Quantikine® ELISA

Hyaluronan Immunoassay

Catalog Number DHYAL0

For the quantitative determination of Hyaluronan concentrations in cell culture supernates, tissue homogenates, serum, and plasma.

This package insert must be read in its entirety before using this product.
For research use only. Not for use in diagnostic procedures.

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INTRODUCTION

Hyaluronan (HA), also called hyaluronic acid or sodium hyaluronate, is a naturally occurring, highly hydrated linear polymer composed of the repeating disaccharide D-glucuronic acid-beta-1, 3-N-acetylglucosamine-beta-1,4 (1-6). It is a glycosaminoglycan (GAG) that is ubiquitously present in the extracellular matrix of all vertebrates. Unlike other GAGs such as chondroitin sulfate, dermatan sulfate, and heparan sulfate, HA lacks sulfate groups and is almost never covalently associated with other proteins (3, 6, 7). Mammalian HA is synthesized by one of three distinct multipass transmembrane HA synthases, HAS1, 2, and 3 (1-4, 7, 8). During HA synthesis, the growing polymer is translocated across the membrane to the extracellular surface as UDP-sugar substrates are added (7). The three HA synthases differ in their expression patterns and rates of synthesis, and may produce HA polymers with different chain lengths (8). In general, HA synthases produce high molecular weight HA (HMW, >500 kDa), while degradation is the main source of low molecular weight HA fragments (LMW, <500 kDa). HA turns over rapidly in mammals via the concerted actions of a family of hyaluronidases, and is cleared by the liver and kidneys (1, 3, 5, 7, 9).

HA is important in the tissues for hydration of the extracellular matrix, cell adhesion and migration (1, 6, 7). It interacts with a large number of hyaluronan-binding proteins, or hyaladherins. In the extracellular matrix, HA binds Link proteins such as aggrecan and HAPLN1 in cartilage, versican in smooth muscle, and brevican in the brain, and neurocan in nervous tissue (1, 6, 7, 10). In the circulation, it binds plasma binding proteins such as ITIH1/2, TSG-6, and HABP1/C1qBP (1, 3, 5, 7, 10). HA binds many cell surface receptors, such as the widely expressed CD44, RHAMM on vascular endothelia and other cells, LYVE-1 on lymphatic endothelia, and the endocytic receptor Stabilin-2 in endothelial sinuses such as in lymph nodes and liver (5-7, 10). Myeloid cell TLR2 and TLR4 also bind LMW HA fragments (11-13).

Cell signaling pathways activated through HA receptors are in part dependent on the size of HA (4, 6, 8, 10-13). While HMW HA is considered anti-angiogenic, anti-inflammatory, and immunosuppressive, LMW HA can be highly angiogenic and pro-inflammatory, and promotes dendritic cell maturation (1, 4-6, 12, 14). HA is mainly produced by fibroblasts, and contributes to fibroblast production of inflammatory mediators and migration to areas of fibrosis (1, 6). Its production by aortic smooth muscle and fibroblasts is increased in the aging arterial wall (6, 15). HA promotes mesodermal and cardiac differentiation from embryonic stem cells, and hematopoietic differentiation from hematopoietic stem cells *in vitro* (1). Plasma HMW HA mainly comes from the lymphatic circulation (7). It is elevated in angiopathy, liver fibrosis, autoimmune diseases such as advanced scleroderma, rheumatoid arthritis and lupus nephritis, end-stage renal failure, idiopathic pulmonary arterial hypertension, adult respiratory distress syndrome, and in diabetics with poor glucose control (1, 15, 16). HA is used therapeutically as a drug carrier, and injected into diseased joints to increase hydration and suppress inflammation (5, 17, 18). It protects tissues by scavenging reactive oxygen species; however, reactive oxygen species can also cause HA fragmentation, contributing to inflammation (1). HMW HA binding to CD44 promotes activity of regulatory T cells, which control inflammation (14, 19). HA promotes epithelial to mesenchymal transition, both during development and during oncogenic transformation (1, 7, 20). Cancer cells frequently produce hyaluronidases which degrade HA, promoting angiogenesis and inflammation (4, 20).

The Quantikine® Hyaluronan Immunoassay is a 4.5 hour solid-phase ELISA designed to measure ≥ 35 kDa Hyaluronan in human, mouse, rat, canine, and porcine cell culture supernates, serum, plasma and tissue homogenate samples. It contains *Streptococcus pyogenes*-derived Hyaluronan and NS0-expressed human Aggrecan. This immunoassay has been shown to accurately quantitate Hyaluronan.

