

HYALURONIC ACID (HA) TEST KIT

For *In Vitro* Diagnostic Use

INTENDED USE

An enzyme-linked binding protein assay for the determination of hyaluronic acid (HA) in human serum or plasma. HA is intended for use as a serologic marker of liver fibrosis to be used in conjunction with other histological, clinical and serological information for predicting advanced fibrosis (Metavir F3-4) in patients with chronic liver disease associated with non-alcoholic steatohepatitis (NASH).

SUMMARY AND EXPLANATION OF THE ASSAY

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 which 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 HA is mainly produced by fibroblasts and other specialized connective tissue cells. It plays a structural role in the connective tissue matrix (proteoglycan) as well as 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 high concentrations in synovial fluid 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/AST, 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 cirrhosis²⁰ 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}

NASH is a type of nonalcoholic fatty liver disease (NAFLD) with an estimated prevalence of 3 – 5% in the general population. It is defined as the presence of hepatic steatosis and inflammation with hepatocyte injury (ballooning) with or without fibrosis.³⁰ Clinical data suggests that elevated HA levels are seen in NASH patients with liver fibrosis.²⁴

PRINCIPLE OF THE TEST

The HA Test Kit measures HA levels and is performed as an enzyme-linked binding protein assay that uses a capture molecule known as the hyaluronic acid binding protein (HABP).^{22, 23, 25} HABP functions as a specific receptor for HA and *in vivo* links HA with the core-protein and other glycosaminoglycans to form proteoglycan aggregate complexes. In this assay, naturally occurring HABP is isolated by affinity purification from bovine nasal cartilage and coated to microwells to specifically capture HA. An enzyme-conjugated version of HABP is also used to measure the HA captured from the human serum or plasma by the HABP coated microwells.²⁸⁻²⁹ HABP is bound, blocked, and stabilized to the bottom and sides of the wells of a microtiter plate. Diluted patient serum or plasma samples are incubated in the wells, allowing any available HA to bind to the immobilized HABP. The plates are then rinsed of any nonbound serum or plasma molecules. Bound HA is quantitated using a second HABP molecule conjugated to an enzyme (horseradish peroxidase). Any unbound conjugated HABP is then rinsed away. Bound conjugated HABP is incubated with a substrate/chromophore system. The final color development is measured spectrophotometrically in optical density units (OD units). HA concentrations of patient serum or plasma are determined from a reference curve resulting from OD units of five HA reference samples of known HA concentration and 0 ng/mL (reagent blank).

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 HAP-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 HAP 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 0.01M PBS, pH 7.35 ± 0.1.
- 1 vial (0.5 mL) HA High Control (acceptable range printed on vial label)
- 1 vial (0.5 mL) HA Moderate Control (acceptable range printed on vial label)
- 1 vial (0.5 mL) HA Low Control (acceptable range printed on vial label)

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. Certain components are labeled with the following:
Irritating to eyes (R 36). Avoid contact with skin (S 24). Avoid contact with eyes (S 25). In case of contact with eyes, rinse immediately with plenty of water and seek medical advice (S 26). If swallowed, seek medical advice immediately and show this container or label (S 46).

Warning 

SPECIMEN COLLECTION AND PREPARATION

Serum or ethylenediaminetetraacetic acid (EDTA) 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
- 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 (20 - 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 and controls, 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. The plate reader should be programmed to blank or zero against air.
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. An automated microplate washing system may 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 (20 - 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.

