PRODUCT INFORMATION & MANUAL

Hyaluronan Valukine™ ELISA

VAL152

For the quantitative determination of natural and recombinant Hyaluronan concentrations

For research use only.
Not for diagnostic or therapeutic procedures.

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Please refer to the kit label for expiry date.
Novus kits are guaranteed for 3 months from date of receipt

Version202107.1

TABLE OF CONTENTS

I. BACKGROUND..... 2

II. OVERVIEW..... 4

III. ADVANTAGES..... 5

IV. EXPERIMENT..... 7

V. KIT COMPONENTS AND STORAGE..... 8

VI. PREPARATION..... 11

VII. ASSAY PROCEDURE..... 14

VIII. REFERENCES..... 16

I. BACKGROUND

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).

II. OVERVIEW

A. PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. An antibody specific for Hyaluronan has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any Hyaluronan present is bound by the immobilized antibody. After washing away any unbound substances, a biotinylated detection antibody specific for Hyaluronan are pipetted into the wells. After washing away any unbound substances, streptavidin-HRP are pipetted into the wells. Following a wash to remove any unbound 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.

B. LIMITATIONS OF THE PROCEDURE

- **FOR RESEARCH USE ONLY NOT FOR USE IN DIAGNOSTIC PROCEDURES.**
- This kit is suitable for cell culture supernate and serum.
- 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, dilute the samples with Reagent Diluent and repeat the assay.
- Any variation in operator, pipetting technique, washing technique, incubation time or temperature, and kit age can cause variation in binding.

III. ADVANTAGES

A. PRECISION

Intra-assay Precision (Precision within an assay)

Three samples were tested twenty times on one plate to assess intra-assay precision.

Inter-assay Precision (Precision between assays)

Three samples were tested in twenty-eight separate assays to assess inter-assay precision.

	Intra-assay Precision			**Inter-assay Precision**		
Sample	1	2	3	1	2	3
Mean (ng/mL)	1.2	4.3	17.9	1.2	4.5	18.4
Standard Deviation	0.1	0.3	1.1	0.1	0.4	1.5
CV%	7.3	6.1	6.2	7.2	8.0	8.1

B. RECOVERY

The recovery of Hyaluronan spiked to different levels throughout the range of the assay in cell culture media was evaluated. The recovery ranged from 84-106.9% with an average of 95.0%.

The recovery of Hyaluronan spiked to different levels throughout the range of the assay in human serum was evaluated. The recovery ranged from 88.1-118.8% with an average of 102.7%.

C. SENSITIVITY

The minimum detectable dose (MDD) of Hyaluronan is typically less than 0.081 ng/mL.

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

D. CALIBRATION

This immunoassay is calibrated against a highly purified form of Hyaluronan (Catalog #GLR004) from R&D Systems®.

E. LINEARITY

To assess the linearity of the assay, different samples were containing or spiked with high concentrations of Hyaluronan and diluted with Reagent Diluent (1×) to produce samples with values within the dynamic range of the assay.

Dilution	Average % of Expected	Range (%)
1:2	100.1	90.3-110.3
1:4	105.2	86.1-114.9
1:8	107.2	106.2-120.9
1:16	100.8	87.9-115.1

F. SAMPLE VALUES

Cell Culture Supernates - TF-1 human blood leukemia cells (1×10^5 cells/mL) and SNU-1 gastric cancer cells (2×10^5 cells/mL) were cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin sulfate for 3 days. MCF-7 human breast cancer cells (4×10^5 cells/mL) were cultured in DMEM 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 cell culture supernate was removed, assayed for human Hyaluronan, and measured ranged from 35.8 to 44.4 ng/mL with an average of 38.8 ng/mL.

Serum - Six human serum samples were evaluated for the presence of Hyaluronan in this assay. All samples measured ranged from 44-126 ng/mL with an average of 77 ng/mL.

