ENGLISH

HYALURONIC ACID (HA) TEST KIT

For *In Vitro* Diagnostic Use

This package insert is for informational use only. When performing the test, please refer to the package insert provided with each kit.

INTENDED USE

An enzyme-linked binding protein assay for the determination of hyaluronic acid (HA) in human serum or plasma. The measurement of HA may be used to assess the degree of liver fibrosis and cirrhosis in chronic liver disease.

SUMMARY AND EXPLANATION OF THE HYALURONIC ACID TEST

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 b-(1-4)-glucoronic acid and b-(1-3)-N-acetylglucosamine moieties. Each dimer is referred to as one unit and has a molecular weight of approximately 450 D. 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]

Serum HA levels can be elevated in various liver diseases characterized by liver fibrosis and cirrhosis, due to decreased hepatic removal and/or increased hepatic production of HA during liver inflammation.[8,9] Increased HA levels have shown a better correlation with the degree of histopathological damage to the liver than conventional liver function tests including ALT/GOT, alkaline phosphatase and bilirubin.[10,11] It has been proposed that the determination of serum HA levels may be useful in distinguishing cirrhotic from non-cirrhotic liver, for assessing the degree of liver fibrosis, and for monitoring liver function.[12-16] It has also been shown that HA levels reflect the extent of hepatic fibrosis in patients with chronic hepatitis C and may be useful in monitoring the response to interferon alpha treatment.[17-19] Similar correlation has been found in patients with alcoholic cirrhosis[20] and primary biliary cirrhosis.[10] HA levels have also been shown to be an early marker of liver damage from toxic agents such as ethanol, acetaminophen, and bacterial lipopolysaccharide, as pathological changes of the SEC in response to these agents precede pathological changes of the hepatocytes.[13,21]

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 human serum or plasma.

PRINCIPLE OF THE TEST

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 serum or plasma and HA reference solutions are incubated in HABP-coated microwells, allowing HA present in samples to react with the immobilized binding protein (HABP). After the removal of unbound serum 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 and hydrogen peroxide is added to develop a colored reaction. The intensity of the color is measured in optical density (O.D.) units with a spectrophotometer at 450 nm. HA levels in patient 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).

REAGENTS

Store at 2 - 8°C. Do Not Freeze.

Each HA 96-microwell Test Kit contains the following reagents (volumes may vary depending on kit size and configuration):

- 12 stabilized HABP-coated 8-well microwell strips with frame.
- 1 bottle (57 mL) Reaction Buffer (blue solution).
- 1 vial (0.5 mL) 50 ng/mL HA Reference Solution.
- 1 vial (0.5 mL) 100 ng/mL HA Reference Solution.
- 1 vial (0.5 mL) 200 ng/mL HA Reference Solution.
- 1 vial (0.5 mL) 500 ng/mL HA Reference Solution.
- 1 vial (0.5 mL) 800 ng/mL HA Reference Solution.
- 1 bottle (13 mL) HRP-conjugated HABP Solution (red solution).
- 1 bottle (13 mL) One-component Substrate Solution (contains 3,3',5,5'-tetramethylbenzidine and hydrogen peroxide, stabilized).
- 1 bottle (15 mL) Stopping Solution (0.36 N sulfuric acid).
- 1 bottle (30 mL) Wash Concentrate [33x Phosphate Buffered Saline (PBS)]; 30 mL reconstitutes to 1 liter of 0.01 M PBS, pH 7.35 ± 0.1.

WARNINGS AND PRECAUTIONS

For *In Vitro* Diagnostic Use

1. Patient serum or plasma samples to be evaluated with this test, like all human blood derivatives, should be handled as potentially infectious material.
2. Do not pipette by mouth.
3. Do not smoke, eat, or drink in areas where specimens or kit reagents are handled.
4. Wear disposable gloves while handling kit reagents and wash hands thoroughly afterwards.
5. Some components of this product contain 0.05% ProClin® as a preservative. ProClin® is a hazardous material in concentrated form. The low concentration used in this product (0.05%) is not considered to be hazardous.
6. One-component Substrate Solution can cause irritation to the eyes and skin. Absorption through the skin is possible. Use gloves when handling substrate and wash thoroughly after handling. Keep reagent away from ignition sources. Avoid contact with oxidizing agents.

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SPECIMEN COLLECTION AND PREPARATION

Serum or heparinized plasma are the preferred sample matrices. 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.

INSTRUCTIONS FOR USE

Materials Provided

Hyaluronic Acid Test Kit; see “Reagents” for a complete listing.

