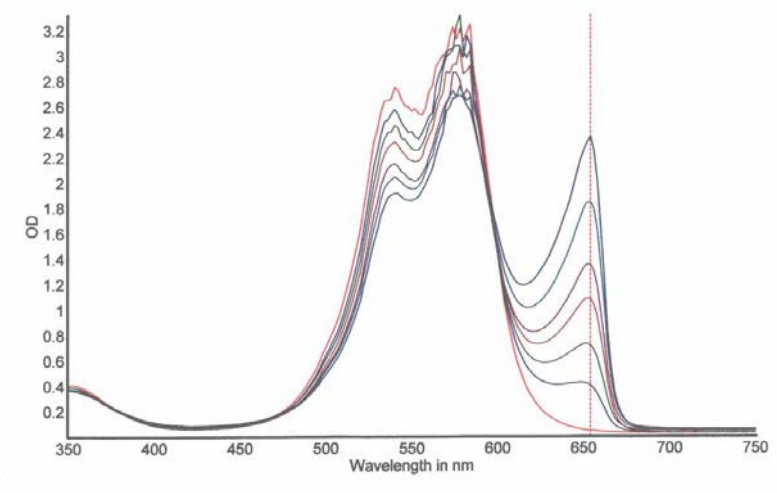
Purple-Jelley
HYALURONAN
[Hyaluronic acid]
Assay



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Purple-Jelley
HYALURONAN
(Hyaluronic acid)
Assay

Assay designed for research work with animal tissues.
The assay is not suitable for blood and urine analysis.

Handle the Assay using Good Laboratory Practice.

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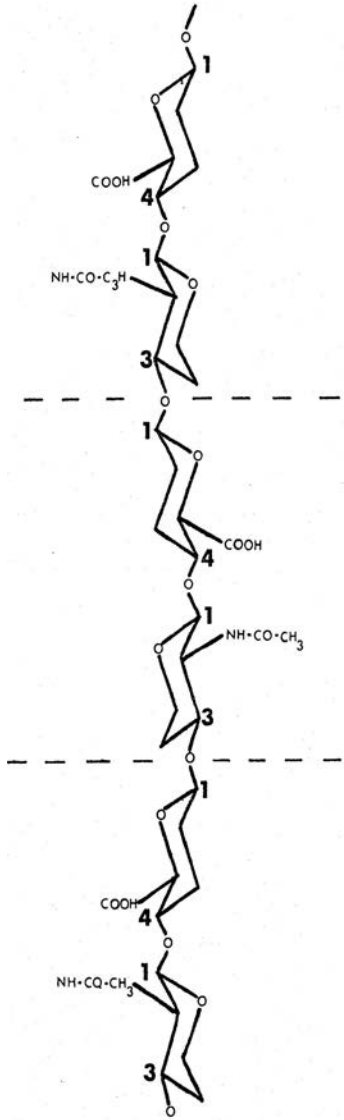

HYALURONAN [HA]

Nature:
A hydrated molecule of HA is a gentle giant of a carbohydrate polymer. It is composed of a long flexible non-branching chain formed with a repeating disaccharide pattern. The disaccharide is made up of alternative uronic acid and aminosugar units.
In the human umbilical cord and synovial fluid, the size of HA is reported to be about 3,000,000 Da. (a flexible chain of 4000 disaccharide units).

Synthesis:
HA is formed by Hyaluronan synthases embedded within the cell membrane. HA is attached to the outside of the cell membrane and can be released from intact cells grown in culture by brief treatment with trypsin. The newly formed HA interacts with the ECM, the cell membrane and with water to form and maintain the formation of a pericellular substrata around the cell, the HA generating a territorial gel, or in cartilage to produce a collagen free lagoon for the cell.

Degradation:
HA is degraded principally by a group of seven hyaluronidases and may account for the variations in the sizes of HA reported. The activity of some hyaluronidases may remain during post mortem acid glycolysis when the animal body is still warm.

Fig. 1 Carbohydrate polymer of HA composed of a repeating disaccharide of an uronic acid and an aminosugar neither of which are sulphated.

The Role of Hyaluronan

HA has the ability to exert control of the water balance of tissues. This property allows selected cells to migrate during growth and is essential for animal development from fertilization to birth.

HA however can have adverse effects in that it will also provide migratory tumour cells the ability to relocate at extended distances from the source of a primary tumour. The wandering tumour cells on relocation may induce metastasis to generate multiple secondary tumours.

Hyaluronan Reviews

Chemistry and Biology of Hyaluronan, Edited by H.G. Garg and C.A. Hales, (2004).
Publisher: Elsevier Oxford, UK.

Hyaluronan in Cancer Biology, Edited by R.Stern, (2009).
Publisher: Academic Press, San Diego, U.S.A.