G. SPECIFICITY

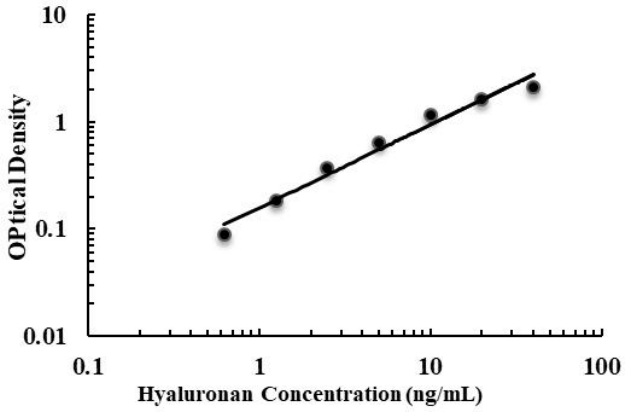
Billirubin, Hemoglobin, and Heparin were assayed at 10, 50, and 1000 µg/mL, respectively, and exhibited no cross-reactivity or interference in this assay.

The low molecular weight (15-40 kDa), medium molecular weight (75-350 kDa), and high molecular weight (>950 kDa) forms of Hyaluronan are all detected in this assay.

IV. EXPERIMENT

EXAMPLE STANDARD

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



ng/mL	OD	Average	Corrected
0	0.052 0.052	0.052	-
0.625	0.136 0.145	0.141	0.089
1.25	0.235 0.236	0.236	0.184
2.5	0.411 0.426	0.419	0.367
5	0.651 0.733	0.692	0.640
10	1.111 1.283	1.197	1.145
20	1.665 1.705	1.685	1.633
40	2.163 2.168	2.166	2.114

V. KIT COMPONENTS AND STORAGE

A. MATERIALS PROVIDED

Parts	Description	Size
Hyaluronan Microplate	96 well polystyrene microplate (12 strips of 8 wells) coated with recombinant human Aggrecan.	1 plate
Hyaluronan Standard	Hyaluronan in a buffered protein base; lyophilized. Refer to the vial label for reconstitution volume.	2 vials
Hyaluronan Detection Antibody	Biotinylated recombinant human Aggrecan lyophilized. Refer to the vial label for reconstitution volume.	1 vial
PBS (10×)	A 10× concentrated PBS	1 vial
Streptavidin-HRP B (40×)	40× Streptavidin conjugated to horseradish peroxidase.	1 vial
Reagent Diluent (5×)	A 5× concentrated buffered protein base used to dilute Standard, Samples, Detection Antibody and HRP.	1 vial
Wash Buffer Concentrate (25×)	A 25× concentrated solution of buffered surfactant with preservatives.	1 vial
Color Reagent A	Stabilized hydrogen peroxide.	1 vial
Color Reagent B	Stabilized chromogen (tetramethylbenzidine).	1 vial
Stop Solution	2 N sulfuric acid.	1 vial
Plate Sealers	Adhesive strip.	3 strips

B. STORAGE

<table>
<tr><td>Unopened Kit</td><td colspan="2">Store at 2-8 °C. Do not use past kit expiration date.</td></tr>
<tr><td rowspan="9">Opened/ Reconstituted Reagents</td><td>Streptavidin-HRP B</td><td rowspan="6">May be stored for up to 1 month at 2-8 °C.*</td></tr>
<tr><td>Diluted Wash Solution</td></tr>
<tr><td>Unmixed Color Reagent A</td></tr>
<tr><td>Unmixed Color Reagent B</td></tr>
<tr><td>PBS (10×)</td></tr>
<tr><td>Stop Solution</td></tr>
<tr><td>Standard</td><td>Prepare fresh for each assay.</td></tr>
<tr><td>Detection Antibody</td><td>Aliquot and store for up to 1 month at -20 °C in a manual defrost freezer. *</td></tr>
<tr><td>Reagent Diluent (5×)</td><td>May be stored for up to 1 month at 2-8 °C.*
Use and discard diluted Reagent Diluent (1×). Prepare fresh for each assay.</td></tr>
<tr><td>Microplate Wells</td><td>Return unused wells to the foil pouch containing the desiccant pack, reseal along entire edge of zip-seal. May be stored for up to 1 month at 2-8 °C.*</td></tr>
</table>

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

C. 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.
- An incubator which can provide stable incubation conditions up to 25°C ±0.5°C.

D. PRECAUTION

- 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.
- The Stop Solution provided with this kit is an acid solution. Wear protective gloves, clothing, eye, and face protection. Wash hands thoroughly after handling.

VI. PREPARATION

A. SAMPLE COLLECTION AND STORAGE

Cell Culture Supernates - Remove particulates by centrifugation and assay immediately or aliquot and store samples at ≤ -20°C. Avoid repeated freeze-thaw cycles. Samples may require dilution with Reagent Diluent (1×).

