Astarte BIOLOGICS

コスモ・バイオ株式会社

Rheumera®

Proteoglycan Detection Kit
Cat# 8000

Assay Kit To Measure Proteoglycan.

INTRODUCTION

Glycosaminoglycans (GAGs) are a major component of the extracellular matrix (ECM) of tissue and are long unbranched polysaccharides containing a repeating disaccharide unit. GAGs consist of N- and/or O-sulfate groups and are covalently linked to core proteins to form proteoglycans. The most common GAGs are hyaluronic acid, dermatan sulfate, chondroitin sulfate, heparin, heparan sulfate, and keratan sulfate. Hyaluronic is unique among the GAGs in that it does not contain any sulfate and is not found covalently attached to proteins as a proteoglycan (it is, however, a component of non-covalently formed complexes with proteoglycans in the ECM).

GAGs are highly negatively charged molecules that impart high viscosity to tissues and liquids. Along with the high viscosity comes low compressibility, which makes these molecules ideal for example as a lubricant (synovial fluid) for joints. GAGs and proteoglycan play an important role in cellular adhesion, growth, migration, and differentiation. They are also involved in regulation of enzymes and tissue remodeling in response to injury, for example, or tissue destruction in diseases such as rheumatoid arthritis. However, certain types of proteoglycans are upregulated in other diseases such as osteoarthritis.

Sulfated GAGs can be measured directly by use of a metachromatic dye, 1,9 Dimethylmethylene blue. The GAG-dye complex results in an absorption spectrum shift which can be measured at A_{525}.

PRINCIPLE OF THE ASSAY

The Rheumera® GAG Assay uses the metachromatic dye 1,9 - dimethylmethylene blue to quantify the amount of sulfated glycosaminoglycans in the standard and test samples. The binding of the sulfated glycosaminoglycans to the dye induces a shift in the absorption spectrum (A_{525}) which is directly proportional to the amount of sulfated glycosaminoglycans. The sample values, µg/ml of sulfated glycosaminoglycans, are determined by the standard curve. The assay detects chondroitin 4 and 6 sulfates, and heparin, keratin, and dermatan sulfates. Hyaluronic acid will not be detected and also will not interfere with the assay.
Various biologic fluids can be tested directly such as synovial fluid, serum, amniotic fluid and urine as well as tissue culture media. Tissue samples such as cartilage, skin, and other organs must first be digested with papain or extracted with 3M guanidinium HCl before testing (see Sample Preparation)

EXAMPLE OF AN ELISA PLATE CONFIGURATION

A Typical Configuration ELISA plate

ELISA Plate **Std(ug/ml)**

	1	2	3	4	5	6	7	8	9	10	11	12
A	1	1	9	9	17	17	25	25	33	33	5	5
B	2	2	10	10	18	18	26	26	34	34	2.5	2.5
C	3	3	11	11	19	19	27	27	35	35	1.25	1.25
D	4	4	12	12	20	20	28	28	36	36	0.63	0.63
E	5	5	13	13	21	21	29	29	37	37	0.31	0.31
F	6	6	14	14	22	22	30	30	38	38	0	0
G	7	7	15	15	23	23	31	31	39	39	41	41
H	8	8	16	16	24	24	32	32	40	40	42	42

A typical ELISA plate configuration where samples and standards are run in duplicate. Ten, 8-well strips, are used for the test samples. Two, 8-well strips, depicted in red, are used for the standards (0 to 5 ug/ml).

REAGENTS INCLUDED

A. 1,9 Dimethylmethylene blue, 100 ml**.**
B. Chondroitin Sulfate Standard (Bovine Trachea) 20µg.

REAGENTS NOT INCLUDED

Papain
3M guanidine hydrochloride
0.05M Tris-HCl or PBS buffers

REQUIRED EQUIPMENT/LABWARE/REAGENTS

Microtiter Plate(s) (96 well format)
Cuvettes
Microplate reader or spectrophotometer with A_{525} nm filter or wavelength capability.
Single and multi-channel pipettes and tips.

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PRECAUTIONS

Follow good laboratory practices including wearing appropriate eye, hand, face, and clothing protection.

The methylene blue dye is acidic and highly irritating to eyes.

