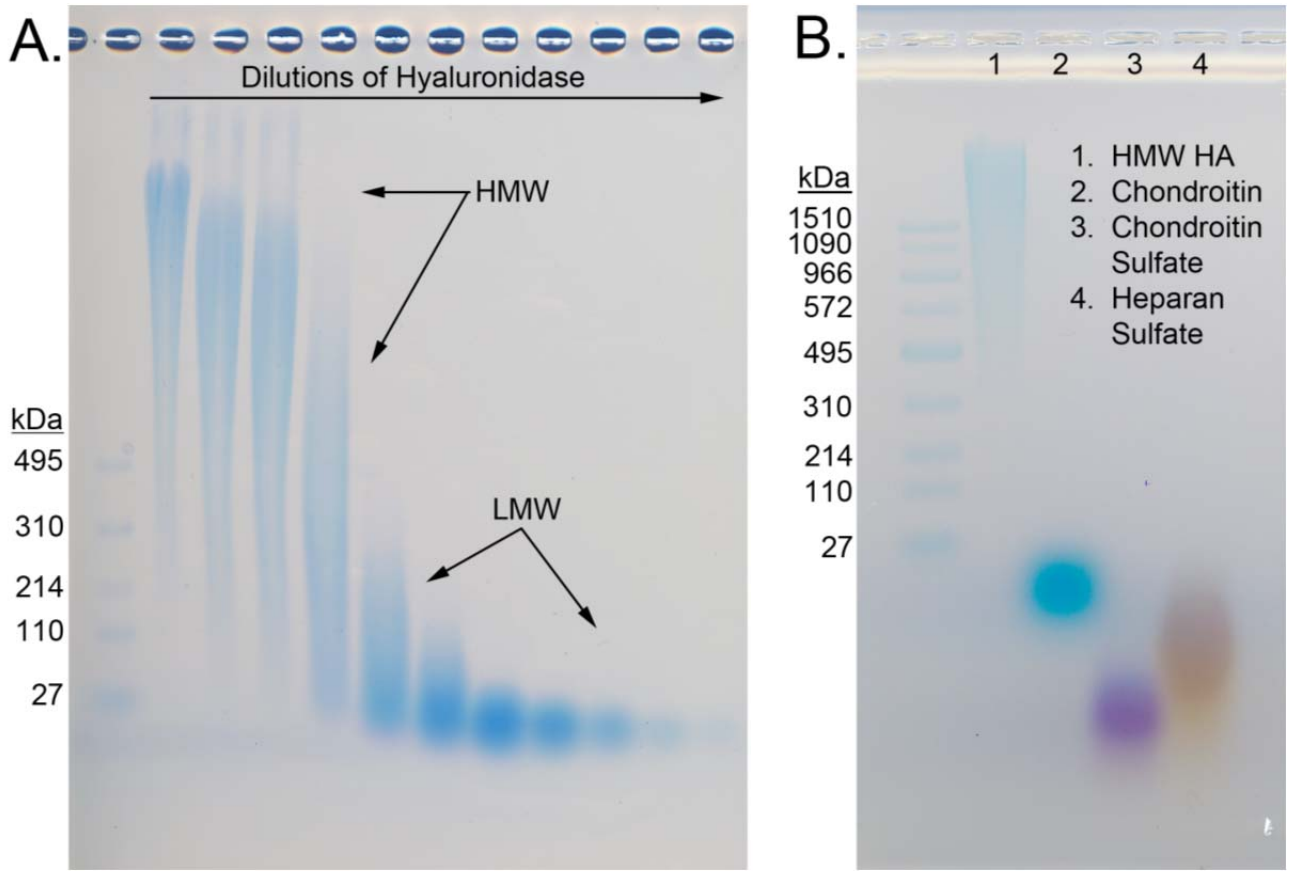
HYALURONAN SIZE ANALYSIS BY AGAROSE GEL ELECTROPHORESIS

***PLEASE ACKNOWLEDGE NHLBI AWARD NUMBER PO1HL107147 WHEN YOU PUBLISH RESULTS USING THIS PROTOCOL**

1.1 OVERVIEW



Hyaluronan Size Analysis by Agarose Gel Electrophoresis. (A) Agarose gel showing high and low molecular weights (HMW and LMW) of hyaluronan following digestion with testicular hyaluronidase. (B) Agarose gel showing different colors of glycosaminoglycans stained with Stains All.

