



QnE Hyaluronic Acid (HA) ELISA Assay

For the quantitative determination of hyaluronic acid in biological fluids
For human and veterinary research use

Catalog# BTP-96200

Introduction

Overview

Hyaluronic acid (HA), also known as hyaluronate or hyaluronan, is a glycosaminoglycan; a high molecular weight polysaccharide with an unbranched backbone composed of alternating sequences of β -(1-4)-glucuronic acid and β -(1-3)-N-acetylglucosamine moieties. Each dimer is referred to as one unit and has a molecular weight of approximately 450Da. The HA molecule can vary in length from less than 10 to more than 1,000 units.¹⁻⁴ Hyaluronic acid is mainly produced by fibroblasts and other specialized connective tissue cells. It plays a structural role in the connective tissue matrix (proteoglycan) and participates in various cell-to-cell interactions. HA is widely distributed throughout the body and can be found as a free molecule in plasma and synovial fluid. In plasma, the half-life of the HA molecule has been estimated to be about 5-6 minutes.^{3,4} HA is found in synovial fluid in high concentrations and is responsible for normal water retention and lubrication of the joint. Synovial HA may pass into plasma via the lymphatic system.⁵ In circulation, HA levels are maintained by an efficient receptor-dependent removal mechanism present in sinusoidal endothelial cells (SEC) of the liver and by the enzymatic action of hyaluronidase.^{6,7}

The HA Test Kit uses a naturally occurring hyaluronic acid binding protein (HABP) from bovine cartilage to specifically capture HA and an enzyme-conjugated version of the HABP to detect and measure the HA captured from the biological fluid. This assay can detect down to a HA concentration of 10ng/ml and a MW of 100,000 Da.

Although the HA molecule is excreted in urine, urine is not a recommended material for testing. The HA molecule is extremely small in urine, usually less than 25 dimers, and the HA assay requires a minimum molecule size of 20 dimers for detection. Although the assay may detect the HA molecule in urine, the results will be variable at this low level.

Principle of the Assay

The HA test kit is an enzyme-linked binding protein assay that uses a capture molecule known as hyaluronic acid binding protein (HABP).^{22,23} Properly diluted specimens and HA reference solutions are incubated in HABP-coated microwells, allowing HA present to react with the immobilized binding protein (HABP). After the removal of unbound molecules by washing, HABP conjugated with horseradish peroxidase (HRP) solution is added to the microwells to form complexes with bound HA. Following another washing step, a chromogenic substrate of tetramethylbenzidine is added to develop a colored reaction. The intensity of the color is measured in optical density (O.D.) units with a spectrophotometer at 450nm. HA levels in specimen samples and control samples are determined against a reference curve prepared from the reagent blank (0 ng/mL) and the HA reference solutions provided with the kit (50, 100, 200, 500, 800 ng/mL).

The reagents supplied in this kit are for Research Use Only.

* 12 x 8-well microstrips coated with stabilized HABP with frame	1 Plate
* Reaction Buffer (blue solution)	57 mL
* HRP-Conjugated HABP (red solution)	13 mL
* Substrate (Tetramethylbenzidine)	13 mL
* Stop Solution (0.36N sulfuric acid)	15 mL
* Wash Buffer, PBS (33x concentrated). Must be diluted before use.	30 mL
* HA Reference Solution, 50 ng/mL	0.5 mL
* HA Reference Solution, 100 ng/mL	0.5 mL
* HA Reference Solution, 200 ng/mL	0.5 mL
* HA Reference Solution, 500 ng/mL	0.5 mL
* HA Reference Solution, 800 ng/mL	0.5 mL
* HA High Control (range on vial)	0.5 mL
* HA Moderate Control (range on vial)	0.5 mL
* HA Low Control (range on vial)	0.5 mL

