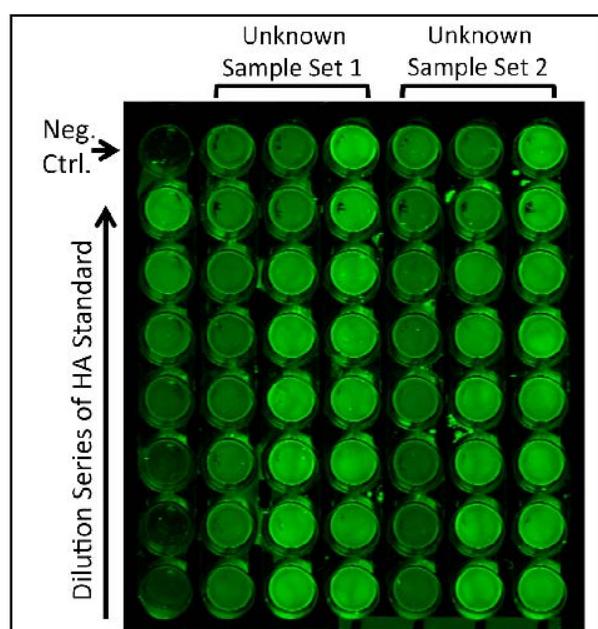


HYALURONAN COMPETITIVE INHIBITION ASSAY

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

1.0 BACKGROUND

ELISA – like assays are sensitive and quantitative approaches to measure levels of hyaluronan (HA) in samples. The expression “ELISA – like” is used instead of “ELISA” (enzyme linked immunosorbent assay) because the assay uses a HA binding protein (HABP) instead of an antibody (so it is technically not an “immunosorbent” assay). This HABP is typically the G1 domain of aggrecan or a combination of G1/Link protein from cartilage. When both G1 and Link are present, this HABP dimer remains bound to HA. If only G1 or Link are present, the HABP monomers will bind off/on to HA. There are two basic approaches to measuring HA by ELISA – like assays: (1) “sandwich” and (2) competitive inhibition approaches. In the former, (a) HABP is bound to a plate (typically by electrostatic forces), (b) samples/standards containing HA are then applied to the well and allowed to bind the HABP attached to the plate, (c) a biotinylated HABP is applied to the well to permit binding to the captured HA (forming an HA sandwich between the HABP bound to the plate and the biotinylated HABP in solution), (d) a streptavidin reporter (typically horseradish peroxidase) is applied, (e) the amount of HA is calculated in each well by its intensity on a spectrophotometer following the application of a color developing reagent that is metabolized by horseradish peroxidase. In competitive inhibition assays, (i) excess biotinylated HABP is added to the samples/standards and allowed to bind HA in the samples/standards, (ii) these samples/standards are then incubated in plates that have been covalently coated with HA, (iii) during this incubation the biotinylated HABP that was in excess of the HA present in each sample/standard binds to the HA in the plate, (iv) a streptavidin reporter (typically horseradish peroxidase) is applied to each well and (v) the amount of HA is calculated in each well by its intensity on a spectrophotometer following the application of a color developing reagent that is metabolized by horseradish peroxidase. In a sandwich assay, the higher



the absorbance on a spectrophotometer the more concentrated the HA is in that sample. In a competitive inhibition assay it is the reverse. The absorbance from both of these assays is not linear so a curved linear regression approach is required to estimate the HA concentration in an unknown sample from the OD values of a standard curve. Sandwich assays are generally more sensitive than competitive inhibition assays but the latter are able to detect a broader range of HA sizes. Both assays require that the amount of HA in the sample not exceed the binding capacity of HABP in the assays, thus concentrated samples may require dilution to prevent saturation. The assay described in this protocol is a “homemade” competitive inhibition assay using commercially available HABP and NH-Covalink plates on which HA is covalently attached.

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2.1 REAGENTS

NH-Covalink Plates (Thermo Scientific; Product #478042)

Plate Sealers (R&D Systems; Product #DY992)

EDC (Thermo Scientific; Product #22980; MW 191.7 g/mol)

Sulfo-NHS (Thermo Scientific; Product #24510; MW 217.14 g/mol)

High Molecular Weight Hyaluronan

Add 50 ml of sterile distilled water to 10 mg of HMW HA (≥ 1000 kDa) (final concentration of 200 $\mu\text{g/ml}$). Incubate overnight at room temperature with continuous end-over-end agitation. Store at 4° C. We recommend research grade sodium hyaluronate from Lifecore Biomedical (1.20 MDa – 1.80 MDa; Product #HA1M-1).

Gentamicin (GIBCO Life Technologies; Product #15750-060)

Biotinylated Hyaluronan Binding Protein (HABP) (EMD/Millipore Product #385911-50UG; Add 100 μl PBS to vial, incubate at RT for 10 min, vortex/spin, make 10 μl aliquots and store at -80° C)

5% Nonfat Dry Milk

Add 2.5 grams of Nonfat Dry Milk to 47.5 ml PBS. Mix by inversion at room temperature until dissolved. Store in 10 ml aliquots at -20° C.

PBS

2M NaCl

Wash Buffer 0.05% Tween 20 in PBS

LI-COR Odyssey Blocker (Product #927-40000) We dilute this blocker 1:2 in PBS and add 1 ml of Tween 20 per liter of the diluted blocker.

