HYALURONIC ACID (HA) TEST KIT
For Research Use Only, Not for use in diagnostic procedures

INTENDED USE
An enzyme-linked binding protein assay for the determination of hyaluronic acid (HA) in human serum or plasma.

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)-glucoronic 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]

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).[8,9,10] 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.[11,12] HABP is bound, blocked, and stabilized to the bottom and sides of the wells of a microtiter plate. Diluted 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 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 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 0.01M 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 Research Use Only. Not for use in diagnostic procedures.

1. 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, ethylenediaminetetraacetic acid (EDTA) plasma 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
- 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 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.

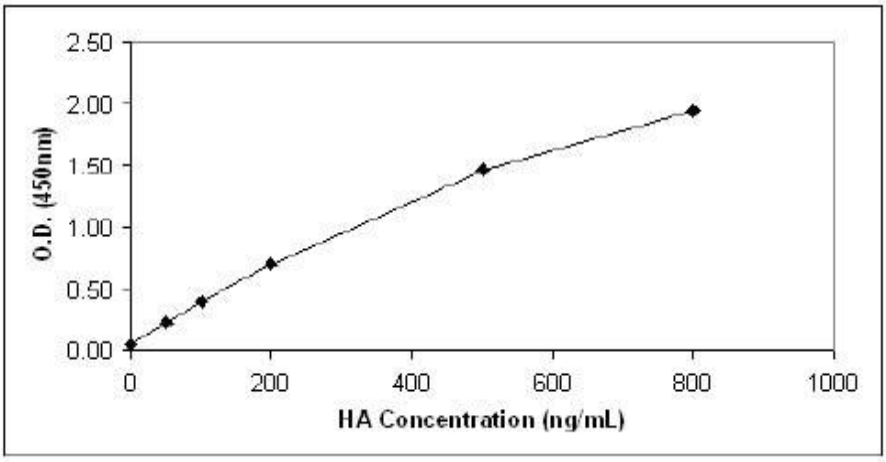
Assay Procedure

1. Assay HA reference solutions, HA controls, and reagent blank in duplicate. Duplicate determinations are also recommended for 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 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 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, HA controls, 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 µL 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 µL 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 µL 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 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 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 ≤ 0.150. Readings greater than 0.150 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.

PERFORMANCE CHARACTERISTICS

Analytical Performance[10]

Expected Values

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 cut-off of 75 ng/mL was established based on the 95th percentile of the population. [30]

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 ± 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-REACTIVITY[10]

The HA Test Kit was evaluated for interference according to CLSI EP7-A2. The following serum/plasma constituents were 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

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.[11,14,15]

As reported in the literature,[14] 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.

