Programs of Excellence in Glycosciences

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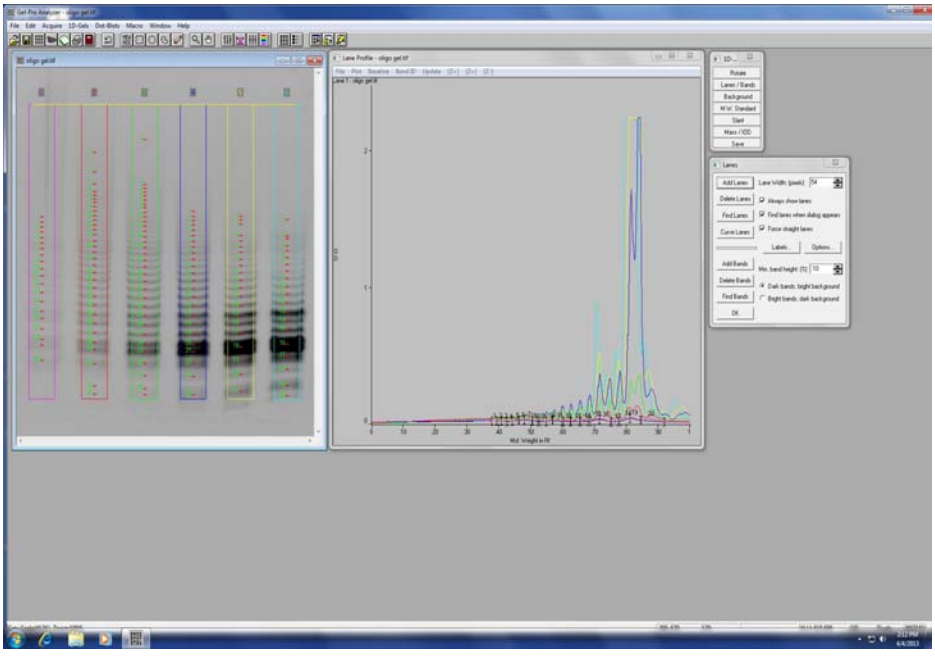
CLEVELAND CLINIC | Hyaluronan Matrices in Vascular Pathologies

FLUOROPHORE-ASSISTED-CARBOHYDRATE ELECTROPHORESIS OF GLYCOSAMINOGLYCANS

1.0 BACKGROUND

Fluorophore – Assisted – Carbohydrate – Electrophoresis (FACE) is a rapid, simple and sensitive method to detect and quantify the mono-and di-saccharide composition, and oligosaccharide profiling, of glycans. It is much less costly than mass spectrometry and capillary electrophoresis because FACE uses standard vertical gel electrophoresis equipment and UV imaging systems common to most life science laboratories. The data produced by FACE is a convenient gel-based output familiar to the broader biological community rather than a graph of plotted peaks. Each band represents a particular glycan monosaccharide, disaccharide or oligosaccharide that can be determined by comparison to a known glycan standard and quantified by standard densitometry software. Because of the gel-based format FACE permits the simultaneous analysis of multiple samples in a single electrophoretic run. Glycans are typically labeled with the fluorophores 2-amino-acridone or ANTS by reductive amination. These glycans can be labeled directly or after previous digestion with glycosidases to produce mono-, di-or oligosaccharide products. This protocol describes a method to measure the internal disaccharides and non-reducing terminal glycosaminoglycan structures, including hyaluronan, chondroitin sulfate and heparan sulfate.



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1.1 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.

Tris-Acetate Gel Solution (Sigma, TRIZMA BASE, T-6791, 500 g, MW 121.1)
Dissolve 4.84 grams of TRIZMA Base into 80 ml water. Bring pH to 7.0 with glacial acetic acid. Adjust volume to 100 ml. Final concentration: 400 mM.