Materials Required But Not Provided

- * Reagent grade water (approximately 1L) to prepare PBS wash solution, and to zero or blank the plate reader during the final assay step
- * Graduated cylinders
- * Precision pipettes capable of delivering between 5 and 1000 microliters, with appropriate tips
- * Miscellaneous glass or plastic ware appropriate for small volume handling
- * Flask, bottle or graduated cylinder, 1 liter
- * Wash bottles, preferably with the tip partially cut back to provide a wide stream, or an automated or semi-automated plate washing system
- * Multichannel pipettes capable of delivering to 8 wells simultaneously (strongly recommended)
- * Microdilution tubes and a 96-well Microdilution tube holder for sample dilutions
- * Plate reading spectrophotometer capable of reading absorbance at 450nm (with a 650nm reference if available)
- * Disposable gloves, powder-free recommended, safety glasses, and other appropriate protective garments
- * Biohazard infectious waste containers

Storage and Stability

All reagents should be stored at 2-8°C and should not be used beyond the expiration date on the label. Once opened, microtitration strips may be stored at 2-8°C until the expiration date on the label, provided that desiccated conditions are maintained. Unused strips should be returned to their original foil pouch along with the sachet of desiccant. Opened pouches should, be securely resealed by folding over the open end and securing it with adhesive tape.

The working strength Wash Buffer should not be stored for longer than 30 days at 2-8°C. If the working strength buffer becomes visibly cloudy or develops precipitate during the 30 days, do not use it. Do not add fresh buffer to old buffer. For maximum shelf life, it is recommended that concentrated wash buffer be freshly diluted before each assay.

Specimen Collection and Preparation

Serum, EDTA, or heparinized plasma is the preferred sample matrix. Blood should be collected by venipuncture. Serum or plasma should be separated from cells by centrifugation. If not tested immediately, the specimens should be stored at 2 – 8°C. If specimens are to be stored for more than 72 hours, they should be frozen at -20°C or below. Specimens containing visible particulate matter should be clarified by centrifugation before testing.

Warnings and Precautions

Research Use Only

The performance characteristics of this product have not been established.

Safety

- * Specimen samples to be evaluated with this test, like all blood derivatives, should be handled as potentially infectious material.
- * Do not pipette by mouth.
- * Do not smoke, eat, or drink in areas where specimens or kit reagents are handled.
- * Wear disposable gloves while handling kit reagents and wash hands thoroughly afterwards.
- * Always follow appropriate biohazard safety precautions.
- * The Substrate Solution and Stop Solution contain ingredients that can irritate the skin and cause eye damage. In case of contact with eyes, rinse immediately with plenty of water and seek medical advice. If swallowed, seek medical advice immediately.

Procedural

- * Allow patient samples and kit reagents to warm to room temperature (18-26°C). Mix well before using; avoid foaming. Return all unused samples and reagents to refrigerated storage (2-8°C) as soon as possible.
- * It is highly recommended that all samples, including reference solutions, and controls, should be assayed in duplicate wells.
- * Set up two wells (if running in duplicate) as reagent blanks. Reaction buffer only is used for the reagent blank to serve as a 0 ng/mL HA reference solution.
- * A single water blank well should be set up on each plate with each run. No sample or kit reagents are to be added to this well. Instead, add 200 µL reagent grade water to the well immediately prior to reading the plate in the spectrophotometer. The plate reader should be programmed to zero or blank against this water well.
- * Good washing technique is critical for optimal performance of the assay. Adequate washing is best accomplished by directing a forceful stream of wash solution from a plastic squeeze bottle with a wide tip into the bottom of the microwells. Wash solution in water blank well will not interfere with the procedure. An automated plate washing system can also be used.
- * Important: Failure to adequately remove residual wash solution can cause inconsistent color development of the substrate solution.
- * Use a multichannel pipette capable of delivering to 8 wells simultaneously when possible. This speeds the process and provides more uniform incubation and reaction times for all wells.
- * Carefully controlled timing of all steps is important. For all incubations, the start of the incubation period begins with the completion of sample or reagent addition.
- * Addition of all samples and reagents should be performed at the same rate and in the same sequence.
- * Incubation temperatures other than room temperature (18-26°C) may contribute to inaccurate results.
- * Avoid contaminating reagents when opening and removing aliquots from the primary vials.
- * Do not use Tween 20 or other detergents in this assay.
- * Do not use kit components beyond expiration date.
- * Do not use kit components from different kit lots.