SAMPLE PREPARATION

The Rheumera® kit can detect both newly synthesized GAGs excreted from cells in culture medium or GAGs incorporated into the extracellular matrix. To measure soluble GAGs, varying dilutions of culture media can be directly applied to the dye (see data below). To measure GAG content in tissue using the Rheumera® kit, the tissue must first be extracted with high salt such as 3M guanidinium HCl or digested with papain. The papain digestion method can also be used to measure GAGs in fluids where contaminating proteins might interfere with the assay. The investigator must determine the best method(s) to use for any particular sample.

The following methods below serve as a guideline:

Cells grown in tissue Culture

Important: perform a count of the cells so that the cell density can be correlated to the amount of GAG measured. To assay the cell culture supernatant, remove the media and proceed directly to testing the sample using varying dilutions. To assay the cell matrix, the following papain digestion protocol can be used:

1. Use 1.0 ml of 20mM sodium phosphate buffer (pH 6.8) containing 1 mM EDTA, 2 mM dithiothreitol and 300ug
Papain. This solution can be placed directly on the cell layer
2. incubate at 60℃ for 30-60 minutes or longer if necessary until the layer is soluble. Add iodoacetic acid to a final concentration,
of 10mM. Add 5 mls of 50mM Tris/HCl (pH 8.0). The sample is ready to test.

Tissue Samples

Important: measure the dry or wet weight of the sample so that it can be correlated to the amount of GAG measured.

High Salt Extraction Method.

1. Mince or homogenize tissue.
2. Add 0.5 ml of 3M guanidine hydrochloride/0.05M Tris-HCl buffer, pH 7.5 and agitate on a rotator/rocker at 4℃ overnight.
3. Dialyze 3M guanidine extracts against 1X TBS and store at -20℃ until measured.

Papain Digestion Method

1. Mince or homogenize tissue.
2. Place tissue in 1.0 ml of 20mM sodium phosphate buffer (pH 6.8) containing 1mM EDTA, 2mM dithiothreitol and 300ug
Papain.
3. incubate at 60℃ for 60 minutes or longer if necessary until tissue is soluble (larger tissue samples will require more time). Add iodoacetic acid to a final concentration, of 10mM. Add 5 mls of 50mM Tris/HCl (pH 8.0). The sample is ready to test.

REAGENT PREPARATION

Solution A. DMB dye. Ready to use.

Standard B. Chondroitin Sulfate (Bovine Trachea) Standard 10 µg. Add 2.0 ml of PBS or the same buffer used for samples. This standard solution is now 10 µg/ml.

For the spectrophotometer method: prepare serial dilutions of the 10 µg/ml standard by mixing 1.0 ml of standard with 1.0 ml of buffer in a separate tube to make a 5 µg/ml solution. Repeat this procedure to make 2.5, 1.25, 0.625, 0.3125 µg/ml solutions. When added to the dye, these standard concentrations will be half of their original concentration. For example, 1.0 ml of 10 µg/ml standard when mixed with 1.0 ml of dye will become 5 µg/ml, and so on. Zero or blank is buffer alone, with no standard.

For the microplate method: prepare serial dilutions of the 10 µg/ml standard by mixing 0.1 ml of standard with 0.1 ml of buffer in a separate tube or microplate to make a 5µg/ml solution. Repeat this procedure to make 2.5, 1.25, 0.625, 0.3125 µg/ml solutions. When added to the dye, these standard concentrations will be half of their original concentration. For example, 0.1 ml of 10 µg/ml standard when mixed with 0.1ml of dye will become 5 µg/ml, and so on. Zero or blank is buffer alone, with no standard.

ASSAY PROCEDURE

It is recommended that test samples and positive controls be run in duplicate.
Prepare Samples and Reagents as described above

For the spectrophotometer method:

1. **Samples.** Add 1.0 ml of sample to 1.0 ml dye in a tube.
2. **Standards.** Add 1.0 ml of each standard to 1.0ml dye in separate tubes. The 10 µg/ml standard will now become 5 µg/ml, the 5 µg/ml standard will now become 2.5µg/ml, and so on
3. **Measure.** Add the 2.0 mls of samples or standards to separate cuvettes, and record readings at A_{525}.