AMAC (2-aminoacridone) Solution (Molecular Probes, *A-6289*, 25 mg, MW 246.7)
Add 2 ml DMSO to 25 mg of AMAC. Let sit at RT with occasional agitation for 30 minutes. Transfer the solubilized AMAC to a 15 ml centrifuge tube. Rinse the vial with another 1 ml DMSO and transfer to the centrifuge tube. Add another 3.89 ml DMSO to the centrifuge tube (final volume at 6.89 ml). Invert until homogeneous. Make a solution of sodium cyanoborohydride (Aldrich, 15,615-9) by adding 770 mg to 9.8 ml water in a 15 ml tube (this is very poisonous so be very careful). Incubate this at RT for 20 min with occasional inversion. Now, add 1.216 ml glacial acetic acid to your 6.89 ml of AMAC. Mix by inversion a few times. Mix 8 ml of the AMAC solution and 8 ml of the sodium cyanoborohydride solution in a 50 ml tube. Mix by inversion a few times. Store in 120 µl and 480 µl aliquots at – 80°C indefinitely. The AMAC is now at 6.25 mM and is ready to directly add to your samples.

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%.

Hyaluronidase, SD (Seikagaku, 100741-1A)
Add 200 µl 100 mM ammonium acetate, containing 0.1% BSA, to 0.5 U of enzyme. Vortex/Spin. Incubate at RT 20 min. Vortex/Spin. Make 10 µl aliquots in PCR tubes. Store at –80° C indefinitely. Final concentration: 2.5 mU/µl

Chondroitinase ABC (Seikagaku, 100330-1A)
Add 400 µl 100 mM ammonium acetate, containing 0.1% BSA, to 10 U of enzyme. Vortex/Spin. Incubate at RT 20 min. Vortex/Spin. Make 10 µl aliquots in PCR tubes. Store at –80° C indefinitely. Final concentration: 25 mU/µl

Heparinase (*Flavobacterium heparinum*) (Seikagaku, 100700-3)
Add 100 µl 100 mM ammonium acetate, containing 0.1% BSA, to 0.1 U of enzyme. Vortex/Spin. Incubate at RT 20 min. Vortex/Spin. Make 10 µl aliquots in PCR tubes. Store at –80° C indefinitely. Final concentration: 1 mU/µl

Heparitinase I (*Flavobacterium heparinum*) (Seikagaku, 100704-1)
Add 100 µl 100 mM ammonium acetate, containing 0.1% BSA, to 0.1 U of enzyme. Vortex/Spin. Incubate at RT 20 min. Vortex/Spin. Make 10 µl aliquots in PCR tubes. Store at –80° C indefinitely. Final concentration: 1 mU/µl

Heparitinase II (*Flavobacterium heparinum*) (Seikagaku, 100705-1)
Add 100 µl 100 mM ammonium acetate, containing 0.1% BSA, to 0.1 U of enzyme. Vortex/Spin. Incubate at RT 20 min. Vortex/Spin. Make 10 µl aliquots in PCR tubes. Store at –80° C indefinitely. Final concentration: 1 mU/µl

Gel Solution to Separate Chondroitin Sulfate and Hyaluronan Disaccharides
In a 1L Erlenmeyer flask, add 500 ml 40% acrylamide (37.5:1) (BioRad, 161-0148), 100 ml Tris-Acetate (400 mM, pH 7.0), 375 ml distilled water and 25 ml of glycerol *(note: Add glycerol by using a 25 ml serological pipette and suspending it above the flask by tape to drip until all the glycerol is removed. This is very important. Slight variations in glycerol content can result in large variations in the migration of the disaccharide bands.)* After thoroughly mixed, make 10 ml aliquots in 15 ml centrifuge tubes and store at 4°C indefinitely. This recipe makes 100 aliquots. Each aliquot makes 2 standard 0.75 mm spacer mini-gels.

Gel Solution to Separate Heparan Sulfate Disaccharides
In a 500 ml Erlenmeyer flask, add 250 ml 40% acrylamide (37.5:1) (BioRad, 161-0148), 25 ml 10X Tris-Borate-EDTA (TBE), 200 ml distilled water and 25 ml of glycerol *(note: Add glycerol by using a 25 ml serological pipette and suspending it above the flask by tape to drip until all the glycerol is removed. This is very important. Slight variations in glycerol content can result in large variations in the migration of the disaccharide bands.)* After thoroughly mixed, make 10 ml aliquots in 15 ml centrifuge tubes and store at 4°C indefinitely. This recipe makes 50 aliquots. Each aliquot makes 2 standard 0.75 mm spacer mini-gels.

