Echelon Biosciences Inc.
675 Arapeen Drive, Suite 302
Salt Lake City, UT 84108
Telephone 866-588-0455
Fax 801-588-0497
echelon@echelon-inc.com
www.echelon-inc.com

For research use only
Not intended or approved for diagnostic or therapeutic use.

Hyaluronan Enzyme-Linked Immunosorbent Assay Kit (HA – ELISA)

Product Number: K-1200

INTENDED USE: THIS PRODUCT IS FOR RESEARCH USE ONLY. NOT INTENDED FOR CLINICAL OR DIAGNOSTIC USE.

Storage:

Kit can be stored unopened at 4 ºC for up to six months. Opened and reconstituted solutions, except the working Substrate Solution, can be used for up to one month when stored at 4 ºC. The working Substrate Solution should be freshly prepared. All components and solutions should be protected from excessive and intense light.

Materials Provided:

Part #	Description	Quantity
K-1201	Detection Plate (12 HA-coated 8-well microwell strips w/ frame)	1 Plate
K-1202	HA Standard (3200 ng/mL HA standard solution)	1 mL
K-1203	HA Detector	< 500 µL
K-1204	Diluent (10X)	10 mL
K-1205	Wash Concentrate (10X)	30 mL
K-1206	Enzyme	< 100 µL
K-1208	Substrate Buffer	11 mL
K-1209	Stop Solution	6 mL
Incubation Plate	Yellow 96-well polypropylene U-bottom	1 Plate
Substrate Pellet	p-Nitrophenyl Phosphate Tablet	1 Pellet
Plate Seals	Clear acetate sheet, 1 side adhesive	2 Seals

Additional Materials Provided by User:

- 37 °C Incubator
- Pipettes (capable of delivering between 5 and 1,000 µL with appropriate tips)
- Multichannel pipettes
- Absorbance microplate reader capable of reading at 405 nm

Background and Product Description:

Hyaluronic acid (HA) is a high molecular weight anionic polysaccharide (1,000-10,000 kD) composed of repeating disaccharides of β(1-4)glucuronic acid and β(1-3)N-acetylglucosamine and is one of several glycosaminoglycan (GAG) components of the extracellular matrix (ECM) of connective tissue (1). Each disaccharide dimer is referred to as one unit and has an approximate molecular weight (MW) of 450 D. Depending on the tissue source, the polymer can consist of 2,000 to 25,000 units (2). HA is an extremely large molecule not necessarily in its molecular weight but in the space that it occupies in solution which lends to its remarkable viscoelastic properties, lending to its importance in joint lubrication (3). Several functions of HA have been described including influencing the hydration and physical properties of tissues; and its ECM (extracellular matrix) interactions which affect tissue structure, assembly and facilitation of cell movement and behavior.

Free HA is transported from the lymph to the circulation with an estimated half life in serum of 2-5 minutes. HA is taken up by the liver in sinusoidal endothelial cells (90%) and the kidneys (10%) where it is degraded and recycled (4,5). Many chronic liver diseases, including infection (hepatitis B or C), toxicity (alcohol and drugs), genetic (hemochromatosis), autoimmunity, and malignancy, result in

Background and Product Description (cont.):

liver inflammation which can progress to liver fibrosis and cirrhosis (6). Each of these cause impairment of liver function and result in a rapid increase in circulating HA levels (4). Data indicates a relationship between HA levels, local inflammation and severity of disease (6).

Recent publications have shown that HA levels in urine are indicative of bladder cancer, that HA levels are directly correlated to liver disease and suggests enhanced breakdown of HA in the lungs of patients with chronic obstructive pulmonary disease (COPD). In addition, serum levels of HA have been found to be elevated in patients with rheumatoid arthritis (7).

Assay Specifics:

The HA-ELISA is a quantitative enzyme-linked immunoassay designed for the in vitro measurement of HA levels in human or animal biological fluids (blood, serum, urine, diffusate, synovial fluid). This simple protocol is a standard competitive ELISA format and requires 3 hours.

The HA-ELISA is a competitive ELISA assay in which the colorimetric signal is inversely proportional to the amount of HA present in the sample. Samples to be assayed are first mixed with the HA Detector, and then added to the HA ELISA plate for competitive binding. An enzyme-linked antibody and colorimetric detection is used to detect the HA detector bound to the plate. The concentration of HA in the sample is determined using a standard curve of known amounts of HA. The enzyme / substrate system is a colorometric assay comprised of alkaline phosphatase / pNPP phosphatase substrate. It should be read at 405 nm.

The size of HA polymers is variable depending on tissue source. However, the sensitivity of the HA ELISA does not depend on the MW of the HA molecule except in the lower MW range (<25 dimers). The HA ELISA works best with HA molecules that are greater than 25 repeating units (dimers) to determine the relative concentration of HA independent of MW.

Reagent Preparation:

1X Diluent (K-1204): The Diluent is supplied as a 10X concentrated solution. Dilute the required amount to a 1X working solution with dH_20 prior to beginning assay. Typically around 30-35 mL of Diluent is required to run a full assay. For 30 mL of 1X Diluent, add 3 mL of the 10X Diluent to 27 mL of dH_20.

HA Standards (K-1202): Make 1:2 serial dilutions of the HA Standard using the Diluent to obtain standards of 1600, 800, 400, 200, 100, and 50 ng/mL (Standards may be diluted in the plate, following the diagram below).

Working Detector (K-1203): Dilute Detector by adding 6 mL Diluent. (Volume provided is small and the bottle may appear empty.)