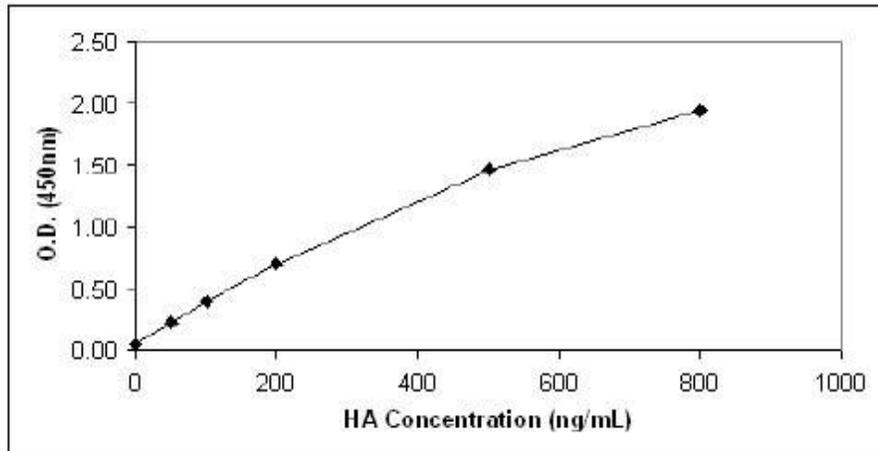
Assay Procedure

1. Assay HA reference solutions, HA controls, 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, controls, or patient samples in subsequent assay steps.
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 patient samples by adding 1 part of the solution or sample to 10 parts Reaction Buffer (blue solution). For example, 30 μ L of sample added to 300 μ L of Reaction Buffer will provide sufficient volume to test in duplicate.
4. Add 100 μ L of diluted HA reference solutions, HA controls, patient samples, and reaction buffer (for reagent blank) to appropriate microwells.
5. Incubate 60 minutes at room temperature (20 - 26°C).
6. After the incubation is complete, carefully invert microwells, and empty 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. 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 plates on absorbent paper to remove residual wash buffer. Do not allow wells to dry out between steps.
7. Add 100 μ L HRP-conjugated HABP Solution (red solution) to all wells.
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 100 μ L One-component Substrate Solution to each 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 μ L Stopping Solution (0.36 N sulfuric acid) to each 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.
12. Blank or zero the plate reader. Read the O.D. of each well at 450 nm (650 nm reference). Optical density (O.D) of wells should be measured within thirty minutes after the addition of stopping solution.

Results

1. Calculate the mean O.D. values for duplicate wells of HA reference solutions, HA controls, reagent blanks and patient samples.
2. Using third-order polynomial regression (recommended), 4-Parameter curve or hand plotting (point to point), 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 patient samples.

Example of reference curve
EXAMPLE ONLY, DO NOT USE



3. Samples with HA concentrations less than 20 ng/mL may be reported as “less than 20 ng/mL”. Samples with HA concentrations greater than 800 ng/mL may be reported as “greater than 800 ng/mL” or may be diluted up to 1:15 and reassayed to obtain more accurate 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 ≤ 0.1 . Readings greater than 0.1 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 0.8 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 0.3.
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 their population of patients. See performance characteristics.

PERFORMANCE CHARACTERISTICS

Expected values²⁴

HA levels were measured in 199 healthy adults across three HA kit lots using CLSI C28-A3c. The mean HA value of this population was determined to be 17.3 ng/mL with a standard deviation of 12.8 ng/mL. Using the non-parametric technique, the four highest and lowest values were eliminated. **The resulting normal range of ≤ 56 ng/mL** includes the remaining 95% of samples.

Data suggests further monitoring may be indicated in patients with HA levels between 56 – 150 ng/mL (see clinical performance data below). **The cut point for clinical decision making³⁰ is 150 ng/mL**, indicating the potential for advanced fibrosis. Liver biopsy may be indicated in individuals with advanced fibrosis³⁰.

Clinical Performance²⁴ Nonalcoholic Steatohepatitis (NASH)

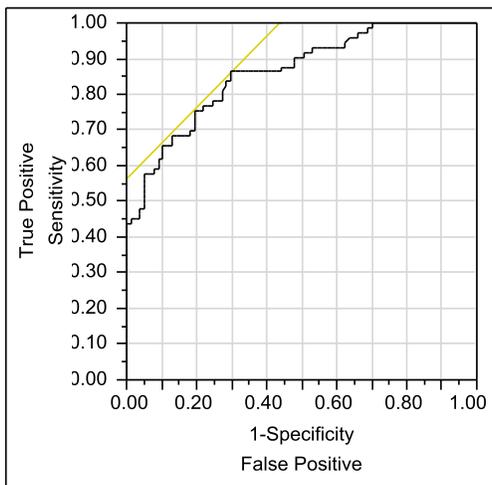
Serum samples from NASH patients were obtained from the National Institute of Health (NIH) – NIDDK division in two separate sets: training set, n = 340 and validation set, n = 300. The training study evaluated clinical performance of the HA assay against liver fibrosis and an appropriate cut point was established to detect advanced fibrosis in a NASH cohort. The data was then validated at the selected cut point. The percent positive (PPV) and negative (NPV) predictive values were calculated using the Receiver Operating Characteristic (ROC) curve method by comparing HA values (ng/mL) against the presence of advanced liver fibrosis (METAVIR F3-F4), assessed by histological examination of liver biopsy specimens. **The cut point for clinical decision making³⁰ is 150 ng/mL.** The HA assay displayed a PPV of 88.0% and NPV of 81.4% for identifying patients with advanced fibrosis at the cut point.