1X TBE (electrophoresis buffer)
We buy this in a 10x stock from our media lab and make 10 liters at a time, stored at 4°C indefinitely. Other suppliers should be fine.

Other Needed Reagents
- Ammonium Persulfate (BioRad, 161-0700, 10 g)(make a 10% solution in water and store in 75 µl aliquots at -80° C indefinitely)
- TEMED (N,N,N'N'-Tetra-methylethylenediamine) (BioRad, 161-0801, 50 ml)

1.2 EQUIPMENT

Electrophoresis Equipment
Most any vertical gel electrophoresis equipment can be used for FACE. We primarily use the BioRad Mini-PROTEAN® Tetra Cell, but we have also used the XCell SureLock® Mini-Cell (Life Technologies). We recommend 0.75 mm spacers, but 0.5 – 1.5 will also work. We recommend 10-well combs for better looking bands, but 15-well combs are more practical for larger sample sets.

With the *XCell*, the gel must be removed from the casting cassette and placed directly on a UV transilluminator for imaging because the plastic of the cassette blocks a significant amount of the UV light. You will need to rinse the AMAC out of the wells before opening the cassette or the gel will be covered in AMAC. Once you remove the gel from the cassette, you only have a few minutes to image the gel before it will to dry out and shrink. Once the gel has been removed from the cassette, you cannot put it back to run it longer.

With the *Tetra Cell*, the gels can be imaged while in the glass plates because glass does not block as much UV light as plastic. On the BioRad system, there is a thicker, taller, glass plate and a thinner shorter glass plate. When imaging, it is important to place the shorter glass plate facing down on the transilluminator, because it permits more penetration of UV light. We've noticed that this shorter plate is made of a type of glass that blocks more UV light than other types of glass. We recommend purchasing "low iron" glass plates from Moliterno (www.tech-glass.com/biorad.htm) choosing the 7.3x10.1 cm plates for the Tetra Cell.

Imaging Equipment
You will need a digital imaging station (preferably with a CCD camera) equipped with a UV transilluminator (preferably with the ability to emit UVB light).

Software
The software that runs the camera should have the ability to identify the saturation of bands. You will need software, such as ImageJ (http://rsb.info.nih.gov/ij/), that can quantify bands on a gel.

1.3 PROTOCOL

1. Proteinase K Digestion: FACE analysis can be done on a wide variety of different tissues and cell types, though there are some important distinctions for cultured cells, conditioned media and tissues.

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 250 µl 10X PK per 100 mg wet weight of tissue.

Incubate the samples at 60° C for 2 hours or until tissue is digested. For tissue samples, vortex the samples every 30 min until the tissue is completely dissociated. Cell culture samples do not need to be vortexed. 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 proceed to the next step or incubate at – 20° C overnight. Next day, centrifuge samples at 14,000 g for 10 minutes at RT. Discard the supernatant by dumping it into a beaker *(Note: the glycosaminoglycans are in the pellet)*. Wash 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 35 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.

3. Digestion of Chondroitin Sulfate and Hyaluronan: Add enzymes of interest 1 µl of chondroitinase ABC and/or 1 µl hyaluronidase SD to each 35 µl sample. Vortex/Spin. Incubate at 37°C overnight (at least 18 hrs).

4. Second Ethanol Precipitation: Add 160 µl of pre-chilled (–20° C) 200 proof ethanol to each sample. Vortex/Spin. Proceed to next step or incubate overnight at –20° C. Centrifuge samples at 14,000 g for 10 minutes at RT. Save supernatant in a separate 1.5 ml tube *(Note: hyaluronan and chondroitin sulfate are now in the supernatant; heparan sulfate, nucleic acids and other carbohydrates are in the pellet)*. Wash the pellet with 100 µl of pre-chilled (–20° C) 75% ethanol and centrifuge as before. Pool this wash with the previous supernatant for each sample. Proceed to step 7 if uninterested in heparan sulfate analysis. Air dry the pellet for 20 min at RT. Resuspend the pellet in 20 µl of 100 mM ammonium acetate and incubate at RT for 20 min. Vortex/spin the pellet. Heat inactivate the enzymes by incubating the samples at 100° C for 5 min, then chill them on ice for 5 min. Then, proceed to step 5. For the supernatants, lyophilize them (using a "Speed-Vac" or equivalent) and proceed to step 7.