Working Enzyme (K-1206): Dilute Enzyme by adding 12 mL Diluent. (Volume provided is small and the bottle may appear empty.)

1X Wash Concentrate Buffer (K-1205): Add the 10X Wash Concentrate to 270 mL deionized water for a 1X Wash Concentrate Buffer solution. Final volume = 300 mL.

Working Substrate Solution (K-1208): Dissolve Substrate Pellet in 11 mL Substrate Buffer. (Dissolve immediately before use.)

HA ELISA Notes:

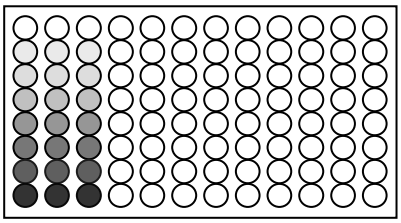
- We suggest the HA Standard dilution series be run in triplicate for best results.
- It is recommended that serum and plasma samples be analyzed with no dilution or a maximum dilution of 1:2 in the provided Diluent.
- **When analyzing biologic samples we advise running a known normal (low) HA sample and a disease (high) HA sample in conjunction with your unknown samples. These will serve as positive and negative controls to distinguish between normal healthy samples and disease samples.**

Assay Procedure:

1. Set up the incubation plate (yellow U-bottom plate) as illustrated. (Each well should contain 150 µL)
    - Add 100 µL of Standards and samples into corresponding wells.
    - Add 150 µL of Diluent to the Blank control wells and 100 µL of Diluent to the Zero HA control wells.
    - Add 50 µL of Working Detector to all wells except the Blank wells.

Standards/ Samples

A. Blank
B. 1600ng/mL
C. 800 ng/mL
D. 400 ng/mL
E. 200 ng/mL
F. 100 ng/mL
G. 50 ng/mL
H. Zero HA



2. Mix the plate gently, cover with plate seal and incubate for one hour at 37 ºC.
3. Following the incubation step, transfer 100 µL of controls and samples from the Incubation Plate to the corresponding wells of the Detection Plate (K-1201).
   ** This is easily accomplished with a multi-channel pipettor.*
4. Once the transfer is complete, mix the Detection Plate by gently tapping. Cover with a plate seal and incubate at 4 ºC for 30 minutes.
5. Shake out the solution from the Detection Plate. Wash the plate four times with 300 µL of 1X Wash Concentrate (K-1205). Ensure all wash buffer is removed from the plate by inverting the plate and blotting it out on absorbent paper.
6. Add 100 µL of Working Enzyme (K-1206) to each well of the Detection Plate.
7. Mix the Detection Plate gently, cover with plate seal and incubate at 37 °C for 30 minutes.
8. Repeat wash step #5.
9. Add 100 µL Working Substrate Solution (K-1208) to each well of the Detection Plate.
10. Incubate the Detection Plate in the dark at room temperature.
11. Measure the absorbance of each well at 405 nm beginning at T = 15 min.
    - The appropriate incubation time should be determined based on the ratio of the Zero HA standard control to the 1,600 ng/mL HA standard control. When the OD_0 / OD_{1600} ratio is > 3.0 the incubation is complete and can be stopped with the Stop Solution (K-1209). This is achieved by reading at 15 min., 30 min. or 45 min. Generally the best results are obtained after 30 minutes of development.
    - The Blank should have an absorbance of $\leq$ 0.20 and the ratio of the Zero HA Control to the 1,600 ng/mL HA Standard should be > 3.0.
12. Stop the reaction by adding 50 µL Stop Solution to each well.
13. Generate a best fit curve for the standards in order to extrapolate relative sample values. (***See figure 1. below as an example)***

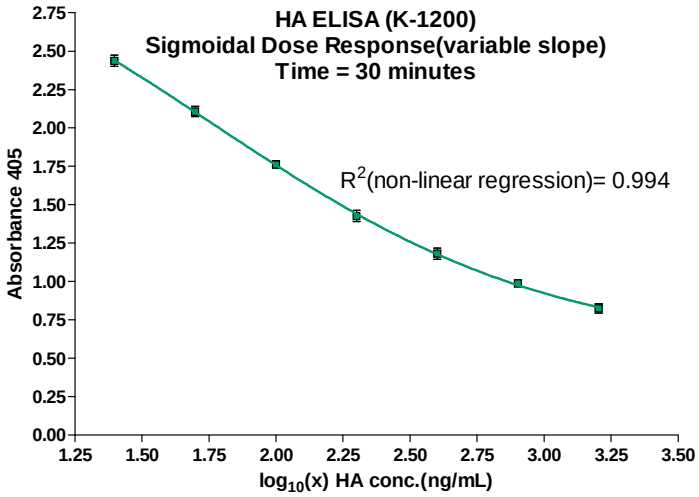


Figure 1. HA competitive ELISA standard curve was generated using non-linear regression analysis with GraphPad prism software. A sigmoidal dose response-variable slope curve (four-parameter) analysis was utilized.

Research:

Reference Values: Normal HA levels in serum from healthy blood donors are less than 120 ng/mL. Serum HA levels are elevated in several disease states including hepatitis (greater than 160 ng/mL) and cirrhosis (greater than 250ng/mL).

** Important Note: The above values are the suggested values based on literature observations. At times, the values measured using the Echelon Hyaluronic Acid ELISA assay have been 2-3 fold higher than those expected from the literature. As a result, we strongly advise users to utilize known reference samples indicative of both normal and disease states in order to establish relevant Hyaluronic acid levels. This will allow the user to differentiate between normal and disease state Hyaluronic Acid samples in a qualitative fashion.

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