Hyaluronan size analysis can be done on a wide variety of different tissues and cell types by agarose gel electrophoresis. This procedure uses a protease and nucleases to remove proteins and nucleic acids from cellular material. Lipids are extracted with detergent and alcohol. Essentially, what remains after the digestions and extractions, are complex carbohydrates (including glycosaminoglycans (GAGs)). Since hyaluronan is so large (usually > 1000 kDa), it separates from other GAGs and glycans on an agarose gel (panel A). Hyaluronan, and the other GAGs, are visualized on the gel by a colored stain (i.e. "Stains All") that stains hyaluronan and chondroitin blue, chondroitin sulfate redish-purple and heparan sulfate yellow (panel B). The amount of cellular material needed to get a strong stain for HA on the agarose gel will vary from sample-to-sample. We recommend starting with at least 25 mg of tissue and 150 cm2 of cultured cells and conditioned media. This procedure produces the best results when the hyaluronan is in the 1-5 µg range.

Please direct questions to Ron Midura (midurar@ccf.org; 216-445-3212).

1.2 REAGENTS

100 mM Ammonium Acetate, pH 7.0
Dissolve 1.927 g ammonium acetate in 250 ml of water. The pH will be 7.0. Make 50 mL aliquots and store at 4° C for >1 year.

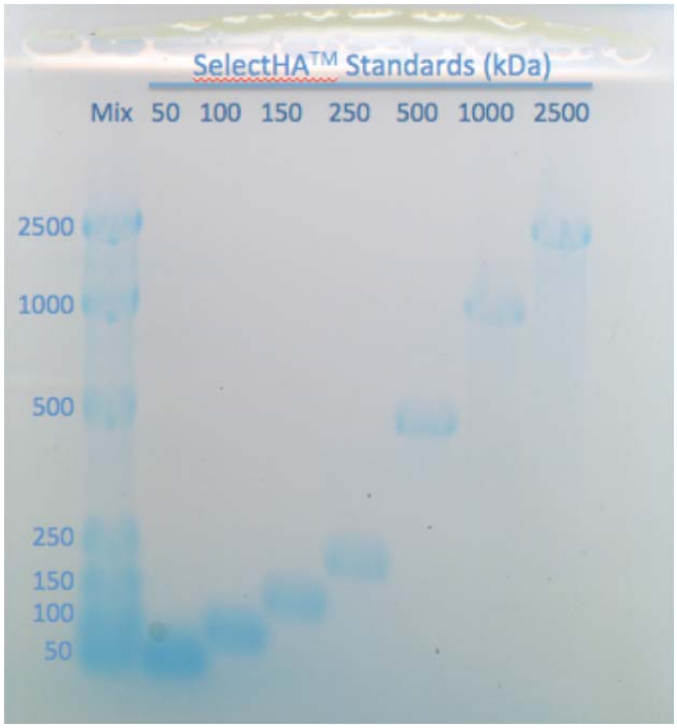
10x Proteinase K (Invitrogen, Storeroom #155490, 100 mg)
Add 10 ml 100 mM ammonium acetate to 100 mg of Proteinase K (PK). Dissolve 30 minutes RT. Transfer to a 50 ml centrifuge tube. Rinse vial with another 10 ml 100 mM ammonium acetate and transfer to the 50 ml centrifuge tube. Add 80 ml of 100 mM ammonium acetate to the solubilized PK (total of 100 ml). Add 10 mg of SDS (or 100 µl of a 10% solution) and stir till soluble. Aliquot at 1500 µl in 2 ml microcentrifuge tubes. Store at –80° C indefinitely. *(Note: Excessive RT incubations, >30 minutes, can result in autolysis.)* Final concentration of the 10x solution is PK at 1 mg/ml with SDS at 0.01%.

Streptomyces Hyaluronidase (Seikagaku, 100741-1A or Calbiochem/EMD/Millipore, 389561)
Add 500 µl 100 mM ammonium acetate to one ampule containing 100 TRU of enzyme. Incubate at RT 20 min. Rotate the ampule to make sure that the liquid comes into contact with the entire surface of the bottom quarter of the ampule. Make 25 µl aliquots in PCR tubes. Store at –80° C indefinitely. Final concentration: 0.2 TRU/µl.