Storage of Tissues

Tissue should be kept frozen to prevent the action of hyaluronidases until it is required for analysis.

Enzyme degradation of HA produces a mix of tetrasaccharide and disaccharide fragments that do not react with the assay dye reagent.

Blood and urine contains HA fragments that have been reduced to tetra-saccharides and disaccharides and are unsuitable for this assay.

In vitro cell culture, in monolayer or in suspension, do not generate sufficient HA for dye analysis.

Purple-Jelley HYALURONAN Assay

Pack Sizes and Storage Conditions

Standard Assay Kit: Product Code H1000 (100 assays)
Economy Assay Kit: Product Code H2000 (400 assays)

All components are stable for one year when stored unopened at 10 to 25^{0}C.
Once opened the Hyaluronan Standard should be ***stored at +4^{0}C***.
The Dye Reagent should be stored in the dark.

Sampling of Tissue, Wet Weight

The protocol is described on the inside front cover of this manual.

The actual weight of each sample should be recorded and the sample placed into 2.0 ml screw capped tubes suitably labelled for subsequent protein removal by the action of Proteinase K.

The Distribution of HA in Tissues

The distribution of HA in tissues as produced in this assay procedure is listed in Table 1 and is given only as an outline guide.

In adult animals the HA percentage gradually increases during ageing as muscle and fat mass decreases.

Table 1. Distribution of HA in tissue

Species	Tissue	Concentration µg/g
Mouse	heart	48
	kidney	11
	lung	14
	leg muscle	20
	skin	307
Rat	heart	83
	kidney	28
	lung	17
	leg muscle	67
	skin	510
Rabbit	heart	138
	kidney	27
	lung	45
	leg muscle	50
	skin	428

Hyaluronan Assay Components

PROTEIN REMOVAL

Protein Digestion Reagent: A buffer containing 50mM TRIS-HCl, pH 7.6 supplied in a tablet format, (one tablet dissolved in 15 ml dH_2O); use within 2 days.

For each tissue sample add 20µl Proteinase K per 400µl of buffer.
A suitable Proteinase K suspension is available from Sigma Aldrich (Code: P4850).

Screw Capped Round Bottomed Centrifuge Tubes, 2 ml capacity for the protein digestion process.

HYALURONAN/HA ASSAY

GAG Precipitation Reagent is a sodium acetate saturated ethanol solution. The kit contains 2 x 170 ml bottles part filled with water saturated suspension of Na acetate.

Add **136 ml Ethanol** (or Industrial Methylated Spirits containing 99% ethanol) to the Na acetate in each bottle. Ensure complete mixing by inverting the bottle and gently shaking the contents.

Leave for 30 min. to allow excess Na acetate to settle out at the bottom of the bottle. Do not shake contents again to avoid solid Na acetate being re-suspended.

Concentrated sodium chloride supplied as a sterile solution in a 20 ml vial (23.3 gm % w/v).

CPC (Cetylpyridinium chloride) supplied as a sterile solution in a 20 ml vial. Warm before use, to ensure complete solution, (2.0 gm % w/v).

HA (Hyaluronan/Hyaluronic Acid Standard) as a sterile solution containing 200µg/ml (4 µg HA/20 µl) in a 10 ml vial. Store at 4°C.

Purple Dye Reagent; 3,3'-Diethyl-9-methyl-4,5,4',5'-dibenzothia carbo-cyanine bromide supplied in a solution containing dimethyl sulfoxide (DMSO) in a 20 ml vial.
THE DYE REAGENT MUST BE PROTECTED FROM LIGHT

REQUIRED FOR THE ASSAY, BUT NOT SUPPLIED

Proteinase K product is available from Sigma Aldrich (Code: P4850).

Ethanol (including Industrial Methylated Spirits) is subject to Government Alcohol Regulations and therefore is not supplied by Biocolor.

The Dye Used to Assay HA

In 1936 Edwin Jelley sent a '*Letter to the Editor'* of Nature (Nature **138,** 1009 -1010) regarding the unusual behaviour of some cyanine dyes.

When dissolved in 5 M NaCl these dyes produce a third absorbance peak at a longer wave-length (650nm) whereas in deionised water only a double peak occurs at ~540 nm and ~570 nm.

The 650 nm peak in concentrated dye solution induces aggregation of the dye molecules and has been described as a '*J-aggregate'* named after Jelley.

The cyanine dye used in the assay is not a planar shaped molecule (Fig 2);

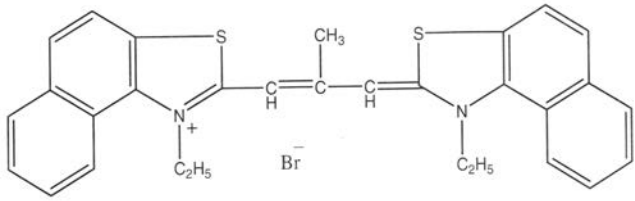

Fig.2 Structure of 3,3'-Diethyl-9-methyl-4,5,4',5'- dibenzothiacarbo-cyanine bromide.

