Echelon Biosciences Inc.

Protocol for Hyaluronic Acid Gel Electrophoresis

This protocol is developed as a general guideline for hyaluronic acid (HA) molecular weight determination by gel electrophoresis. We recommend using this guideline as a starting point. Optimize the protocol to fit your needs.

Materials:

- Hyaluronic Acid Samples to be test (see HA Sample Preparation for more information)
- Hyaluronic Acid Standards (Echelon Select-HA™ Ladder, Cat. No. HYA-LOLAD-20, HYA-HILAD-20, HYA-MGLAD-20)
- High quality agarose or pre-poured polyacrylamide gel
- 1X Tris-Borate-EDTA (TBE) Buffer or 1X Tris-Acetate-EDTA (TAE) Buffer
- 1X Sample Buffer – 0.02% Bromophenol blue, 2 M sucrose in 1X TBE
- Stains-All Solution – 0.005% Stains-All in 50% ethanol, filtered
- Destaining Solution – 30% Ethanol
- Ultrapure Water

Protocol:

1. Prepare agarose solution with the selected buffer system in an Erlenmeyer flask. Refer to Table 1 for the recommended agarose concentration, gel dimension and buffer system.
2. Melt the agarose using microwave. Do not overheat. Stop heating when the agarose solution is clear. Let the solution warm to touchable temperature.
3. Pour the agarose solution into the gel-casting system of your choice. Insert comb and let the gel set. Once set, cover the gel with the selected buffer. Let gel set overnight at room temperature.
4. If necessary, dilute HA samples with ultrapure water. Mix 15 µL of HA samples or standards with 5 µL 1X Sample Buffer (1:4 dilution). Refer to Table 1 for recommended HA samples and HA standards concentration.
5. Carefully remove comb from gel and place the gel into the electrophoresis apparatus of your choice. Pour the selected buffer to cover the gel.
6. Load 5-20 µL the prepared HA samples or standards (step 4) into the gel. Refer to Table 1 for recommended sample loading concentration & volume.
7. Place electrophoresis unit cover on. Ensure the negative electrode is connected to the top of the gel and the positive electrode to the bottom of the gel.
8. Run gel with decided time and voltage. Refer to Table 1 for recommended running time and voltage.
9. Immediately remove gel after the electrophoresis is completed. Transfer gel into a glass container. Pre-wet the glass container with Stains-All Solution to prevent gel sticking to the glass container.
10. Pour enough Stains-All Solution to cover the gel. Cover the glass container with lid or plastic wrap. Stain gel overnight at room temperature. Protect the glass container from light by wrapping the entire glass container with aluminum foil.
11. Carefully remove the Stains-All Solution. Stains-All Solution can be re-used once.
12. Pour enough Destaining Solution to cover the gel. Cover the glass container with lid or plastic web. Destain the gel at room temperature for minimum overnight. Protect the glass container from light by wrapping the entire glass container with aluminum foil. Change Destaining Solution at least once to facilitate the destaining process
13. When gel background is reduced, and bands are clear. Scan or image gel for record. Gel can be stored in the dark in the Destaining Solution for several days.
14. Discard the gel, Stains-All Solution and Destaining Solution as biohazard waste.

HA Sample Preparation

In order to measure the HA molecular weight using gel electrophoresis, HA is needed to be isolated from biological samples. The following protocol is generated based on published literature. Optimization is highly recommended to fit your needs. A summary of HA concentration and MW distribution in biological samples can be found in Table 2. Spiked biological fluids or solid tissue with monodispersed HA (Echelon Cat. No. HYA-50KEF-1, HYA-500KEF-1, HYA-601KEF-1, HYA-1000KEF-1) can be used to check for potential HA degradation during the isolation process.

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Table 1. Recommended Gel Electrophoresis Condition for HA MW Determination[1-2]

HA MW	Gel/Buffer	Gel Dimension	Chamber Dimension	HA Sample Conc.	HA Sample Vol.	Select-HA™ Standards	Voltage & Run Time
>1500 kDa	0.5% Agarose in TAE	10 cm L x 6.2 cm W x 6.5 cm H	20 cm L x 15 cm W	0.3-0.5 mg/mL	10-20 µL	5 µL/lane	20V for 0.5 hour follow by 40V for 3.5 hours
100-1500 kDa	0.5-1% Agarose in TBE	10 cm L x 6.2 cm W x 6.5 cm H	25.5 cm L x 9.2 cm W	0.5 mg/mL	10-20 µL	5 µL/lane	20V for 0.5 hour follow by 40V for 3.5 hours
30-1000 kDa	1.5-2% Agarose in TBE	10 cm L x 6.2 cm W x 6.5 cm H	25.5 cm L x 9.2 cm W	0.5 mg/mL	10-20 µL	5 µL/lane	Pre-electrophoresis at 40V for 20 minutes. Then, 20V for 0.5 hour follow by 40V for 4 hours
10-500 kDa	3-4% Agarose in TBE	10 cm L x 6.2 cm W x 6.5 cm H	25.5 cm L x 9.2 cm W	0.5 mg/mL	10-20 µL	5 µL/lane	Pre-electrophoresis at 40V for 20 minutes. Then, 20V for 0.5 hour follow by 40V for 4 hours
4-100 kDa	4-20% Polyacrylamide in TBE	Pre-Cast	Invitrogen Xcell SureLock Mini Cell System	0.1-0.2 mg/mL	3-5 µL	2-3 µL/lane	400 V for 28-40 minutes