Reagent Preparation

Wash Solution (PBS): Dilute 30 mL of 33x PBS Wash Concentrate to 1 liter with reagent grade water. Store unused PBS solution at 2-8°C. Discard if the solution shows signs of microbial or other contamination.

Assay Procedure

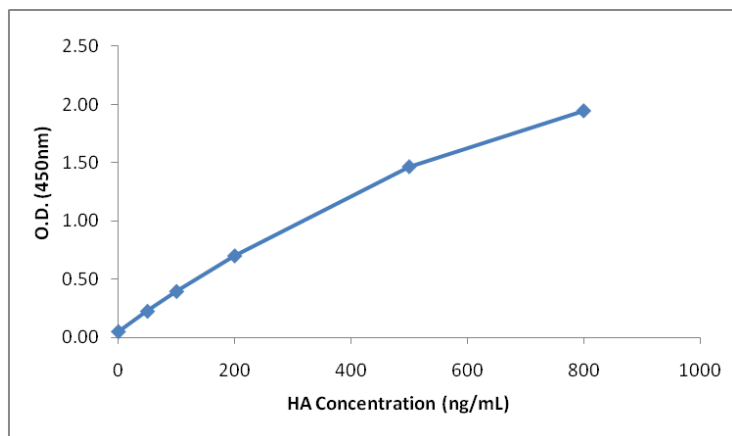
1. We highly suggest that the assay HA reference solutions, HA controls, and reagent blank are performed in duplicate for best results. Duplicate determinations are also recommended for samples. Reaction Buffer without specimen is used for the reagent blank, which represents the 0 ng/mL HA reference solution. The reagent blank will be treated the same as reference solutions, controls, or samples in subsequent assay steps. A water blank well should be included with each plate; it is to remain empty until 200µL of reagent grade water is added at the completion of the assay, immediately prior to reading the plate. The water blank well is to be used to zero the plate reader.
2. Remove any microwell strips that will not be used in the run from the frame, and reseal in the foil pouch.
3. Prepare HA reference solutions, HA controls and samples by making a 1:11 dilution with the Reaction Buffer (blue solution). For example, 30µL of HA reference solutions, HA controls or samples added to 300µL of Reaction Buffer will provide sufficient volume to test in duplicate. If testing in singlet, 15µL of the HA reference solutions, HA controls, or samples is added to 150µL of the Dilution Buffer.
4. Add 100µL of diluted HA reference solutions, HA controls, samples, and reaction buffer only (for reagent blank) to appropriate microwells. Leave the water blank well empty.
5. Incubate 60 minutes at room temperature (18-26°C).
6. After the incubation is complete, carefully invert microwells, and empty the contents into a suitable container. Do not allow samples to contaminate other microwells. Wash wells 4 times with working wash solution (PBS), filling wells completely. PBS in the water blank well will not interfere with the procedure. Invert microwells between each wash to empty fluid. Use a snapping motion of the wrist to shake the liquid from the wells. Tap and/or blot plate on absorbent paper to remove residual wash buffer. Do not allow wells to dry out between steps.
7. Add 100µL HRP-conjugated HAPB Solution (red solution) to all wells except the water blank.
8. Incubate for 30 minutes at room temperature.
9. After the incubation is complete, carefully invert microwells and empty conjugate solution. Wash 4 times with PBS and tap or blot as described in Step 6. Do not allow the wells to dry out.
10. Add 100µL Substrate Solution to each well (except the water blank well) and incubate for 30 minutes at room temperature. Blue color will develop in wells with positive samples.
11. Add 100µL Stopping Solution (0.36 N sulfuric acid) to each well (except the water blank well) to stop the enzyme reaction. Be sure to add stopping solution to wells in the same order and at the same rate as the substrate solution. Do not add stopping solution to the water blank well. Instead, add 200 µL of reagent grade water to the water blank well.
12. Blank or zero plate reader against the water blank well. Read the O.D. of each well at 450nm (650nm reference). Optical density (O.D.) of the wells should be measured within one hour after the addition of stopping solution.