REFERENCES

1. Sundblad L. The chemistry and biology of compounds containing aminosugars. In *The Amino Sugars*. EA Balazs and REW Jeanloz Editors, Academic Press, N.Y. (1965).
2. Laurent TC. Structure of hyaluronic acid. *In Chemistry and Molecular Biology of the Intracellular Matrix*. EA Balazs Editor, Academic Press, N.Y. (1970).
3. Fraser JRE, Laurent TC, Pertoft H, et al. Plasma clearance, tissue distribution and metabolism of hyaluronic acid injected intravenously in the rabbit. *Biochem J* 200:415- 424 (1981).
4. Fraser JRE, Laurent TC, Engstrom-Laurent A, et al. Elimination of hyaluronic acid from the blood stream in the human. *Clin Exp Pharmacol Physiol* 11:17-25 (1984).
5. Balazs EA, Watson D, Duff IF, et al. Hyaluronic acid in synovial fluid. I. Molecular parameters of hyaluronic acid in normal and arthritic human fluids. *Arthritis Rheum* 10:357-376 (1967).
6. Engstrom-Laurent A, Hallgren R. Circulating hyaluronate in rheumatoid arthritis: relationship to inflammatory activity and the effect of corticosteroid therapy. *Ann Rheum Dis* 44:83-88 (1985).
7. Tamaki S, Ueno T, et al. Evaluation of hyaluronic acid binding ability of hepatic sinusoidal endothelial cells in rats with liver cirrhosis. *Gastroenterology* 111:1049-1057 (1996).
8. Chichibu K, Matsuura, T, Shichijo S, et al. Assay of serum hyaluronic acid in clinical application. *Clin Chimica Acta* 181:317-324 (1989).
9. Lindqvist U, Chichibu K, Delpech B, et al. Seven different assays of hyaluronan compared for clinical utility. *Clin Chemistry* 38:127-132 (1992).
10. Data on file (Corgenix Inc.)
11. Santos ME, Kondo T, Wieczorek A, Collier D, Lopez LR. Clinical utility of serum hyaluronic acid levels in liver disease. *Clinical Chemistry* 41:S71 (1995).
12. Sharif M, George E, Shepstone L, et al. Serum Hyaluronic Acid level as a predictor of disease progression in Osteoarthritis of the knee. *Arthritis Rheum* 38:760-767 (1995).
13. Engstrom-Laurent A, Feltelius N, Hallgren R, et al. Raised serum hyaluronate levels in Scleroderma: an effect of growth factor induced activation of connective tissue cells? *Ann Rheum Dis* 44:614-620 (1985).
14. Santos ME, Kondo T, Wieczorek A, Lopez LR. Increased serum hyaluronic acid levels in rheumatoid arthritis. *Arthritis Rheum* 37:S247; 525 (1994).
15. Engstrom-Laurent A, Hallgren R. Circulating hyaluronic acid levels vary with physical activity in healthy subjects and in rheumatoid arthritis patients: relationship to synovial mass and morning stiffness. *Arthritis Rheum* 1333-1338 (1987).
16. Engstrom-Laurent A, Loof L, Nyberg A, et al. Increased serum levels of hyaluronate in liver disease. *Hepatology* 5:638-642 (1985).
17. Freborug T, Delpech B, Bercoff E, et al. Serum hyaluronate in liver diseases: study by enzymoimmunological assay. *Hepatology* 6:392-395 (1986).
18. Nyberg A, Engstrom-Laurent A, Loof L. Serum hyaluronate in primary biliary cirrhosis: a biochemical marker for progressive liver damage. *Hepatology* 8:142- 146 (1988).
19. Wong VS, Hughes V, Trull A, et al. Serum hyaluronic acid is a useful marker of liver fibrosis in chronic hepatitis C virus infection. *J Viral Hepatitis* 5:187-192 (1998).
20. McHutchison JG, Blatt LM, Medina MD, et al. Measurement of serum hyaluronic acid in patients with chronic hepatitis C and its relationship to liver histology. *J Gastroenterol Hepatol* 15:945-951 (2000).
21. Bramley PN, Rathbone BJ, Forbes MA, et al. Serum hyaluronate as a marker of hepatic derangement in acute liver damage. *J Hepatology* 13:8-13 (1991).
22. Ueno T, Inuzuka S, Torimura T, et al. Serum hyaluronate reflects hepatic sinusoidal capillarization. *Gastroenterology* 105:475-481 (1993).
23. Plevris JN, Haydon GH, Simpson KJ, et al. Serum hyaluronan – a non-invasive test for diagnosing liver cirrhosis. *Eur J Gastro & Hepatology* 12:1121-1127 (2000).
24. Guéchot J, Serfaty L, et al. Prognostic value of serum hyaluronan in patients with compensated HCV cirrhosis. *J. Hepatology* 32:447-452 (2000).
25. Ueno T, Inuzuka S, Sata M, et al. Serum hyaulronate predicts response to interferon alpha therapy in patients with chronic hepatitis C. *Hepato-Gastroenterology* 42:522-527 (1995).
26. Yamada M, Fukuda Y, et al. Serum hyaluronic acid reflects the effect of interferon treatment on hepatic fibrosis in patients with chronic hepatitis C. *J Gastroenterol Hepatol* 11:646-651 (1996).
27. Hashimoto O, Ueno Y, et al. Long-term improvement of hepatic fibrosis in chronic hepatitis C treated with interferon- a. *Hepatology Res*. 10:200-216 (1998).
28. Tran A, Hastier P, Barjoan EM, et al. Non invasive prediction of severe fibrosis in patients with alcoholic liver disease. Gastroenterol Clin Biol 24:626-630 (2000).
29. Deaciuc IV, Spitzer JJ. Hepatic sinusoidal endothelial cell in alcoholemia and endotoxemia. *Alcoholism: Clin & Exp. Research* 20:607-614(1996).
30. Chalasani N, Younossi Z, Lavine JE, Diehl AM, Brunt EM, Cusi K, Charlton M, Sanayal AJ. The Diagnosis and Management of Non-Alcoholic Fatty Liver Disease: Practice Guideline by the American Association for the Study of Liver Diseases, American College of Gastoenterology, and the American Gastroenterological Association. *Hepatology* 2005-2023 (2012).

SYMBOL LEGEND

							
Manufacturer	Batch Code	Use by/ Expiry Date	Temperature Limitation	Warning	Caution	Catalog Number	Consult Instructions for Use/ Package Insert

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.

Manufactured and Distributed under license from Chugai Diagnostics Science Co., Ltd. by:

Corgenix, Inc.
11575 Main Street, Suite 400
Broomfield, Colorado 80020, USA
© 2021, Corgenix, Inc.

US Patent Number 5,019,498
Japanese Patent Number (JP) 2073041
European Patent Number (EP) 283779

10679 13
Effective: 2021-05-17

THIS PAGE INTENTIONALLY LEFT BLANK

THIS PAGE INTENTIONALLY LEFT BLANK