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. Recombinant human (rh) Aggrecan has been pre-coated onto a microplate. Standards, controls, and samples are pipetted into the wells and any Hyaluronan present is bound by the immobilized rhAggrecan. After washing away any unbound substances, enzyme-linked rhAggrecan is added to the wells. Following a wash to remove any unbound rhAggrecan-enzyme reagent, a substrate solution is added to the wells and color develops in proportion to the amount of Hyaluronan bound in the initial step. The color development is stopped and the intensity of the color is measured.

LIMITATIONS OF THE PROCEDURE

- FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.
- The kit should not be used beyond the expiration date on the kit label.
- Do not mix or substitute reagents with those from other lots or sources.
- If samples generate values higher than the highest standard, further dilute the samples with calibrator diluent and repeat the assay.
- Any variation in diluent, operator, pipetting technique, washing technique, incubation time or temperature, and kit age can cause variation in binding.
- Variations in sample collection, processing, and storage may cause sample value differences.
- This assay is designed to eliminate interference by other factors present in biological samples. Until all factors have been tested in the Quantikine® Immunoassay, the possibility of interference cannot be excluded.

TECHNICAL HINTS

- When mixing or reconstituting protein solutions, always avoid foaming.
- To avoid cross-contamination, change pipette tips between additions of each standard level, between sample additions, and between reagent additions. Also, use separate reservoirs for each reagent.
- To ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary.
- Substrate Solution should remain colorless until added to the plate. Keep Substrate Solution protected from light. Substrate Solution should change from colorless to gradations of blue.
- Stop Solution should be added to the plate in the same order as the Substrate Solution. The color developed in the wells will turn from blue to yellow upon addition of the Stop Solution.

PRECAUTIONS

Hyaluronan is detectable in saliva. Take precautionary measures to prevent contamination of kit reagents while running this assay.

Some components in this kit contain a preservative which may cause an allergic skin reaction. Avoid breathing mist.

Color Reagent B may cause skin, eye, and respiratory irritation. Avoid breathing fumes.

Wear protective gloves, clothing, eye, and face protection. Wash hands thoroughly after handling. Refer to the MSDS on our website prior to use.

MATERIALS PROVIDED & STORAGE CONDITIONS

Store the unopened kit at 2-8 °C. Do not use past kit expiration date.

PART	PART #	DESCRIPTION	STORAGE OF OPENED/ RECONSTITUTED MATERIAL
Hyaluronan Microplate	894137	96 well polystyrene microplate (12 strips of 8 wells) coated with recombinant human Aggrecan.	Return unused wells to the foil pouch containing the desiccant pack. Reseal along entire edge of the zip-seal. May be stored for up to 1 month at 2-8 °C.*
Hyaluronan Conjugate	894138	12 mL of recombinant human Aggrecan conjugated to horseradish peroxidase with preservatives.	May be stored for up to 1 month at 2-8 °C.* ***Must be stored in its original container.***
Hyaluronan Standard	894139	Hyaluronan in a buffered protein base with preservatives; lyophilized. *Refer to the vial label for reconstitution volume.*	May be stored for up to 1 month at 2-8 °C.*
Assay Diluent RD1-14	895180	12 mL of a buffered protein base with preservatives. *May contain a precipitate. Mix well before and during use.*	
Calibrator Diluent RD5-18	895335	2 vials (21 mL/vial) of a buffered protein base with preservatives.	
Wash Buffer Concentrate	895003	21 mL of a 25-fold concentrated solution of a buffered surfactant with preservative. *May turn yellow over time.*	
Color Reagent A	895000	12 mL of stabilized hydrogen peroxide.	
Color Reagent B	895001	12 mL of stabilized chromogen (tetramethylbenzidine).	
Stop Solution	895174	23 mL of a diluted hydrochloric acid solution.	
Plate Sealers	N/A	4 adhesive strips.	

* Provided this is within the expiration date of the kit.

OTHER SUPPLIES REQUIRED

- Microplate reader capable of measuring absorbance at 450 nm, with the correction wavelength set at 540 nm or 570 nm.
- Pipettes and pipette tips.
- Deionized or distilled water.
- Squirt bottle, manifold dispenser, or automated microplate washer.
- 500 mL graduated cylinder.
- Horizontal orbital microplate shaker (0.12" orbit) capable of maintaining a speed of 500 ± 50 rpm.
- Test tubes for dilution of standards and samples.
- Hyaluronan Controls (optional; R&D Systems®, Catalog # QC104).