5. Digestion of Heparan Sulfate: Mix equal portions of Heparinase, Heparitinase I and Heparitinase II together and add 1 µl of this mixture to each 20 µl sample. Incubate at 37°C overnight (at least 18 hrs).

6. Third Ethanol Precipitation: Add 80 µl of pre-chilled (–20° C) 200 proof ethanol to each sample. Vortex/Spin. Proceed to the next step or incubate overnight at –20° C. Centrifuge samples at 14,000 g for 10 minutes at RT. Save supernatant in a separate 1.5 ml tube *(Note: heparan sulfate is now in the supernatant; nucleic acids and other carbohydrates are in the pellet)*. Wash the pellet with 100 µl of pre-chilled (–20° C) 75% ethanol and centrifuge as before. Pool this wash with the previous supernatant for each sample. Air dry the pellet for 20 min at RT. Resuspend the pellet in 20 µl of 100 mM ammonium acetate and incubate at RT for 20 min. Vortex/spin the pellet. Heat inactivate the enzymes by incubating the samples at 100° C for 5 min, then chill them on ice for 5 min. If desired, DNA levels can be measured from this resuspended pellet (see Sample Normalization in 1.7).

7. Labeling with AMAC: Lyophilize supernatants (using a "Speed-Vac" or equivalent). The volume of labeling solution needed for each sample will vary depending upon the amount of your starting material. The following are recommended volumes: (i) add 1 µl of the AMAC solution per cm^2 of culturing area (for both cell

layer and conditioned media), (ii) add 1 µl of the AMAC solution for every 2.5 mg of tissue wet weight. Add less AMAC to dilute down later since concentration is not an option. Incubate the sample at 37° C for 18 hrs in the dark.

If your signal on the gel is too strong, you can dilute the samples with the AMAC solution and re-run the gel. If your signal is too week, the only thing you can do is to add more volume of your sample to the gel (see "Loading the Samples" in step 3 of 1.4 for precautions).

1.4 GEL PREPARATION

See the Equipment section (1.2) for recommendations on gel apparati.

1. Gel Casting: Transfer the amount of gel solution you need to a clean tube. You will need about 5 ml/gel for a gel with 0.75 mm spacers. For each 5 ml, add 25 µl ammonium persulfate *(10%)* and 5 µl TEMED. Mix by inversion and add to your pre-assembled casting plates. Polymerization is usually complete within 12 min.

2. Chilling the Gels: Place the gels you just cast into their gel apparatus. Quickly, add electrophoresis buffer to the inner/outer chamber and rinse the gel wells before the residual acrylamide polymerizes (failure to do so will make an uneven well surface resulting in a distorted banding pattern). The buffer levels in the outer chamber should be as high as possible (typically just below the wells) for better cooling of the gels. Place the gel box in a secondary container (such as a rat cage box or plastic substitute). Surround the gel apparatus with ice up to the top of the apparatus. Pour water into the ice to create a slurry until the water level reaches about halfway up the gel apparatus. If possible, place a stir bar in the bottom of the apparatus and place it on a stir plate to make cooling more even. Cool to 4° C before running the gel (typically takes 2 hrs). Replace melted ice with fresh ice before running the gel. The temperature of the gel is extremely important for optimum band separation.

3. Loading the Samples: Unlike protein gels in which >30 µl sample volume per lane is common, FACE can only accommodate much smaller sample volumes. This is because the "stacking" phenomenon that concentrates the samples in protein gels is largely absent in FACE. We recommend adding no more than 2-5 µl of sample per lane. Add them directly to the well (a "loading buffer" is not necessary). Keep in mind that the greater the volume, the thicker, and less resolved, the bands will be. It is also important to accurately add a standard to at least one lane (see 1.8 for standard preparation). Taking the ratio of the IOD of the sample disaccharides to the standard disaccharides will enable an estimation of the concentration of disaccharides in your samples. We also recommend adding an AMAC "blank" to one of your wells (to identify any impurities in the AMAC solution that may be, and often are, present). Furthermore, adding an AMAC blank to each unoccupied well will prevent lane "smiling."