Materials Required but not Supplied

- 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 450 nm (with a 650 nm reference if available)
- Disposable gloves, powder-free recommended

Procedural Notes

1. 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.
2. All samples, including reference solutions, should be assayed in duplicate wells.
3. Set up two wells as reagent blanks. Reaction buffer (without serum) is used for the reagent blank to serve as a 0 ng/mL HA reference solution.
4. 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.
5. 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.
6. IMPORTANT: Failure to adequately remove residual wash solution can cause inconsistent color development of the substrate solution.
7. 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.
8. 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.
9. Addition of all samples and reagents should be performed at the same rate and in the same sequence.
10. Incubation temperatures other than room temperature (18 - 26°C) may contribute to inaccurate results.
11. Avoid contaminating reagents when opening and removing aliquots from the primary vials.
12. Do not use Tween 20 or other detergents in this assay.
13. Do not use kit components beyond expiration date.
14. 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. The pH of the final solution should be 7.35 ± 0.1. Store unused PBS solution at 2 - 8°C. Discard if the solution shows signs of microbial or other contamination.

Assay Procedure

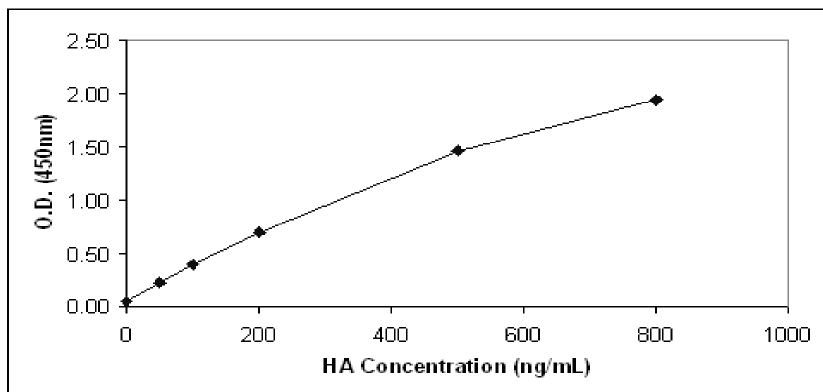
1. Assay HA reference solutions and reagent blank in duplicate. Duplicate determinations are also recommended for patient samples. Reaction Buffer without serum is used for the reagent blank, which represents the 0 ng/mL HA reference solution. The reagent blank will be treated the same as reference solutions or patient samples in subsequent assay steps. A water blank well should be included with each plate; it is to remain empty until 200 µL 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 and patient samples by adding 1 part of the solution or sample to 10 parts Reaction Buffer (blue solution). For example, 30 µL of sample added to 300 µL of Reaction Buffer will provide sufficient volume to test in duplicate.
4. Add 100 µL of diluted HA reference solutions, patient samples, and reaction buffer (for reagent blank) to appropriate microwells. Leave the water blank well empty.
5. Incubate 60 minutes at room temperature.
6. After the incubation is complete, carefully invert microwells, and dump 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. Pound and/or blot on absorbent paper to remove residual wash buffer. Do not allow wells to dry out between steps.
7. Add 100 µL HRP-conjugated HABP 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 dump conjugate solution. Wash 4 times with PBS and pound or blot as described in Step 6. Do not allow the wells to dry out.
10. Add 100 µL One-component Substrate Solution to each well (except the water blank well) and incubate for 30 minutes at room temperature. Add substrate solution to wells at a steady rate. Blue color will develop in wells with positive samples.
11. Add 100 µL 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 450 nm (650 nm reference). Optical density (O.D) of wells should be measured within one hour after the addition of stopping solution.

Results

1. Calculate the mean O.D. values for duplicate wells of HA reference solutions, reagent blanks and patient 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 patient samples.

Example of reference curve

EXAMPLE ONLY, DO NOT USE



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.

Quality Control

1. The mean O.D. value of the Reagent Blank should be less than 0.100. 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. Each laboratory should periodically confirm the normal cut-off and prevalence values for their population of patients.

NORMAL RANGE[24]

Serum samples from 100 healthy blood donors (Population A) were tested with three HA kit lots. The mean HA value of this population was determined to be 28.5 ng/mL with a standard deviation of 24.0 ng/mL. A normal cut-off of 75 ng/mL was established based on the 95th percentile of the normal population.