The table below outlines the test characteristics of the HA test kit at 150 ng/mL:

	Validation Set
Positive Predictive Value	88.0%
Negative Predictive Value	81.4%

The following figure shows the ROC (area under the curve) to assess advanced fibrosis (F3 and F4) in NASH patients:

Validation Set:



Area Under Curve = 0.86070

The area under the curve for the validation set is 0.86070, supporting clinical performance of the HA assay to assess advanced fibrosis in NASH patients.

Analytical Performance²⁴

Precision:

The precision was assessed as specified in CLSI EP5-A2 using high, moderate and low serum samples.

	Low	Moderate	High
Actual Value (ng/mL)	53.5	147.1	721.7
Repeatability Estimate (%CV)	13.5%	9.3%	8.1%
Estimate of Within-Lab Precision (%CV)	16.2%	13.0%	14.5%
Between Day Precision (%CV)	NC	5%	7%
Between Run, Within-Day (%CV)	11%	8%	10%

NC: Not calculable or <0

Reproducibility:

Reproducibility data was calculated according to CLSI EP5-A2 and ISO PDTR 22971 (Practical Guide to ISO 5725-2: 1994, TC 69/SC 6.)

The summary of data from all testing sites is summarized below:

	Low	Moderate	Low Positive	Moderate Positive
Actual Value (ng/mL)	32.3	122.0	372.4	613.1
Repeatability Estimate (%CV)	7.7%	5.9%	8.6%	6.9%
Repeatability Limit	7.0	20.2	90.1	117.8
Between Lab Estimate (%CV)	3.2%	3.2%	4.9%	3.6%
Reproducibility Estimate (%CV)	8.3%	5.0%	7.1%	5.8%
Reproducibility Limit	7.5	17.1	73.9	100.2

Linearity:

The linearity of the Hyaluronic Acid Test Kit was assessed as specified in CLSI EP6-A and has been demonstrated to be linear from 20 ng/mL to 1300 ng/mL within $\pm 10\%$ in this interval.

Limit of Blank (LoB) / Limit of Detection (LoD):

Based on 360 determinations (180 blank and 180 positive) using CLSI EP17-A, the LoD for the HA Test Kit is 11 ng/mL with a 95% probability of obtaining a positive response (a result that exceeds LoB) at this level and a 95% probability of obtaining a negative response on blank samples. A LoB of 7 ng/mL was used.

INTERFERENCE AND CROSS-REACTIVITY²⁴

The HA Test Kit was evaluated for interference according to CLSI EP7-A2. The following serum/plasma constituents, when added to serum with low, moderate and high HA levels. No interference was found at the levels below:

Hemoglobin	200 mg/mL	20,000 mg/dL	N/A
Conjugated Bilirubin	0.5 mg/mL	50 mg/dL	855 mmol/L
Free Bilirubin	0.5 mg/mL	50 mg/dL	855 mmol/L
Triglycerides	50 mg/mL	5000 mg/dL	56.5 mmol/L

Cross-reactivity between HA and IgM Rheumatoid Factor was evaluated. IgM Rheumatoid Factor, when added to serum with low, moderate and high levels of HA according to NCCLS EP7-A2, was found to cross-react at <2% at all levels with IgM Rheumatoid Factor.

Cross-reactivity between HA and a mixture of glycosaminoglycan compounds (chondroitin-6-sulfate, chondroitin-4-sulfate, chondroitin-2,6-sulfate and chondroitin-4-6-sulfate) was evaluated. This mixture when added to serum with low, moderate and high HA levels according to NCCLS EP7-A2, was found to cross-react at <2% at all levels with glycosaminoglycan compounds.

LIMITATIONS OF THE TEST

The Hyaluronic Acid levels obtained with this assay can be used to assess advanced fibrosis 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 studies show that age has an increased effect on HA levels in healthy individuals although the effect was minimal. The rate of increase was shown to be approximately 0.36 ng/mL per year in healthy individuals.

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.

For Technical or Customer Service in the United States, phone 1-800-729-5661. Outside the United States phone 303-457-4345, fax 303-457-4519, email: technicalsupport@corgenix.com or contact a Corgenix authorized distributor.

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