For the microplate method:

1. **Samples.** Add 0.1 ml of sample to 0.1 ml dye in a microplate well.
2. **Standards.** Add 0.1 ml of each standard to 0.1 ml dye in separate microplate wells (see ELISA plate configuration). The 10 µg/ml standard will now become 5 µg/ml, the 5 µg/ml standard will now become 2.5 µg/ml, and so on.
3. **Measure.** Read the plate in a micoplate reader at A_{525}.

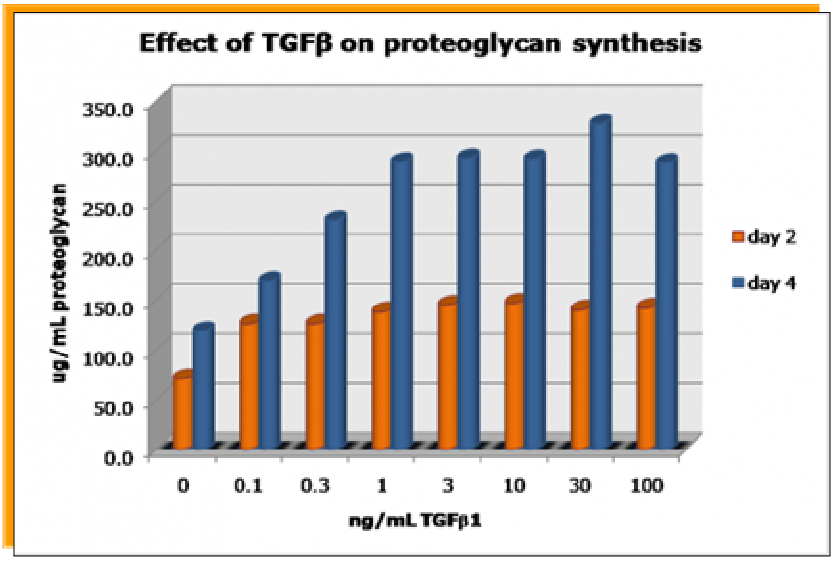


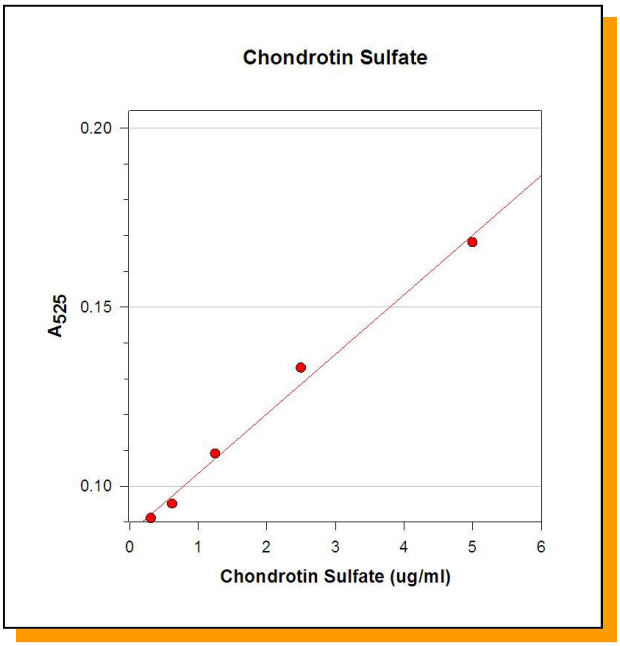
Figure Legend. Bovine chondrocytes were cultured for 2 or 4 days with varying amounts of TGF-β. Culture supernatants were measured for the presence of proteoglycan by testing for GAG content using the Rheumera® **Proteoglycan Detection Kit (Cat# 8000).**

CALCULATION OF RESULTS

1. Average the duplicate OD values for the standards and test samples.

2. Plot the OD values of the chondroitin sulfate standards against their known concentrations (5 - 0.3125 µg/ml) using a linear graph. The graph below shows a representative typical experiment where the standard range is from 5 - 0.3125 µg/ml.

3. The µg/ml of chondroitin sulfate in test samples can be calculated using regression analysis.



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