HA normal range = 0 - 75 ng/mL

EXPECTED VALUES

Clinical Specificity:

Healthy Individuals

Serum samples obtained from healthy individuals were tested on the HA Test Kit to determine the clinical specificity of the assay. A second population of 100 normal serum samples (Population B) and 180 serum samples obtained from healthy individuals were evaluated for HA concentration. Relative to the established normal range of $\leq$ 75 ng/mL, nine of the 280 serum samples were found to be positive, resulting in a overall clinical specificity of 96.8%.

Clinical Sensitivity

Liver Disease (Cirrhosis)

A total of 62 serum samples obtained from patients followed in a Hepatic Disease Clinic were tested with the HA Kit to determine the clinical sensitivity of the assay.[25] Forty-nine patients were diagnosed with cirrhosis (mostly alcohol related), and 13 patients were undergoing detoxification (detox) treatment for alcohol abuse with no clinical or laboratory evidence of liver disease. The percentage of samples with HA levels > 100 ng/mL for the two groups compared to the healthy controls tested at the same time are shown in the table below:

patients	n	Mean HA value	% positive
cirrhosis	49	371.6 ng/mL	38/49 = 77.6%
detox	13	51.4 ng/mL	2/13 = 15.4%
healthy	100	19.8 ng/mL	0/100 = 0%

The mean HA values for the cirrhosis and detox groups were found to be statistically different when compared to the healthy control group ($p < 0.001$).

PERFORMANCE CHARACTERISTICS[24]

Detection limit:

The minimum detectable HA concentration that this test kit is able to accurately measure has been established at 10 ng/mL.

Precision:

The HA assay was evaluated for intra-assay (16 replicates) and inter-assay value precision (3 assays, 16 replicates per assay) across multiple HA kit lots. The average coefficient of variation (CV%) obtained are summarized in the following table:

mean HA value of Sample	*Intra-assay CV%*	*Inter-assay CV%*
100 ng/mL	4.7 %	7.0 %
200 ng/mL	3.6 %	5.7 %
400 ng/mL	4.2 %	6.2 %

Recovery:

The recovery of the HA assay was determined by testing 11 samples of known HA concentration on three HA product lots. Two sets of dilutions were prepared and tested in duplicate. Percent (%) recovery was calculated for each lot by dividing the mean HA value obtained from testing by the known (expected) value. The results for all three lots are summarized in the table below:

	HA Lot #1		HA Lot #2		HA Lot #3	
Sample#	Expected value	Mean % recovery	Expected value	Mean % recovery	Expected value	Mean % recovery
1	600.0	102.1%	600.0	102.0%	600.0	97.7%
2	400.0	99.5%	400.0	100.3%	400.0	98.7%
3	292.2	106.4%	313.3	100.6%	314.9	101.0%
4	250.0	100.3%	250.0	99.2%	250.0	102.3%
5	211.3	107.5%	224.2	99.5%	224.6	102.3%
6	130.3	91.7%	135.1	97.4%	134.2	102.4%
7	100.0	101.3%	100.0	101.4%	100.0	99.9%
8	89.9	96.9%	90.6	95.7%	89.0	104.9%
9	68.2	101.0%	64.8	105.1%	62.7	122.6%
10	50.0	100.6%	50.0	101.1%	50.0	99.7%
11	25.0	95.1%	25.0	96.3%	25.0	96.4%
	Mean:	100.2%	**Mean:**	99.9%	**Mean:**	102.5%

Curve fit:

The curve fit for the HA assay was determined by calculating the coefficient of determination (r^2) of the curve obtained from the HA reference solutions (from 0 to 800 ng/mL) provided with the kit. Using third order polynomial regression, the r^2 was consistently > 0.999 across multiple HA lots.

INTERFERENCE AND CROSS-REACTIVITY[24]

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.

Cross-reactivity between HA and various other glycosaminoglycan compounds (chondroitin-4-sulfate, chondroitin-6-sulfate, dermatan sulfate, heparan sulfate and keratan sulfate) was evaluated. No reactivity was observed, demonstrating the specificity of HABP for HA.

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 THE TEST

The Hyaluronic Acid levels obtained with this assay can be used to assess the degree of fibrosis or cirrhosis in patients with chronic liver disease. Each physician must interpret these results in light of the patient's history, physical findings, and other diagnostic procedures.

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.

Warranty

This product is warranted to perform as described in this package insert. Corgenix, Inc. disclaims any implied warranty of merchantability or fitness for a particular use, and in no event shall Corgenix, Inc. be liable for consequential damage.

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