If using tissue homogenate samples, the following are also required:

- Cell Lysis Buffer 2 (R&D Systems®, Catalog # 895347).
- PBS

SAMPLE COLLECTION & STORAGE

The sample collection and storage conditions listed below are intended as general guidelines. Sample stability has not been evaluated.

Cell Culture Supernates - Remove particulates by centrifugation and assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Note: *Animal serum used in the preparation of cell culture media may contain high levels of Hyaluronan. For best results, do not use animal serum for growth of cell cultures when assaying for Hyaluronan production. If animal serum is used as a supplement in the media, precautions should be taken to prepare the appropriate control and run the control in the immunoassay to determine the baseline concentration of Hyaluronan.*

Tissue Homogenates - Prior to assay, tissues must be homogenized according to the directions in the Sample Values section.

Human Serum - Use a serum separator tube (SST) and allow samples to clot for 30 minutes at room temperature before centrifugation for 15 minutes at 1000 x g. Remove serum and assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Mouse Serum - Allow blood samples to clot for 2 hours at room temperature before centrifuging for 20 minutes at 2000 x g. Remove serum and assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Rat/Porcine/Canine Serum - Allow blood samples to clot for 2 hours at room temperature before centrifugation for 30 minutes at 1000 x g. Remove serum and assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Plasma - Collect plasma using EDTA or heparin as an anticoagulant. Centrifuge for 15 minutes at 1000 x g within 30 minutes of collection. Assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Note: *Citrate plasma has not been validated for use in this assay.*

SAMPLE PREPARATION

Canine, human, rat, and porcine serum and plasma samples require a 4-fold dilution. A suggested 4-fold dilution is 50 µL of sample + 150 µL of Calibrator Diluent RD5-18.

Mouse serum and plasma samples require a 40-fold dilution. A suggested 40-fold dilution is 10 µL of sample + 390 µL of Calibrator Diluent RD5-18.

REAGENT PREPARATION

Bring all reagents to room temperature before use.

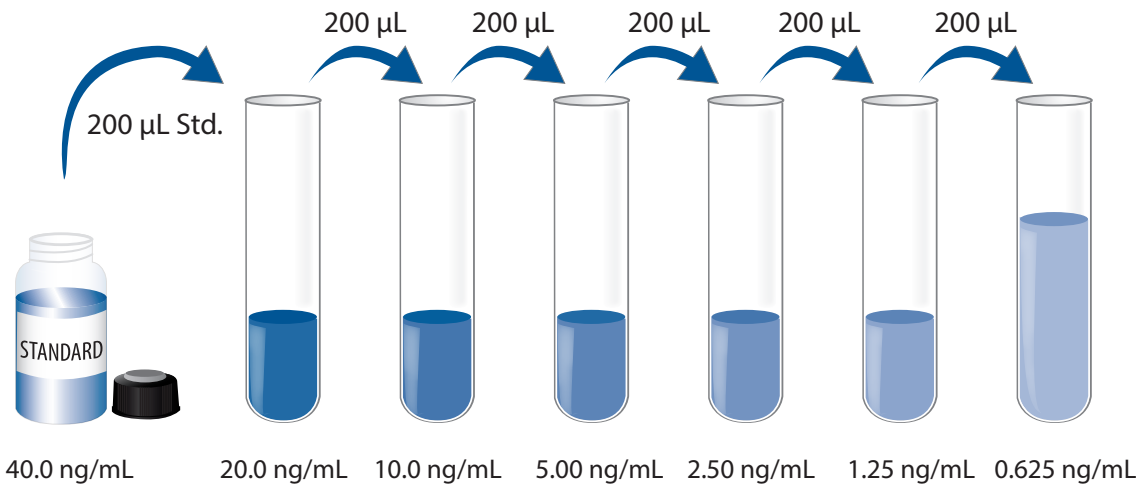
Note: *Hyaluronan is detectable in saliva. It is recommended that a face mask and gloves be used to protect kit reagents from contamination.*

Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Add 20 mL of Wash Buffer Concentrate to deionized or distilled water to prepare 500 mL of Wash Buffer.

Substrate Solution - Color Reagents A and B should be mixed together in equal volumes within 15 minutes of use. Protect from light. 100 µL of the resultant mixture is required per well.