Milk (20-30 mL)[3]

1. Heat milk in boiling water for 10 minutes. Cool on ice for 10 minutes.
2. Add Proteinase K to final concentration at 0.5 mg/mL.
3. Incubate at 60 °C overnight. Cool on ice for 10 minutes.
4. Centrifuge at 3,000 x g for 15 minutes at 4 °C.
5. Carefully collect the lower layer into a sterile tube.
6. Repeat Step 4-5 once.
7. Centrifuge at 25,000 x g for 10 minutes at 4 °C. Collect the supernatant.
8. Heat in boiling water for 10 minutes. Cool to room temperature.
9. Repeat Step 7.
10. Dialyze against diH2O overnight using 3.5 kDa cutoff dialysis membrane/cassette.
11. Concentrate sample using centrifugal evaporator.

Serum (0.1 mL)[4]

1. Mix 1-part serum with 3-part of cold ethanol with 1.3% (w/v) sodium acetate.
2. Vortex to mix and incubate on ice for 30 minutes.
3. Centrifuged at 12,000 x g for 20 minutes at 4 °C.
4. Discard supernatant and reconstitute pellet with 0.4 mL diH2O.
5. Repeat Step 1-4 three times.
6. Resuspend the pellet with 0.4 mL 10 mM CaCl2 with 3 mg/mL Proteinase K.
7. Incubate at 50 °C overnight. Inactive Proteinase K by boiling for 15 minutes.
8. Repeat Step 1-4 three times.
9. Resuspend the pellet with diH2O

Tissue (300-350 mg)[5]

1. Incubate issue in 2 mL 0.15 M Tris, 0.15 M NaCl, 0.01 M CaCl2, and 5 mM deferoxamine mesylate, pH 8.3, containing 40 units of Proteinase K at 55 °C overnight.
2. Inactive Proteinase K by boiling for 20 minutes.
3. Centrifuge at 21,000 rpm for 15 minutes at 4 °C.
4. Collect supernatant.

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Table 2. HA Concentration and MW Distribution in Biological Samples[6]

Species	Sample Type	Concentration	Molecular Weight
Bovine	Nasal cartilage	1200 μg/g	
Bovine	Vitreous		500-800 kDa
Human	Amniotic Fluid - 16 weeks		330 kDa
Human	Amniotic Fluid - 40 weeks		Mix of high & low MW
Human	Aqueous Human	1 μg/mL	
Human	Articular Cartilage	500-2500 μg/g	>2000 kDa
Human	Eye Vitreous	200 μg/mL	
Human	Knee Joint	2-3 mg/mL	
Human	Lymph Fluid	0.1-18 μg/mL	Mix of high & low, median at 800 kDa
Human	Milk	200-800 ng/mL	440 kDa (5% at <100 kDa)
Human	Organs	1-100 μg/g	
Human	Serum - Healthy	10-100 ng/mL	100-300 kDa
Human	Serum - Hepatic Cirrhosis	>46.5 ng/mL	
Human	Serum - Rheumatoid Arthritis	0.07-200 μg/mL	
Human	Serum - Ankylosing spondylitis	7-13 μg/mL	
Human	Serum - Osteoarthritis	0.01-2.3 μg/mL	
Human	Serum - Untreated cancer	2-fold of normal	
Human	Synovial Fluid - Healthy		6000-7000 kDa
Human	Synovial Fluid - Rheumatoid Arthritis & Osteoarthritis		Vary can be <500 Da
Human	Skin	400-500 μg/g	4000-6000 kDa
Human	Urine	100-300 ng/mL	Low MW
Rabbit	Cornea	1.3 μg/g	
Rabbit	Brain	65 μg/g	
Rabbit	Heart	200 μg/g	
Rabbit	Intestine (Large)	200 μg/g	
Rabbit	Intestine (Small)	130 μg/g	
Rabbit	Kidney	103 μg/g	
Rabbit	Muscle	27 μg/g	
Rabbit	Liver	1.5 μg/g	
Rabbit	Lung	80-90 μg/g	
Rabbit	Skin	500 μg/g	4000-6000 kDa
Rabbit	Vitreous		2000-3000 kDa

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