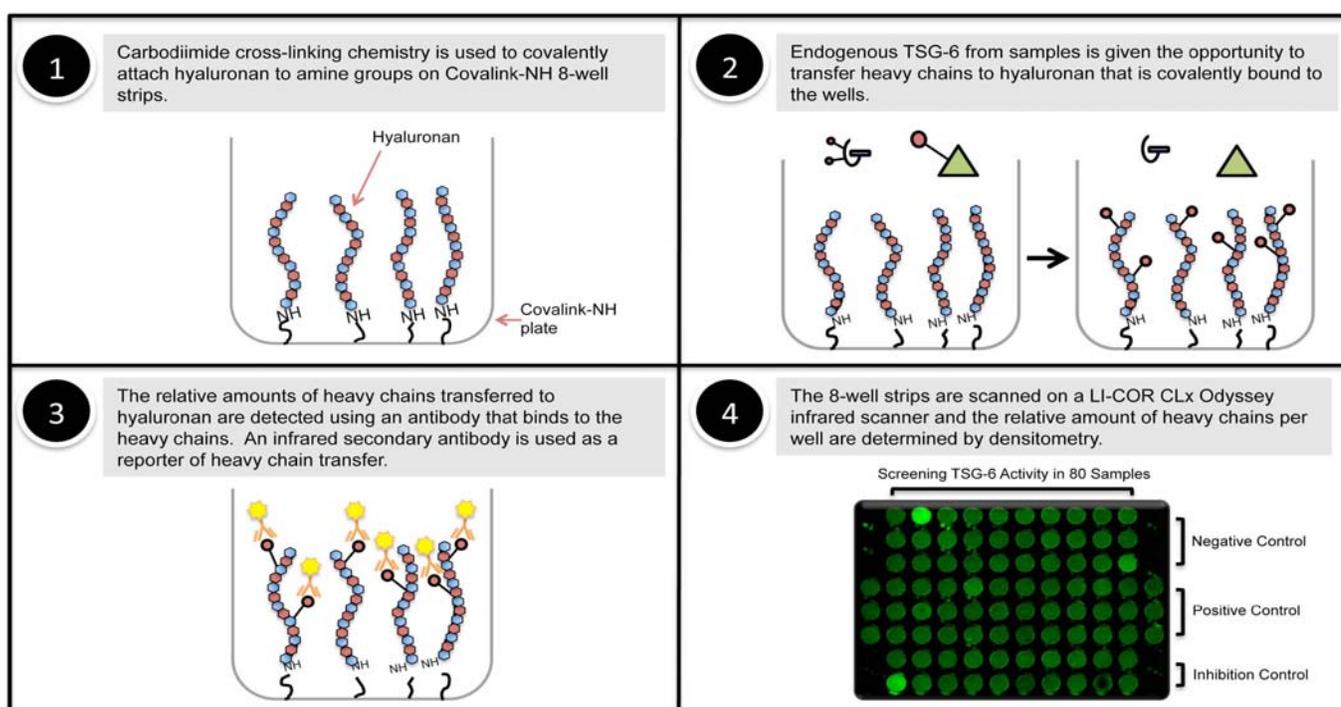


TSG-6 ACTIVITY PROTOCOL

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

1.1 BACKGROUND

Tumor-necrosis-factor-stimulated-gene-6 (TSG-6) is an enzyme that transfers heavy chains (HCs) from the chondroitin sulfate chain of inter-alpha-inhibitor to the hyaluronan (HA) glycosaminoglycan. Specifically, a C-terminal aspartate residue of the HCs, which was attached to the 6th carbon of a N-acetylgalactosamine residue of the non-sulfated region of bikunin's chondroitin sulfate chain, is transferred to the 6th carbon of N-acetylglucosamine residues on HA to form a covalent HC-HA complex. The function of this HC modification of HA is under investigation but includes making HA "sticky" for leukocytes. In other words, the transfer of HCs to HA significantly improves leukocyte adhesion to HA matrices. In this protocol we describe a technique to measure TSG-6 activity in samples. The following figure summarizes the approach of this method:



This protocol is largely based on the protocol by **Wisniewski, et al.** (2014) *Osteoarthritis Cartilage* 22(2):235-41.

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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 (≥ 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)

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

0.02 M Sodium-EDTA pH 8.0

Recombinant TSG-6 (R&D Systems; Product #2104-TS; resuspend at 0.125 $\mu\text{g}/\mu\text{l}$ in PBS which is 400 μl PBS per 50 μg of TSG-6 to make a concentrated stock; *note that the working stock is a 1:7.5 dilution of the concentrated stock*)

Human Serum (*any non-EDTA containing serum should work; we recommend serum from Equitech-Bio, Inc., Kerrville, TX*)

Inter-Alpha-Inhibitor Antibody (Dako A0301; Rabbit Polyclonal; This antibody can be obtained through the Cleveland Clinic Shared Resource Core <http://pegnac.sdsc.edu/cleveland-clinic/sharedresources/>)

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 Donkey Anti-Rabbit Secondary Antibody (Product #926-32213)

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)

- 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 3x's with PBS
- i) Store with 2600 μ l 50 μ g/ml Gentamicin in PBS
- j) Seal and Store at 4° C

2 TSG-6 Activity Incubation

- a) Block each well with 100 μ l of 5% nonfat dry milk for 1 hr at room temperature.
- b) Wash wells with 100 μ l PBS 3x.
- c) Add 4 μ l human serum to each 100 μ l sample. Vortex/Spin.
- d) Add the samples from 2c to each well.
- e) Include negative controls (100 μ l of PBS containing 4% human serum) and positive controls (100 μ l PBS containing 4% human serum and 1 μ l of recombinant TSG-6 (diluted 1:7.5 in PBS) to the appropriate wells.
- f) Incubate for 2 hrs at 37° C. (Note: The incubation can be extended overnight to detect low levels of TSG-6 though this will somewhat increase the background)
- g) Stop the reaction by adding 50 μ l of 0.02 M EDTA into each well (this step is optional; EDTA will stop TSG-6 activity because TSG-6 activity requires divalent cations).

3. TSG-6 Activity Detection

- a) Aspirate each well and wash with Wash Buffer 3x. (*Complete removal of liquid at each step is essential to avoid well-to-well variation.*)
- b) Add 100 μ L of the Inter-alpha-Inhibitor antibody diluted in Odyssey Primary Blocker (at 1:1000) to each of the wells. Seal the strips and incubate at room temp for 1 hour.
- c) Aspirate each well and wash with Wash Buffer 3x.
- d) Dilute the appropriate amount of LI-COR Donkey Anti-Rabbit secondary antibody 1:2000 in Odyssey primary blocker. Add 100 μ L of this secondary solution to each of the wells, seal the strips and incubate at room temp for 45 minutes.
- e) Aspirate each well and wash with Wash Buffer 3x.
- f) 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
- g) 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.