



## HYALURONIDASE ACTIVITY PROTOCOL

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

### 1.1 BACKGROUND

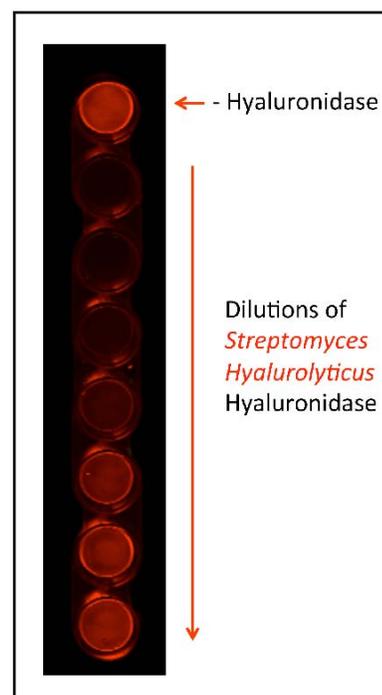
Hyaluronan is a large (>2500 MDa), linear glycosaminoglycan that can be broken down into smaller sizes as a result of developmental and pathological processes. This can occur enzymatically (i.e. by endogenous or bacterial hyaluronidases), by free radicals or by chemical-based depolymerization (i.e. acid hydrolysis). The production of hyaluronan fragments is biologically important, not only in the study of hyaluronan turnover in healthy and diseased tissues, but also because hyaluronan fragments have been shown to have bioactivity that is different than high molecular weight HA bioactivity. In general, smaller fragments of hyaluronan are thought to be pro-inflammatory while larger sizes are considered to possess anti-inflammatory properties. The assay described in this protocol involves incubating a sample in a hyaluronan – coated well under conditions permissive for hyaluronan degradation, followed by detection of the remaining hyaluronan in the well by hyaluronan binding protein (HABP) fluorescence. For the sake of simplicity we titled this assay a “hyaluronidase activity protocol” but it should be noted that loss of hyaluronan signal in this assay does not distinguish hyaluronan degradation by a hyaluronidase compared to, for example, free radicals.

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### 1.2 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 ( $\geq 1000$  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)

Hyaluronidase (*Streptomyces hyalurolyticus*; EMD/Millipore Product #389561); add 500  $\mu\text{l}$  PBS to 100 unit ampule, let incubate at RT for 10 min, swirl ampule to mix, store 25  $\mu\text{l}$  aliquots at -80° C; final concentration 0.2 U/ $\mu\text{l}$ )

Biotinylated Hyaluronan Binding Protein (HABP) (EMD/Millipore Product #385911-50UG); Add 100  $\mu\text{l}$  PBS to vial, incubate at RT for 10 min, vortex/spin, make 10  $\mu\text{l}$  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 (primary 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)

## 1.3 EQUIPMENT

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

LI-COR Odyssey Infrared Scanner (CLx)

## 2.0 PROTOCOL

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

- Dissolve 5 mg of sulfo-NHS in 500  $\mu\text{l}$  sterile distilled water (final concentration 10 mg/ml).
- Add 97.52  $\mu\text{l}$  of the sulfo-NHS from step 1a to 5.3 ml HA at 200  $\mu\text{g/ml}$ .
- Dissolve 16.5 mg EDC in 44.7 ml H<sub>2</sub>O (final concentration 0.37 mg/ml)

- d) Mix 5.3 ml HA/sulfo-NHS with 5.3 ml EDC
- e) Add 100ul 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 µl of 2M NaCl.
- h) Wash wells with 100 µl PBS 3x.
- i) Store with 200µl 50 µg/ml Gentamicin in PBS
- j) Seal and Store at 4° C

## 2. Hyaluronidase Activity Incubation

- a) Wash wells with 100 µl PBS 3x
- b) Positive Controls: Dilute *Streptomyces* hyaluronidase at 1:1000 in PBS and add 50 µl per appropriate well for positive controls (*do not* add it to the wells where your samples will be added). Incubate at 37° C from 2-18 hrs. Don't use NUNC Immunowasher immediately after treatment with hyaluronidase or you may contaminate the washer.
- c) Negative Controls: Add 50 µl PBS to appropriate wells for a negative control. Incubate at 37° C from 2-18 hrs. (steps b and c should be done at the same time as the incubation of your samples in step d).
- d) Incubation of Samples: Apply 50-100 µl of each sample (unknown) to the appropriate well and incubate at 37° C from 2-18 hrs.
- e) Wash wells with 100 µl PBS 3x
- f) Wash wells with 100 µl 2M NaCl 3x to remove any proteins that may have bound to the HA on the plate that would interfere with the binding of HABP in step i)
- g) Wash wells with 100 µl PBS 3x
- h) Blocking: Add Li-Cor Blocking Buffer for 1 hr at room temperature
- i) Incubation with HABP: Add 50 µl/well HABP to Odyssey Primary Blocker at a 1:200 dilution
- j) Wash wells with 100 µl PBS 3x
- k) Dilute Li-Cor streptavidin 1:2000 in Odyssey blocker. Add 50 µl/well, cover and incubate at room temperature for 45 min.
- l) Wash wells with 100 µl PBS 3x m) Add 100 µl/well PBS to scan on the Odyssey scanner

## 3. Scanning and Quantification Using the Odyssey Scanner

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
- x. Edit brightness and contrast
- xi. Select the Image tab on the top toolbar
- xii. 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
- xiii. Select Plate wells on the bottom toolbar. It will show the total signal for each well in the 700 nm

and 800 nm channel. We are interested in the 800 nm (green channel).

- xiv. Select all of the data and can copy to excel
- xv. Select Grid sheet to see the grid layout of the quantification. Under column select total and channel select 800.
- xvi. Select all data and can copy to excel.