Hyaluronan Standard - **Refer to the vial label for reconstitution volume.** Reconstitute the Hyaluronan Standard with Calibrator Diluent RD5-18. This reconstitution produces a stock solution of 40.0 ng/mL. Mix the standard to ensure complete reconstitution and allow the standard to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions.

Pipette 200 µL of Calibrator Diluent RD5-18 into each tube. Use the stock solution to produce a dilution series (below). Mix each tube thoroughly before the next transfer. The undiluted Hyaluronan Standard (40.0 ng/mL) serves as the high standard. Calibrator Diluent RD5-18 serves as the zero standard (0 ng/mL).



ASSAY PROCEDURE

Bring all reagents and samples to room temperature before use. It is recommended that all standards, samples, and controls be assayed in duplicate.

Note: *Hyaluronan is detectable in saliva. It is recommended that a face mask and gloves be used to protect kit reagents from contamination.*

1. Prepare all reagents, working standards, and samples as directed in the previous sections.
2. Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, and reseal.
3. Add 50 µL of Assay Diluent RD1-14 to each well. *May contain a precipitate. Mix well before and during use.*
4. Add 50 µL of standard, control, or sample* per well. Cover with the adhesive strip provided. Incubate for 2 hours at room temperature on a horizontal orbital microplate shaker (0.12" orbit) set at 500 ± 50 rpm. A plate layout is provided to record standards and samples assayed.
5. Aspirate each well and wash, repeating the process four times for a total of five washes. Wash by filling each well with Wash Buffer (400 µL) using a squirt bottle, manifold dispenser, or autowasher. Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.
6. Add 100 µL of Hyaluronan Conjugate to each well. Cover with a new adhesive strip. Incubate for 2 hours at room temperature on the shaker.
7. Repeat the aspiration/wash as in step 5.
8. Add 100 µL of Substrate Solution to each well. Incubate for 30 minutes at room temperature **on the benchtop. Protect from light.**
9. Add 100 µL of Stop Solution to each well. Gently tap the plate to ensure thorough mixing.
10. Determine the optical density of each well within 30 minutes, using a microplate reader set to 450 nm. If wavelength correction is available, set to 540 nm or 570 nm. If wavelength correction is not available, subtract readings at 540 nm or 570 nm from the readings at 450 nm. This subtraction will correct for optical imperfections in the plate. Readings made directly at 450 nm without correction may be higher and less accurate.

*Samples may require dilution. See Sample Preparation section.

CALCULATION OF RESULTS

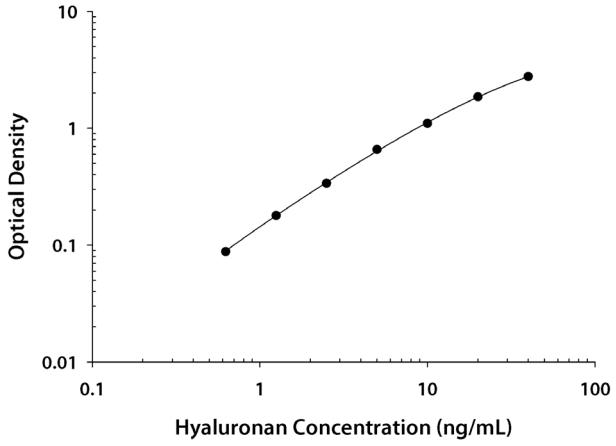
Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density (O.D.).

Create a standard curve by reducing the data using computer software capable of generating a four parameter logistic (4-PL) curve-fit. As an alternative, construct a standard curve by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis and draw a best fit curve through the points on the graph. The data may be linearized by plotting the log of the Hyaluronan concentrations versus the log of the O.D. and the best fit line can be determined by regression analysis. This procedure will produce an adequate but less precise fit of the data.

If samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

TYPICAL DATA

This standard curve is provided for demonstration only. A standard curve should be generated for each set of samples assayed.



(ng/mL)	O.D.	Average	Corrected
0	0.035 0.036	0.036	—
0.625	0.122 0.126	0.124	0.088
1.25	0.213 0.216	0.215	0.179
2.50	0.371 0.376	0.374	0.338
5.00	0.681 0.702	0.692	0.657
10.0	1.102 1.169	1.136	1.100
20.0	1.863 1.923	1.893	1.857
40.0	2.788 2.817	2.803	2.767

PRECISION

Intra-assay Precision (Precision within an assay)
Three samples of known concentration were tested twenty times on one plate to assess intra-assay precision.