Interpretation of Results

1. Calculate the mean O.D. values for duplicate wells (if run in duplicate) of HA reference solutions, HA controls, reagent blanks and samples.
2. Using either third-order polynomial regression (recommended), linear regression or hand plotting, calculate the best fit curve using the mean O.D.s of the 0 ng/mL (reagent blank), 50, 100, 200, 500, and 800 ng/mL reference solutions. A new curve must be plotted with each assay run. From this six point curve, calculate the resulting HA concentrations (ng/mL) in the HA controls and samples. See sample graph in next section.
3. Samples with HA concentrations greater than 800 ng/mL may be reported as "greater than 800 ng/mL" or they can be further diluted and re-assayed to obtain more accurate HA results. Results from the second assay for these samples must be multiplied by the dilution factor to obtain the final HA concentration.
4. Assure that all quality control parameters have been met (see Quality Control) before reporting test results

Example of a reference curve

EXAMPLE ONLY, DO NOT USE



Performance Characteristics

The performance characteristics of this assay have not been established; it is strictly a Research Use Only product, not for diagnostic or clinical Use. Each facility must establish its own performance range based upon their internal findings. Preliminary studies indicate that the HA normal range for human serum or plasma on this assay is 0-75 ng/mL. If using specimens other than human serum or plasma, we advise to run a known normal HA sample and a disease state HA sample with the unknown samples. These will serve as positive and negative controls to distinguish between normal samples and disease state samples. The initial specimen dilution scheme may need to be modified to accommodate a non-serum/plasma matrix. Feel free to contact us and we may be able to provide you with preliminary research data from various researchers that may assist in your own research.

Quality Control

1. The mean O.D. value of the Reagent Blank should be 100. Readings greater than 0.100 may indicate possible contamination of the One-component Substrate or other reagents.
2. The mean O.D. value of the 500 ng/mL HA reference solution should be 0.800 or greater.
3. Duplicate O.D.s should be within 20% of each other for samples with a mean O.D. reading of greater than 0.300.
4. The values obtained for the HA Controls should be within the ranges printed on each container label. Testing variables in each laboratory, including equipment and technique, may influence control recovery; each laboratory should consider establishing its own acceptable range for the HA Controls.
5. Each laboratory should periodically confirm the normal cut-off and prevalence values for samples.

Interference and Cross Reactivity

1. The following serum/plasma constituents were tested for interference in the HA assay and found to have no effect: free and conjugated bilirubin, hemoglobin, and plasma lipoprotein (chyle). Heparin has also been studied at concentrations from 1.0 ng/mL through 1.0 mg/mL and no interference/cross-reactivity was observed.
2. Cross-reactivity between HA and various other glycosaminoglycan compounds (chondroitin-4-sulfate, chondroitin-6-sulfate, dermatan sulfate, heparin sulfate and keratin sulfate) was evaluated. No reactivity was observed, demonstrating the specificity of HABP for HA.

3. Cross-reactivity between HA and IgM Rheumatoid Factor was evaluated. IgM Rheumatoid Factor levels measured by ELISA demonstrated no correlation with HA levels ($R = 0.145$).

Limitations of Use

For Research Use Only. The performance characteristics of this product have not been established.

Serum HA levels can be elevated during synovial inflammation and cartilage destruction as seen in rheumatoid arthritis (RA), due to increased production and passage into circulation. Elevated serum levels of HA have also been reported in some patients with more advanced or active osteoarthritis (OA), progressive systemic sclerosis (PSS) and systemic lupus erythematosus (SLE), and are believed to result from growth factor activity in connective tissue cells and synovial involvement.²⁶⁻²⁸

As reported in the literature,²⁶ our studies show that age has a positive effect on HA levels in healthy individuals although the effect was minimal. The rate of increase was shown to be approximately 0.5 ng/mL per year in healthy individuals. A diurnal variation in serum HA levels is described in another study,²⁹ however this was not confirmed with our data.

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Warranty

This product is warranted to perform as described in the labeling provided that: the product is stored and used as directed; used before the expiration dating; and adequate quality control is performed. No other warranty is implied, nor are we liable for any consequential damages arising out of the aforesaid warranty.

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