LI-COR Streptavidin (Product #926-32230)

2.2 EQUIPMENT

NUNC Immunowasher (*Thermo Scientific; 8-channel (470174) and 12-channel (470175) are available*)

LI-COR Odyssey Infrared Scanner (*CLx*)

3.0 PROTOCOL

1. Covalent Transfer of Hyaluronan to NH-Covalink Plates (96-well protocol)

- a) Dissolve 5 mg of sulfo-NHS in 500 μ l sterile distilled water (final concentration 10 mg/ml).
- b) Add 97.52 μ l of the sulfo-NHS from step 1a to 5.3 ml HA at 200 μ g/ml.
- c) Dissolve 16.5 mg EDC in 44.7 ml H₂O (final concentration 0.37 mg/ml)
- d) Mix 5.3 ml HA/sulfo-NHS with 5.3 ml EDC
- e) Add 100 μ l of mixture per well of twelve 8-well NH-Covalink strips.
- f) Incubate for 2 hrs at room temperature and store at 4° C overnight
- g) Wash the wells twice with 200 μ l of 2M NaCl
- h) Wash wells with 100 μ l PBS 3x
- i) Store with 200 μ l 50 μ g/ml Gentamicin in PBS
- j) Seal and Store at 4° C

2. Preparation of Hyaluronan Standards

- a) Set up seven PCR tubes
- b) Add 6 μ l PBS to tubes #1-6 and 17.5 μ l PBS to #7
- c) Add 3 μ l HMW-HA 1mg/mL to tube #1, pipet up/down
- d) Transfer 3 μ l from #1 to #2, mix, and consecutively continue transfer up to tube #6
- e) Add 17.5 μ l HABP to tube #7 (HABP diluted 1:2)
- f) Prepare seven 1.5 mL tubes
- g) Add 95 μ l PBS to tubes #1-6, and 100 μ l to #7
- h) Add 5 μ l of each HMW-HA dilutions to each of 6 tubes respectively; vortex/spin
- i) Add 5 μ l HABP 1:2 to each of the 7 tubes
- j) Incubate at RT for 1 hour (this, and the following steps, are done while you are incubating your samples, as described in section 3)
- k) Rinse an 8-well strip HA Covalink 3x w/PBS
- l) Add 100 μ l Odyssey blocking buffer; block 1 hour at RT
- m) Add 100 μ l HMW-HA dilution series to each well (1-6)
- n) Add 100 μ l PBS "positive" control to well #7 (this will tell you if your HABP detection works)
- o) Add 100 μ l PBS only to well #8 (this will show your background)
- p) Incubate 1 hour at RT
- q) Rinse 3x with 100 μ l/well Wash Buffer (PBS/0.05% Tween 20)
- r) Add 100 μ l/well Li-Cor streptavidin construct at 1:2000 in Odyssey Licor blocking solution
- s) Incubate for 45 minutes at RT
- t) Wash wells 3x with wash buffer (PBS/0.05% Tween-20)
- u) Scan strips on Odyssey Scanner

3. Competitive Inhibition Assay

- a) Set up a 1.5mL tube for each of your samples (unknowns)
- b) Add 99 μ l PBS to each of these tubes
- c) Add 1 μ l of each sample to the appropriate tube (note: if your samples contain HA > 0.5 μ g/ml you will need to dilute them)

- d) Dilute HABP 1:2 with PBS such that you have enough to add 5 μ l of the diluted HABP per sample
- e) Add 5 μ l of the diluted HABP to each sample (from step c)
- f) Add 5 μ l of diluted HABP 95 μ l PBS as a positive control (this will tell you if your HABP detection works)
- g) Incubate 1 hour at RT
- h) Prepare the appropriate number of 8-well NH-HA Covalink strips (you will need 1 well per sample; more if you want to obtain replicates of each sample)
- i) Add 100 μ l/well of the diluted Odyssey blocking solution to each well and incubate 1 hr at RT
- j) Aspirate blocking solution from wells
- k) Add 100 μ l/well of each sample already incubated with HABP in steps a-g
- l) Incubate 1 hour at RT
- m) Wash wells 3x with 100 μ l washing buffer (PBS/0.05% Tween-20)
- n) Add 100 μ l/well LiCor streptavidin at 1:2000 in Odyssey Licor secondary blocking solution
- o) Incubate for 45 minutes
- p) Wash wells 3x with Wash Buffer (PBS/0.05% Tween-20)
- q) Scan strips on Odyssey Scanner

4. Scanning and Quantification Using the Odyssey Scanner

- a) Scan the wells on an Odyssey Scanner.
 - i. Open Odyssey Image Studio
 - ii. Select work area or create a new one if you haven't already
 - iii. Select Custom Plate
 - iv. Place the L shaped ruler on slide scanner
 - v. Place strips in plate holders (*note: you will need to paint the bottom of the blue plate holders with flat black paint to minimize autofluorescence*)
 - vi. Edit the size of the box to your scan area
 - vii. Set Focus Offset to 3.0
 - viii. Scan
 - ix. Brightness and Contrast can be edited after the scan
- b) To quantify the signal in each well
 - i. Edit brightness and contrast
 - ii. Select the Image tab on the top toolbar
 - iii. Adjust the placement of the 96well grid. The wells should have the right spacing so use the mouse when it is a cross to just relocate the grid circles over the wells
 - iv. Select Plate wells on the bottom toolbar. It will show the total signal for each well in the 700 and 800 channel. We are interested in the 800 (green channel).
 - v. Select all of the data and can copy to excel
 - vi. Select Grid sheet to see the grid layout of the quantification. Under column select total and channel select 800.
 - vii. Select all data and can copy to excel.