Sample Loading Solution
0.2% bromophenol blue in 10 M formamide

Hyaluronan Molecular Weight Ladders
We purchased 50, 100, 150, 250, 500, 1000 and 2500 K Select HA (1 mg/vial). Resuspended these at 5 mg/ml in 200 µl of water, pool 20 µl from each HA size into a single tube, lyophilized on a vacuum concentrator and resuspended in 1 ml of 10 M formamide with 0.2% bromophenol blue to give a 0.7 mg/ml solution of an HA ladder. Loading 10 µl in a single lane provides 7 µg of HA per lane, which produces a strong signal on a gel.

Gel Staining Solution
Make a 400x stock by adding 12.5 mg of Stains-All (Sigma-Aldrich, E9379) to 5 ml of 200 proof ethanol. Store this solution in the dark at RT. If a precipitation is observed, discard and remake. To make the working stock, add 0.5 ml of the 400x stock to 200 ml of 30% ethanol. The working stock should be made fresh each time.



Other Needed Reagents

- Agarose (SeaKem HGT Agarose, Cambrex, 50041).
- 10X TAE buffer
- Benzonase (EMD Millipore, 70664-3)
- PicoGreen dsDNA quantification reagent (Molecular Probes, P-11496)
- *10 M Formamide (Sigma-Aldrich, F-4761, 25.18 M)*

1.3 EQUIPMENT

Microcentrifuge (capable of 14,000 g)

Vacuum Concentrator (such as a Savant SpeedVac or Eppendorf Vacufuge)

Electrophoresis Equipment
Most any horizontal vertical gel electrophoresis equipment can be used. We prefer the OWL EasyCast™ system (B1 or B1A). You will need a power supply capable of 100 volts (constant), 100 mA and 75 W.

Imaging Equipment
You will need a gel documentation system equipped with a light box or a good scanner.

1.4 PROTOCOL

1. Proteinase K Digestion: If you want to analyze pure HA, skip to step 6. For all others, proceed as follows:

a) Cultured Cells: Remove (and save) the conditioned media and wash the cells at least 1X with PBS. Add 1 ml of 1X Proteinase K (PK) per 8 cm^2 of a cell monolayer.
b) Conditioned Media: Add 10X PK to the media to give a final concentration of 1X PK.
c) Tissues: Add 1 ml 10X PK per 25 mg wet weight of tissue.

Incubate the samples at 60° C for 4 hours. For tissue samples, vortex the samples every 30 min until the tissue is completely dissociated. After digestion, and at the completion of each of the following steps, the samples can be stored at –20° C indefinitely.

2. First Ethanol Precipitation: Add 4 volumes of pre-chilled (–20° C) 200 proof ethanol to each sample and incubate at – 20° C overnight. Next day, centrifuge samples at 14,000 g for 10 minutes at RT. Discard the supernatent by dumping it into a beaker *(Note: the glycosaminoglycans are in the pellet)*. Wash the pellet by adding 4 volumes of pre-chilled (–20° C) 75% EtOH and vortex. Centrifuge samples at 14,000 g for 10 minutes as before. Discard wash as before, but this time you will need to remove residual ethanol with a pipette. Air dry the pellet at RT for 20 minutes *(do not use a vacuum and do not over dry; this will make the pellet more difficult to re-dissolve).* Add 100 µl 100 mM ammonium acetate to each sample. Vortex/Spin. Incubate at RT for 20 minutes. Vortex/Spin. Heat inactivate the PK by incubating the samples at 100° C for 5 min, then chill them on ice for 5 min. *(Note: Before proceeding to the next step, we recommend normalizing your samples by measuring the DNA content from 1 µl of your 100 µl sample using the PicoGreen dsDNA procedure.)*

3. Digestion of Nucleic Acids: Add 1 µl of Benzonase (EMD Millipore, 70664-3 25U/µl). Let sit overnight at 37° C. Heat kill the enzymes on a boiling water bath for 5 min. Spin. Add 400 µl cold 200 proof ethanol and let sit overnight at -20° C.