Individual dye molecules may form a stacking format to produce a supra-molecular complex as seen in peak 3 (Image on front cover of the Manual).

Further studies in the1960s notably by Kay *et al*. (J. Physical Chem. **68**, 1896 – 1906) found many biological polymers including proteins, DNA, polar lipids and glycosaminoglycans could induce the third peak when using high dye concentrations.

The dye was renamed '*Stains-all'* by Dhlberg, Dingman and Peacock in 1969 (J. Mol. Biol. **41**, 139).

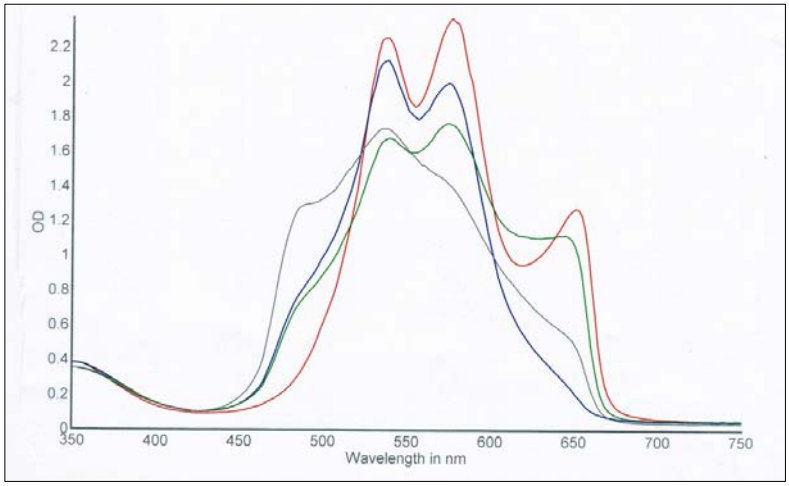

Fig. 3 Absorbance Spectrum of Stains-All when mixed with glycosaminoglycans. Sulfated glycosaminoglycans need to be removed before measuring isolated HA via a 2-step critical electrolyte salting out process (CEC).

RED line spectrum: HA (2 µg) only
BLUE line spectrum: chondroitin 4-sulfate (2 µg) only
GREEN line spectrum: equal quantities of HA and chondroitin 4-sulfate
GREY line spectrum: HA plus twice the concentration of chondroitin 4-sulfate

Assay Protocol – Supplementary Notes

(Numbers in brackets correspond to similarly numbered stages in the 'General Protocol')

[1] Removal of tissue protein is essential for isolating HA from potentially dye binding proteins.

Proteinase K (EC 3.4.21.64), a broad serine Proteinase was selected in preference to Papain.

Proteinase K was used at 55˚C and pH 7.6, from 3 hours for soft tissue.

For hard tissues, including cartilage, it was used overnight.

[2-8] Recovery of GAG from the protein digest. This requires a sequence of precipitation steps (2-8 in protocol) using (a) *ethanol saturated with Na acetate* and (b) *CPC containing NaCl*. Industrial Methylated Spirits containing 99% ethanol is suitable and will require a Government Alcohol Licence for your laboratory.

Assay protocol Supplementary Notes (continued)

For comfort breaks (coffee/lunch/etc.) select the end of section [4] and/or [6] and/or [8]. The same breakpoints apply to overnight stops where the precipitate should be stored at 4°C.

[9] Colour Stability. No decrease of Absorbance readings at 655 nm occurred over one hour from mixing the dye and HA, providing the microwell plates were wrapped in aluminum foil between Absorbance readings.

[10] Standard Curve (Fig. 4)

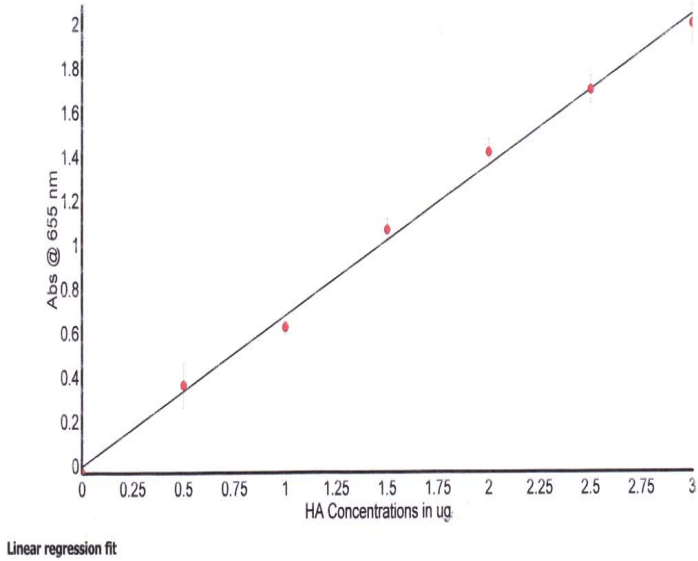

Fig.4 A Typical Straight Line Assay Standard Curve. HA Ref Std, 0 – 3µg.