4. Electrophoresis: Run the gel at 500 V constant voltage. The amperage should start at 18/gel and go to 7/gel by the end (if not, adjust your starting voltage). Electrophoresis should take about 1 hour and 15 minutes (more or less depending on the length of the gel).

1.5 IMAGING OF THE GEL

See Equipment section (1.2) for guidelines regarding the differences in imaging for different gel apparati and the other equipment needed. You will need at least one image in which the intensity of the bands are not saturated.

1.6 BAND QUANTIFICATION

We use ImageJ (http://rsb.info.nih.gov/ij/) to quantify our bands. (1) Open the gel in ImageJ. (2) Click the rectangle tool and draw a rectangle around your first lane. (3) Under "Analyze" select "Gels" and "Select 1st Lane." (4) Drag the rectangle you just created to the next lane. (5) Under "Analyze" select "Gels" and "Select 2nd Lane." (6) Repeat for the other lanes. (7) When you get to the last lane, under "Analyze" select "Gels" and

"Plot Lanes." (8) Click the line tool and draw a line across the bottom of each peak you created. (9) Click the magic wand tool and touch the magic wand within each peak you drew the line under. Copy and paste these numbers (IOD) into Excel. Divide the IOD of your unknown band by the IOD of the known standard band to convert IOD to a mass or molar amount.

1.7 SAMPLE NORMALIZATION

We use DNA analysis to "normalize" the intensity of the disaccharide bands of samples that are cellular in origin. This is done simply by dividing the disaccharide IOD (or mass/mole) by the DNA concentration of that sample. We recommend the PicoGreen dsDNA quantification Reagent (Molecular Probes, P-11496; store at 4^{o} C).

1.8 STANDARDS PREPARATION

CHONDROITIN SULFATE AND HYALURONAN STANDARDS

Reagents

-Unsaturated Chondro-Disaccharide Kit (C-kit) (Seikagaku #400571-1)
-Unsaturated Dermato/Hyaluro-Disaccharide Kit (D-kit) (Seikagaku #400572-1)
-Maltose 999 (Seikagaku #400509-1)
-Maltotetrose (Seikagaku #400519-1)
-Maltotriose (Seikagaku #400530-2)
-Mercury Acetate 17.5 mM (MW 318.68) (Sigma-Aldrich #456012) in a solution of Sodium Acetate 50 mM, pH 5.0 (MW 136.1)(Sigma-Aldrich #S-7670)
-AG 50W-X8 Resin (make a 50% slurry in water) (BioRad #142-1451)
-FACE Dilution Buffer (add 1.964 g sodium cyanoborohydride to 25 ml water; to this solution add 21.5 ml DMSO and 3.75 ml glacial acetic acid)

	Saccharide	MW	Single Aliquot (µl)	Pooled Aliquot (µl)
1	GalNAc	221	11.05	68.51
2	Maltotetrose	666	33.3	206.46
3	Maltotriose	504	25.2	156.24
4	Maltose	342	17.1	106.02
5	Glucose	180	9.01	55.86
6	ΔDiHA	401	20.1	124.62
7	ΔDi0S	401	20.1	124.62
8	6S-galNAc	323	16.2	100.44
9	4S-galNAc	323	16.2	100.44
10	ΔDi6S	503	25.2	156.24
11	ΔDi4S	503	25.2	156.24
12	ΔDi2S	503	25.2	156.24
13	ΔDi4,6S	605	30.3	187.86
14	ΔDi2,6S	605	30.3	187.86
15	ΔDi2,4,6S	707	35.4	219.48
16	ΔDi2,4S	605	30.3	187.86

1. Note that there is 250 µg of each disaccharide in kits C and D. Bring vials to RT. Also notice that Di-0S, Di-4S, and Di-6S are found in both kits (i.e. they are duplicated). One set will be labeled with AMAC directly. The other set will be treated with mercury to generate Gal-Nac, 4S-Gal-Nac and 6S-Gal-Nac respectively. Resuspend one set with 100 µL of the mercury and sodium acetate solution. Incubate at RT for 30 minutes. Add 30 µl of the diluted resin, vortex, and spin at 200 g for 5 minutes. Save supernatant and wash resin with

100 µL water. Vortex and spin at 200 g for 5 minutes. You should have about 200 µL of pooled supernatants containing the modified disaccharides.