Inter-assay Precision (Precision between assays)
Three samples of known concentration were tested in twenty separate assays to assess inter-assay precision. Assays were performed by at least three technicians using two lots of components.

	Intra-Assay Precision			Inter-Assay Precision		
Sample	**1**	**2**	**3**	**1**	**2**	**3**
n	20	20	20	20	20	20
Mean (ng/mL)	1.53	4.81	14.3	1.68	4.68	14.98
Standard deviation	0.11	0.26	0.52	0.08	0.23	0.72
CV (%)	7.2	5.4	3.6	4.8	4.9	4.8

RECOVERY

The recovery of Hyaluronan spiked to levels throughout the range of the assay in various matrices was evaluated.

Sample Type	Average % Recovery	Range
Cell culture media (n=2)	104	96-116%
Serum* (n=10)	98	81-119%
EDTA plasma* (n=10)	97	83-106%
Heparin plasma* (n=10)	99	80-124%

*Two samples each from human, mouse, rat, canine, and porcine were evaluated and no significant difference in recovery was observed.

SENSITIVITY

Sixty-four assays were evaluated and the minimum detectable dose (MDD) of Hyaluronan ranged from 0.027-0.200 ng/mL. The mean MDD was 0.068 ng/mL.

The MDD was determined by adding two standard deviations to the mean O.D. value of twenty zero standard replicates and calculating the corresponding concentration.

CALIBRATION

This immunoassay is calibrated against *Streptococcus pyogenes*-derived Hyaluronan with a molecular weight range of 75-350 kDa (mean=132 kDa).

LINEARITY

To assess the linearity of the assay, samples containing high concentrations of Hyaluronan were serially diluted with calibrator diluent to produce samples with values within the dynamic range of the assay. Samples were diluted prior to assay.

Human		Cell culture supernates (n=4)	Serum (n=4)	EDTA plasma (n=4)	Heparin plasma (n=4)
1:2	Average % of Expected	99	104	101	96
	Range (%)	95-100	100-107	91-111	93-102
1:4	Average % of Expected	98	106	102	94
	Range (%)	97-101	100-111	92-115	90-107
1:8	Average % of Expected	102	105	99	100
	Range (%)	99-107	99-114	90-114	90-114
1:16	Average % of Expected	109	106	105	103
	Range (%)	103-113	94-114	95-119	93-123

Mouse		Cell culture supernates (n=4)	Tissue homogenates (n=4)	Serum (n=4)	EDTA plasma (n=4)	Heparin plasma (n=4)
1:2	Average % of Expected	97	99	103	99	100
	Range (%)	95-101	96-101	97-110	94-105	96-104
1:4	Average % of Expected	95	94	99	101	99
	Range (%)	89-103	82-101	95-104	94-108	96-108
1:8	Average % of Expected	98	99	100	105	100
	Range (%)	88-112	82-106	92-109	94-116	92-113
1:16	Average % of Expected	101	98	106	107	103
	Range (%)	90-120	86-105	96-112	99-114	93-113

Rat		Cell culture supernates (n=4)	Serum (n=4)	EDTA plasma (n=4)	Heparin plasma (n=4)
1:2	Average % of Expected	97	112	102	101
	Range (%)	94-100	108-117	94-107	98-104
1:4	Average % of Expected	93	111	106	102
	Range (%)	89-96	107-116	97-111	99-106
1:8	Average % of Expected	94	112	108	104
	Range (%)	90-96	109-114	95-117	99-110
1:16	Average % of Expected	98	111	110	109
	Range (%)	96-103	108-115	98-120	104-114

LINEARITY *CONTINUED*

Porcine		Cell culture supernates (n=3)	Serum (n=4)	EDTA plasma (n=4)	Heparin plasma (n=4)
1:2	Average % of Expected	95	102	102	100
	Range (%)	92-100	99-106	97-109	96-109
1:4	Average % of Expected	95	102	101	99
	Range (%)	91-98	99-105	94-107	87-111
1:8	Average % of Expected	94	100	99	106
	Range (%)	89-100	95-102	91-105	94-118
1:16	Average % of Expected	88	102	101	106
	Range (%)	74-97	96-109	92-109	101-108

Canine		Cell culture supernates (n=4)	Serum (n=4)	EDTA plasma (n=4)	Heparin plasma (n=4)
1:2	Average % of Expected	102	94	98	100
	Range (%)	99-106	90-100	92-104	96-103
1:4	Average % of Expected	103	89	98	93
	Range (%)	94-111	85-94	89-106	87-101
1:8	Average % of Expected	104	87	103	94
	Range (%)	97-110	80-93	92-114	87-99
1:16	Average % of Expected	105	87	105	90
	Range (%)	94-113	83-90	94-116	87-120

SAMPLE VALUES

Serum/Plasma - Samples were evaluated for the presence of Hyaluronan in this assay.