Note: Microplate Readers vary in their design and performance so this Figure should be considered as a guide only. The HA Standard aliquots sampled were adjusted to 20µl with water before mixing with 200µl Dye Reagent to produce an Absorbance value less than 2.0.

General Hyaluronan Assay Protocol

Detection Limit: 0.2 µg **Hyaluronan Abbreviated to HA**

Proteins are first removed by enzymatic digestion.

Protein Removal

[1] Prepare tissue sample, typically 50 mg ± 10 mg for skin and cartilage and 250 mg ± 50 mg for soft tissues.

Chop the test samples into small fragments using a surgical scalpel and record the weights.

Transfer samples to 2.0 ml screw capped round bottom micro-centrifuge tubes. Add 400µl TRIS-HCl buffer (pH 7.6) containing 20µl Proteinase K.
Digest tissue protein **overnight** at 55^0 C.
Centrifuge extracts @13000 *x g* for 10 min**. DISCARD RESIDUES**

Recovery Of GAG

[2] Transfer 400µl of supernatants to a set of 1.5 ml **conical** microcentrifuge tubes. Add 1.0 ml **GAG Precipitation Reagent** to each tube. Mix and then leave undisturbed for 15 minutes. Centrifuge @13000 *xg* for 10 min. **RETAIN RESIDUES**

[3] To the residues add 360µl water to each tube. Extract for 15 minutes by mixing with a vortex mixer at intervals to fully dissolve the GAG. The pellet must be completely dislodged from tube wall and fully dispersed. It may be necessary to manually dislodge the pellet using a fine tipped pipette tip.

Add 40µl concentrated **NaCl** and mix before adding 95µl **CPC** (Cetylpyridinium chloride). Leave for 30 minutes.
Centrifuge @13000 *x g* for 10 min. **DISCARD RESIDUES**

[4] Ensure the complete transfer of the supernatants to a new set of 1.5 ml conical centrifuge tubes.
Add 1.0 ml GAG Precipitation Reagent to each tube.
Mix and leave undisturbed for 15 minutes.
Centrifuge @13000 *x g* for 10 min. **RETAIN RESIDUES**

[5] Add 300µl water to each residue, extract for 15 min., with mechanical mixing or occasional vortexing. Add 33µl concentrated NaCl and mix before adding 77µl **CPC.** Leave 30 minutes before centrifuging at 13000 *x g* for 10 min. **DISCARD RESIDUES**

HA Isolation

[6] Transfer supernatants to a set of 1.5 ml conical microcentrifuge tubes. Add 1.0ml **GAG Precipitation Reagent** to each tube.
Mix and leave undisturbed for 15 min. before centrifuging at 13000 *x g* for 10 min. **RETAIN RESIDUES**

[7] To the residues add 500µl ethanol (+98%) **without** sodium acetate. **Do not mix.** Centrifuge at 13000 *x g* for 5 min then drain and discard supernatant. Leave tubes inverted on absorbent paper to drain dry.

[8] Add 100µl water to each residue and use intermittent mixing to complete the solubility of the HA pellets.
Allow 30 min. for HA to fully hydrate.

Measurement of HA

[9] Transfer 20µl aliquots of Standards/Reagent Blanks/Test Samples to the flat bottom wells of a 96-microwell plate.

Add 200µl of **Dye Reagent.** Mix by gentle rotation of the plate on the lab bench. Full colour development occurs within 10 min. and is stable for one hour when stored in the dark.
IMPORTANT: Avoid exposure to light, *hold microplate within a light proof drawer or box until transfer to the microplate reader.*

[10] Using a microplate spectrophotometer read the Absorbance values at 655 nm and print data obtained.

Data Analysis

With the aid of a Standard Curve (see page 8) convert the Abs @ 655 nm into µg HA. Multiply this value by five (20µl x 5 = 100µl).

The 100µl of purified HA [Step 8] was recovered from x mg of wet tissue (see **[1]** for the recorded wet weights of each tissue sample).

Convert the µg HA extracted from x mg wet tissue to µg HA contained in one gram of wet tissue.

If a test sample volume of 20µl produces an Absorbance value greater than 2.0 then use a smaller aliquot obtained in Step 8 made up to 20µl with water before adding 200µl Dye Reagent.