2. Add 250 µL ultrapure water to the remaining disaccharides. Vortex and let sit 20 minutes. Vortex and spin.

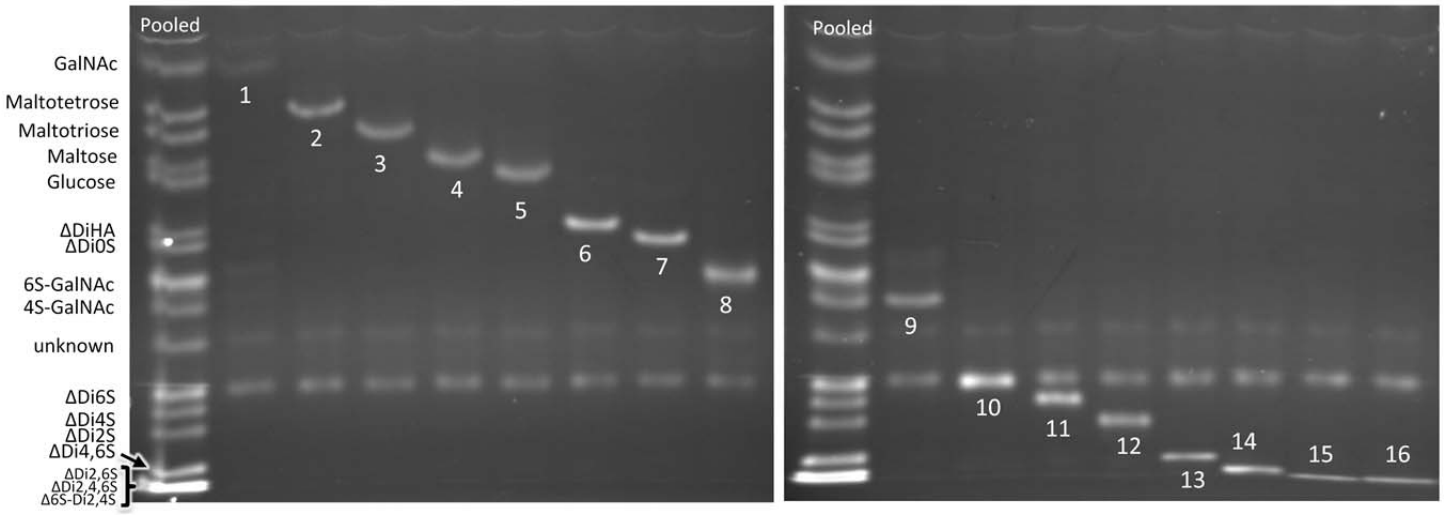
3. Make a 1 mg/ml solution of glucose, maltose, maltotriose and maltotetrose in pure water. Go ahead and make 10 ml to be more accurate (but this is way more than you need).

4. Transfer one aliquot of each saccharide to an individual 1.5 ml tube according to the "single aliquot" volumes listed in the table above. These will be labeled separately and stored separately in case they need to be loaded separately on a gel. Dry them in a speed-vac.

5. Pool the aliquots of each saccharide in a 15 ml polypropylene centrifuge tube according to the volumes listed in the "pooled aliquot" section of the table. Your final volume should be 2295 µl. (*Note: All left over GAGs and an small aliquot of the other sugars should be dried on a speed-vac and stored at –80° C for a rainy day*). Gently mix by bringing 500 µl into a p1000 tip over and over again (do not vortex or you will minimize recovery). Transfer 369.75 µl of these pooled saccharides to 6 different 15 ml tubes. There should be a small amount of volume left in the original 15 ml tube (just discard it). Freeze them at –80° C and lyophilize them in a lyophilizer. Store them at –80° C indefinitely.

6. Label the individual samples in #4 with 413 µl AMAC as you would for normal samples. Label one of the pooled aliquots from #5 in 5.0 ml of AMAC and incubate for 16-18 hour incubation at 37° C.