Species	Serum (ng/mL)			EDTA plasma (ng/mL)			Heparin plasma (ng/mL)		
	Mean	Range	SD	Mean	Range	SD	Mean	Range	SD
Human (n=14)	28	5.12-92	25	29	5.12-83	24	25	4.06-67	21
Mouse (n=23)	520	195-887	209	457	132-893	241	462	140-704	152
Rat (n=20)	88	24.5-136	34	110	76.7-148	20	99	58.1-154	24
Porcine (n=15, 17, 5 respectively)	133	62.4-200	42	147	55.5-388	86	76	37-128	44
Canine (n=20, 15, 20 respectively)	37	7.8-106	29	21	7.4-64	16	38	5.4-101	29

SD=Standard Deviation

Cell Culture Supernates:

COLO 205 human colorectal adenocarcinoma cells (3 x 10^4 cells/mL) were cultured in RPMI supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin sulfate for 4 days. An aliquot of the culture supernate was removed, assayed for Hyaluronan, and measured 294 ng/mL.

A-72 canine fibroma cells (3 x 10^5 cells/mL) were cultured in L-15 media supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin sulfate for 3 days. An aliquot of the culture supernate was removed, assayed for Hyaluronan, and measured 355 ng/mL.

PK-15 porcine kidney epithelial cells (0.5 x 10^5 cells/mL) were cultured in MEM NEAA supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin sulfate for 3 days. An aliquot of the culture supernate was removed, assayed for Hyaluronan, and measured 572 ng/mL.

Kidneys from mice and rats were removed, rinsed in 1X PBS, and kept on ice in 1X PBS. Organs were then homogenized using a tissue homogenizer and seeded into media containing RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin sulfate. Cells were stimulated with 1.0 µg/mL lipopolysaccharide. Aliquots of the cell culture supernates were removed, assayed for Hyaluronan, and measured 134 ng/mL and 282 ng/mL respectively.

Tissue Homogenates - Kidneys from mice were rinsed with PBS and homogenized with a tissue homogenizer in PBS. An equal volume of Cell Lysis Buffer 2 was added and tissues were lysed at room temperature for 30 minutes with gentle agitation. Debris was then removed by centrifugation. An aliquot of the homogenate was removed, assayed for Hyaluronan, and measured 3754 ng/mL.

SPECIFICITY

The factors listed below were prepared at 400 ng/mL in calibrator diluent and assayed for cross-reactivity. Preparations of the following factors at 400 ng/mL in a mid-range human Hyaluronan control were assayed for interference. No significant cross-reactivity or interference was observed.

Recombinant human:

Aggrecan (aa 20-675)
Aggrecan (aa 1346-2204)
CD44H
CD44v2
CD44v3-10
HABP1/C1qBP
Layilin
LYVE-1
Stabilin-1
Stabilin-2
Versican

Recombinant mouse:

HABP1/C1qBP
Layilin
LYVE-1

Recombinant rat:

CD44 (aa 1-223)
CD44 (aa 22-271)

Recombinant porcine:

CD44

One bovine serum sample and one goat serum sample were assayed and measured 51.1 ng/mL and 267 ng/mL, respectively.

Recombinant human Brevican and recombinant mouse Brevican cross-react at approximately 0.6% and 0.3%, respectively.

Recombinant human HAPLN1 cross-reacts at approximately 1.7%.

Recombinant mouse CD44 cross-reacts at approximately 0.2%.

Recombinant mouse Neurocan cross-reacts at approximately 0.3%.

Recombinant human Neurocan interferes at concentrations > 50 ng/mL.

Recombinant human TSG-6 and recombinant mouse TSG-6 interfere at concentrations > 12 ng/mL and 25 ng/mL, respectively.

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PLATE LAYOUT

Use this plate layout to record standards and samples assayed.