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 (~30% of wet weight).

Papain Extraction Reagent.

To 100ml of 0.2M sodium phosphate buffer ($\text{Na}_2\text{HPO}_4 - \text{NaH}_2\text{PO}_4$), pH 6.4

add 0.1M sodium acetate, (8.2 g)

0.01M Na_2EDTA , (3.7 g)

0.005M cysteine HCl, (790 mg)

When the above components have dissolved the papain suspension can be introduced.

Enzyme suspension of ~ 800 μl crystallized papain, (page 2), provides 15 to 20mg papain per 100ml extraction buffer.

Check pH and adjust if required before storing at 4°C , (shelf life is 10 days@ 4°C).

PAPAIN EXTRACTION OF sGAG FROM TISSUE SAMPLES, (*in-vivo*)

The test sample, (20-50mg), with 1 ml Papain Reagent are placed in 1.5 ml labelled microcentrifuge tubes.

Protein digestion is then carried out at 65°C , using a thermally regulated metal heating block, or water bath.

A digestion time of 3 hours @ 65°C is sufficient to break up soft tissue aliquots of 20 to 50 mg, (wet weight), with occasional tube removal and mixing.

A three hour protocol permits sGAG assays to be carried out on the same day (Fig. 7). Hard tissues, such as cartilage and bone, require overnight incubation @ 65°C .

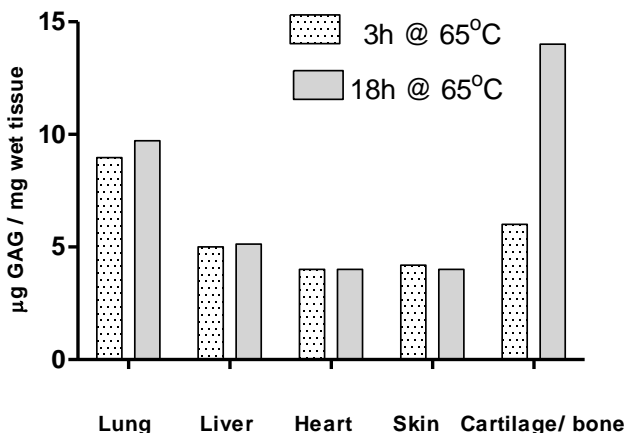


Fig. 7: Recovery of sGAG from mouse tissues, with 3 and 18 hours of hot papain.

PAPAIN EXTRACTION OF sGAG FROM CELL CULTURE SAMPLES, (*in-vitro*)

Retain cell medium from T-flask or microwell plate. 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.

Initially try using 50µl of centrifuged digested extract with 1ml Blyscan Dye and proceed with the assay if a colour change can be observed (Fig. 8).

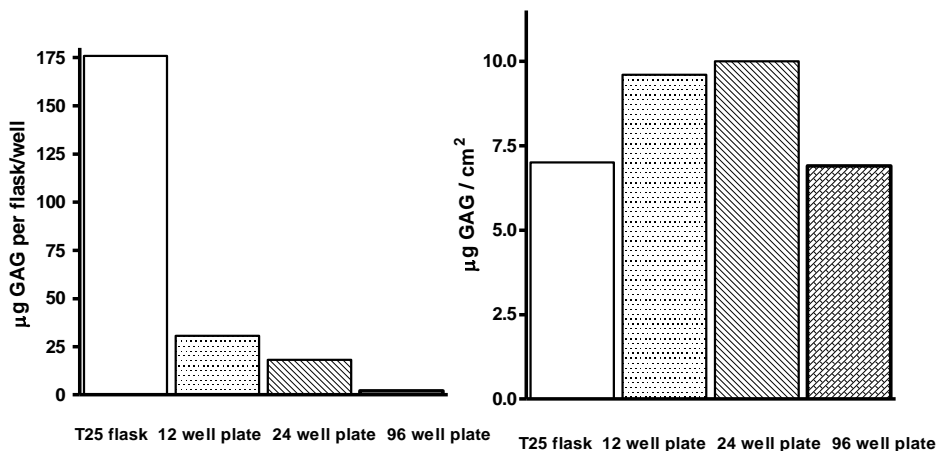


Fig. 8: A CHO cell line culture for 72 hours, without a culture medium change, (cells confluent).

PAPAIN EXTRACTION OF EXTERNAL AND INTERIOR CELL sGAG.

There is little sGAG released into the culture medium until cell confluency occurs. This discovery led to the development of a cell based sGAG assay protocol.

[1] sGAG contents of spent cell culture medium.

Centrifuge to remove debris and assay sGAG in supernatant.

(a) Wash cells with PBS and drain. To release anchored cells use Papain Extraction Reagent for exactly 60 seconds @ 37°C.

(b) Tap flask or wells and transfer the cell suspension to 1.5 ml microcentrifuge tube(s) and immediately place in ice-water bath. Ensure cell suspension is well mixed when sampling aliquots for [2], [3] & [4].

[2] sGAG attached to the external surface of the cell (Fig. 9).

Take an aliquot (50 μ l) of cell suspension. Add 1000 μ l Blyscan Dye. Shake for 30 mins @ room temperature. Complete the assay.

[3] sGAG matrix associated (Fig. 9).

Centrifuge an aliquot of cell suspension for 10 mins @ 10K x g. Add 100 μ l of supernatant to 1000 μ l Blyscan Dye. Shake for 30mins @ room temperature. Complete the assay.

[4] sGAG external & internal cell contents (Fig. 9).

To a 1.5ml microcentrifuge tube add an aliquot of cell suspension and 500 μ l of Papain Extraction Reagent. Incubate contents at 65°C for 3 hours, then cool to room temperature.

To 100 μ l of the papain digested cells add 1000 μ l Blyscan Dye. Shake for 30 mins @ room temperature and complete the assay.

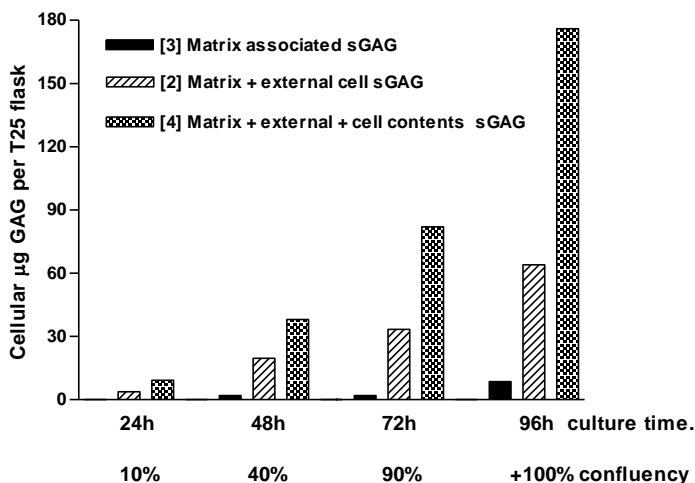


Fig. 9: CHO cells cultured in a T-25 flask.

SYNOVIAL FLUID, URINE, CHROMATOGRAPHY FRACTIONS, etc.

Direct analysis of water based samples are possible provided they are transparent. Any turbidity or suspension 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 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; 25µl plus 75µl water and with 100µl sample in 1.5ml microcentrifuge tubes. Add 1ml Blyscan Dye and gentle shake for 30 mins. Complete the assay as described on the inside cover of this manual. Results are usually expressed as mg sGAG /100ml or as sGAG mg/mM of creatinine.

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