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 Loading Buffer – 0.02% Bromophenol blue, 2 M sucrose in 1X TBE
- Stains-All Solution – 0.005% Stains-All in 50% ethanol (Protect from light. Stains-All is light sensitive)
- Destaining Solution – 30% Ethanol
- Ultrapure Water

Protocol:

1. Reconstitute ladder if needed.
   - Reconstitution of HiLadder and LoLadder:
     Centrifuge the tube for a few seconds to collect the Select-HA™ solids in the bottom of the tube. Carefully open and add 100 µL of sterile water directly to the bottom of the tube. Allow two hours at 4 ºC for sample hydration. Mix well before use.
   - The Mega Ladder requires no reconstitution.
2. Prepare agarose solution in an Erlenmeyer flask with the selected buffer system. Refer to Table 1 for the recommended agarose concentration, gel dimension and buffer system.
3. Melt the agarose using microwave oven, avoid overheating. Stop heating once the agarose solution is clear, then cool to a touchable temperature.
4. Pour the agarose solution into the gel-casting system. Insert comb and allow the gel to set. Once set, cover the gel with the selected buffer and let it set overnight at room temperature.
5. If necessary, dilute HA samples with ultrapure water. Mix HA samples or Select-HA Ladder with 1X Loading Buffer at a 4:1 ratio. For example, mix 8 µL sample with 2 µL 1X Loading Buffer. Refer to Table 1 for recommended amounts for HA samples and HA standards.
6. Carefully remove comb from gel, then place the gel into the electrophoresis apparatus of choice. Pour the selected buffer until the gel is fully covered.
7. Load the prepared HA samples or Select-HA standards (step 4) into the gel. Refer to Table 1 for loading concentration & volume.
8. Secure the electrophoresis unit cover. Confirm that the negative electrode is connected to the top of the gel and the positive electrode to the bottom.
9. Run gel for the designated time and voltage. Refer to Table 1 for recommended settings.
10. Immediately remove gel after the electrophoresis is completed. Transfer the gel into a pre-wetted glass container with Stains-All Solution to prevent gel sticking.
11. 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.

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12. Carefully remove the Stains-All Solution.
13. Pour enough Destaining Solution to cover the gel. Cover the glass container with lid or plastic wrap. 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.
14. 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.
15. Discard the gel, Stains-All Solution and Destaining Solution as biohazard waste.

Table 1. Recommend Gel Electrophoresis Condition for HA Determination[1,6]

HA MW	Gel/Buffer	Gel Dimension	Chamber Dimension	HA Sample Amount	Select-HA Ladder	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	3-10 µg/lane	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	≤10 µg/lane	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	≤10 µg/lane	5 µL/lane	Pre-electrophoresis at 40V for 20 minutes. Then, 20V for 30 minutes 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	≤10 µg/lane	5 µL/lane	Pre-electrophoresis at 40V for 20 minutes. Then, 20V for 30 minutes follow by 40V for 4 hours
4-100 kDa	4-20% Polyacrylamide in TBE	Pre-Cast	Invitrogen Xcell SureLock Mini Cell System	0.3-1 µg/lane	2-3 µL/lane	400 V for 28-40 minutes

Sample Preparation

In order to measure the HA molecular weight using gel electrophoresis, HA needs 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.

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 diH_2O overnight using 3.5 kDa cutoff dialysis membrane/cassette.
11. Concentrate sample using centrifugal evaporator.

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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 diH_2O.
5. Repeat Step 1-4 three times.
6. Resuspend the pellet with 0.4 mL 10 mM $CaCl_2$ 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 diH_2O

Tissue (300-350 mg)[5]

1. Incubate issue in 2 mL 0.15 M Tris, 0.15 M NaCl, 0.01 M $CaCl_2$, 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.

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 humor	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	

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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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