7. Samples are ready to be directly loaded on the gel. Store at –80° C for > a year. Over a period of years, the standards will develop extra bands (for unknown reasons) and need to be relabeled. 5 µl of each standard applied to the gel will give 50 pmol carbohydrate.



Chondroitin Sulfate and Hyaluronan FACE Standards: The banding pattern of pooled, and individual CS and HA standards were analyzed by FACE as described in this protocol. Saccharides are numbered in the following order: (1) GalNAc, (2) Maltotetrose, (3) Maltotriose, (4) Maltose, (5) Glucose, (6) ΔDiHA, (7) ΔDi0S, (8) 6S-GalNAc, (9) 4S-GalNAc, (10) ΔDi6S, (11) ΔDi4S, (12) ΔDi2S, (13) ΔDi4,6S, (14) ΔDi2,6S, (15) ΔDi2,4,6S, (16) Δ6S-Di2,4S. Note that the AMAC solution, itself, contains an unknown band that migrates at the same place as ΔDi6S, and between ΔDi6S and 4S-GalNAc. Note that the bottom three CS disaccharides migrate together as a single band using this gel solution.

HEPARAN SULFATE STANDARDS

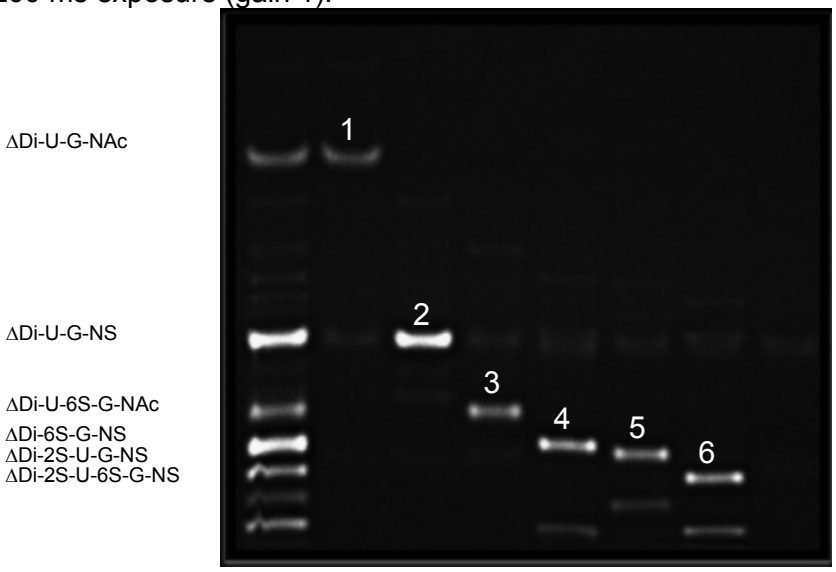
Reagents
Unsaturated Heparan/Heparin-Disaccharide Kit (H-Kit) (Seikagaku, 400575) AMAC (see 1.1)

Procedure
1. There should be 200 nmol of disaccharide per each of the 6 vials in the H-Kit (ΔDi-0S, ΔDi-GlcNAc-NS, ΔDi-6S-GlcNAc, ΔDi-6S-GlcNAc-NS, ΔDi-2S-U-GlcNAc-NS, ΔDi-2S-U-6S-GlcNAc-NS). Add 100 µl of sterile MilliQ water to each vial, incubate at RT for 20 min, and vortex/spin.

2. Transfer 10 µl of each standard to a new tube. Store the other 90 µl at -80^{o} C. Lyophilze the 10 µl aliquots on a Speed-Vac (or equivalent).

3. Add 80 µl of the AMAC solution, vortex/spin and incubate overnight at 37^{o} C. Each standard is now at 0.25 nmol/µl (keep in mind that this is 6x more concentrated than necessary to make a strong signal on a gel).

4. Pool 40 µl from each labeled disaccharide into a single tube (now you have 240 µl of HS standard at 41.7 pmol/µl). Store in 10 µl aliquots and store at -80^{o} C indefinitely. Loading 2 µl per lane gives a strong signal at a 200 ms exposure (gain 1).