4. Second Ethanol Precipitation: Centrifuge samples at 14,000 g for 10 minutes at RT. Discard the supernatent. Wash with 1 ml cold 75% ethanol and centrifuge as before. Air dry 20 min, and use a p200 tip to remove any residual ethanol. Resuspend in 20 µl 100 mM ammonium acetate (pH 7.0) and transfer to a 200 µl PCR tube. *[Note: Before proceeding to the next step, we recommend that you quantify the amount of hyaluronan in your sample by transferring 1 µl of your 20 µl sample to a separate tube for FACE analysis (loading the entire 1 µl sample should yield a strong band on a FACE gel).]*

5. Digestion with Hyaluronidase: In this step, you will digest half of your sample with hyaluronidase as a negative control to prove that the hyaluronan staining seen on your gel is truly hyaluronan. Divide your 20 µl sample in half by transferring 10 µl to another tube. Add 1 µl *Streptomyces* hyaluronidase (Seikagaku, 100740-1, 0.2 TRU/ul stock) to ½ of your sample (i.e. one of the tubes containing 10 µl of your sample). Incubate overnight at 37oC. Heat inactivate the enzymes on a boiling water bath for 5 minutes. Spin.

6. Resuspension in Formamide: Lyophilize your samples on a centrifugal vacuum concentrator and resuspend them in 10 µl of 10 M formamide. Let sit overnight at 4° C. Before loading gel, add 2 µl of the sample loading solution (0.2% bromophenol blue in 10 M formamide) to each 10 µl sample and each 9 µl of pooled hyaluronan standards (see 1.2). *(Note: The advantage of formamide is that it sinks in water, making it suitable for loading on a gel, and it also denatures any enzymes (such as hyaluronidases) that could unintentionally degrade/modify your samples. If desired, formamide can be substituted with most any buffer, using 20% glycerol to make it sink in the well.)*

1.5 GEL PREPARATION

See the Equipment section (1.3) for recommendations on gel apparati.

1. Gel Casting: Cast a 0.5 cm thick 1% agarose gel (SeaKem HGT Agarose, Cambrex, 50041) in 1x TAE and pre-run the gel for 6 hours at 80 V (constant voltage). *(Note: This pre-run removes impurities from the agarose that stain strongly with Stains-All).*

2. **Loading the Gel:** From the samples and standards prepared in step 6 of 1.3, load 11 µl of hyaluronan standards to at least one lane and 12 µl of sample. *(Note: If you did not use formamide for your sample buffer, do not place samples that were treated with hyaluronidase next to samples that were not treated with hyaluronidase.)*

3. Running the Gel: Run the gel at 100 V (constant voltage) until the tracking dye has migrated about 75% the length of the gel (typically 1-1.5 hr). *(Note: If you run the gel more than this, you will most likely run the other non-hyaluronan GAGs off the gel. Chondroitin and sulfated GAGs are smaller than HA and migrate faster than the dye front. When present, these GAGs can function as a loading control).*

4. Staining of the Gel: Equilibrate the gel in a dish/box with 30% ethanol in water *(this step prevents the formation of Stains-All precipitates in the gel).* Let rock for 1 hour at RT. Decant and replace with the working stock of Stains-All solution (see 1.2 for preparation). Cover the dish/box with foil to prevent light from entering and incubate the gel with the stain overnight. The next day, decant the stain and replace with water. Equilibrate the gel in water for about an hour at RT in the dark. Equilibration in water prevents ethanol/water droplets from forming on the surface of the gel that are visible when imaging. When the gel stops floating to the surface (i.e. it sinks instead) it is ready for destaining. The gel will have a pink background as a result of slightly over staining. Briefly (less than 1 hour) expose the gel to light to decrease background, as needed. Once the desired destaining has been achieved, place the gel back in the dark. The gel is now ready for imaging, but it can be kept for many months in the dark in water without losing much of its stain.

1.6 IMAGING OF THE GEL

We typically scan the gel on a standard color scanner, but color photographs on a light box also work. Grayscale images are fine, but you will lose the ability to distinguish between the colors that different GAGs stain. Pouring some water on the top of the gel after it has been transferred to the scanner (or light box) will prevent puddles of water from accumulating on the top of the gel (that can be seen on the image).