Heparan Sulfate FACE Standards: The banding pattern of pooled, and individual HS standards were analyzed by FACE as described in this protocol. Saccharides are numbered in the following order: (1) ΔDi-U-G-NAc, (2) ΔDi-U-G-NS, (3) ΔDi-U-6S-G-NAc, (4) ΔDi-6S-G-NS, (5) ΔDi-2S-U-G-NS, (6) ΔDi-2S-U-6S-G-NS.

HYALURONAN STANDARD

Reagents:
Sodium Hyaluronate (LifeCore Biomedical, 1.7 kDa, #80190)
Chondroitin (Seikagaku, 400640-1A)
Hyaluronidase SD (see 1.1)
Streptomyces Hyaluronidase (Seikagaku, 100740-1; alternatively, EMD Millipore, 389561)
Chondroitinase ABC (see 1.1)
D-Glucose (Gibco, 15023-021)

Procedure:
1. To make HA disaccharides, add 100 µl of Sodium Hyaluronate (1 mg/ml) to a 1.5 ml tube. Add 5 µl of hyaluronidase SD, vortex/spin and incubate overnight at 37^{o} C.

2. To make HA tetramers and hexamers, add 100 µl Sodium Hyaluronate (1mg/ml) to two separate 1.5 ml tubes. Add 50 µl Streptomyces hyaluronidase to each of these two tubes, vortex/spin and incubate overnight at 37° C.
3. To make "0S" chondroitin disaccharides, add 100 µl of chondroitin (1 mg/ml) to a 1.5 ml tube. Add 5 µl of chondroitinase ABC, vortex/spin and incubate overnight at 37° C.

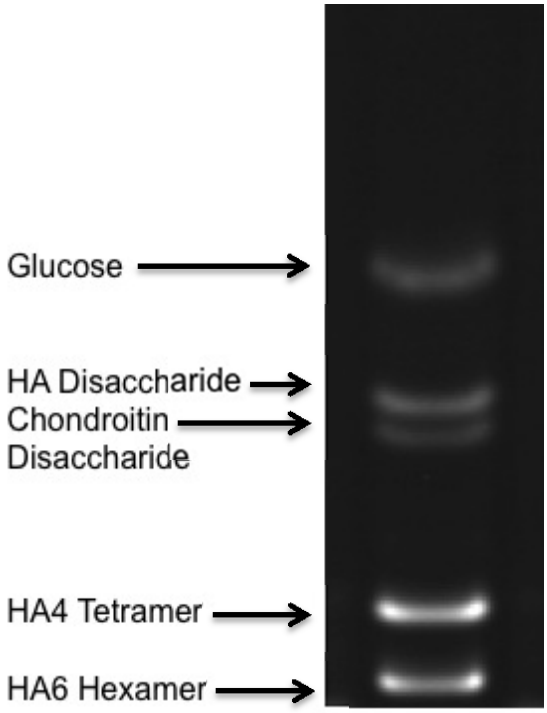
4. Next day, heat inactivate the enzymes from steps 1-3 at 100° C for 10 min.
5. Pool all of the samples from tubes 1-3 into a single tube.

6. To add glucose to your HA standard, make a 1 mg/ml solution of glucose in sterile MilliQ water. Add 100 ul of this 1 mg/ml glucose solution to the pooled tube in step #5.

7. Add 500 µl sterile MilliQ water to the pooled tube (final volume 1 ml). Vortex/spin. Make 100 µl aliquots into ten separate 1.5 ml tubes.

8. Speed-vac all ten of the pooled tubes from #7 to dryness. Store 9 of them in the -80° C. Add 1 ml of AMAC to the remaining tube. Incubate overnight at 37° C. Make 10 µl aliquots and store at -80° C indefinitely.

9. Each µl of this standard gives 10 ng of glucose, disaccharide HA, and disaccharide chondroitin. The HA tetramer and hexamer represent 8 and 12 ng/µl, respectively.



Hyaluronan FACE Standards: The banding pattern of pooled HA standards were analyzed by FACE as described in this protocol. Saccharides are numbered in the following order: (1) Glucose, (2) HA Disaccharide, (3) Chondroitin Disaccharide, (4) HA4 Tetramer, (5) HA6 Hexamer.

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