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.

B. SAMPLE PREPARATION

Cell Culture Supernates samples require a 10-fold dilution. A suggested 10-fold dilution is 20 µL of sample + 180 µL of Reagent Diluent (1×).

Serum samples require a 20-fold dilution. A suggested 20-fold dilution is 10 µL of sample + 190 µL of Reagent Diluent (1×).

C. REAGENT PREPARATION

Note: *Bring all reagents to room temperature before use.*

Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Dilute 20 mL of Wash Buffer Concentrate (25×) into deionized or distilled water to prepare 500 mL of Wash Buffer.

PBS (1×)- Use deionized or distilled water to prepare PBS (1×).

Reagent Diluent (1×) - Dilute Reagent Diluent (5×) with PBS (1×) to prepare Reagent Diluent (1×).

Detection Antibody- Reconstitution Volume refer to vial label with Reagent Diluent (1×). Aliquot and store if needed. Dilute stock solution in Reagent Diluent (1×) to the working concentration of 400 ng/mL. Prepare at least 15 minutes prior to use.

Streptavidin-HRP B (1×) **-** Dilute to the working concentration specified on the vial label using Reagent Diluent (1×).

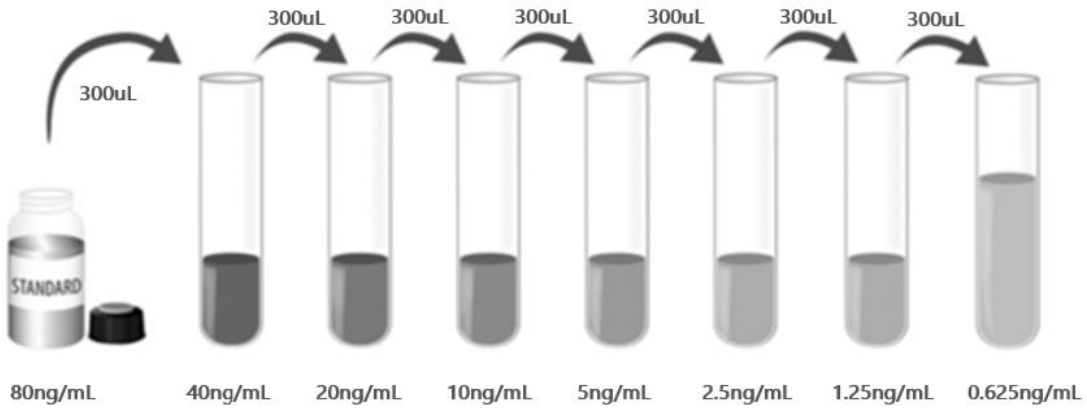
Substrate Solution - Color Reagent A and B should be mixed together in equal

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 the reconstitution volume*. This reconstitution produces a stock solution of 80 ng/mL. Allow the standard to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions.

*if you have any question, please seek help from our Technical Support.

Pipette 300 µL of the appropriate Reagent Diluent (1×) into each tube. Use the stock solution to produce a dilution series (below). Mix each tube thoroughly before the next transfer. The 40 ng/mL standard serves as the high standard. The Reagent Diluent (1×) serves as the zero standard (0 ng/mL).



D. 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.
- It is recommended that the samples be pipetted within 15 minutes.
- 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. Wells that are green in color indicate that the Stop Solution has not mixed thoroughly with the Substrate Solution.

VII.ASSAY PROCEDURE

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

1. Prepare all reagents and working standards 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 100 µL of standard, or prepared sample per well. Cover with the adhesive strip provided. Incubate for 2 hours **in an incubator at 25°C.** A plate layout is provided for a record of standards and samples assayed. (Samples may require dilution. See Sample Preparation section.)
4. Aspirate each well and wash, repeating the process three times for a total of four washes. Wash by filling each well with Wash Solution (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.
5. Add 100 µL of the Detection Antibody diluted in Reagent Diluent, to each well. Cover with a new adhesive strip and incubate 2 hours **in an incubator at 25°C.**
6. Repeat the aspiration/wash as in step 4.
7. Add 100 µL of the working dilution of Streptavidin-HRP B to each well. Cover the plate and incubate for 20 minutes **in an incubator at 25°C**. **Avoid placing the plate in direct light.**
8. Repeat the aspiration/wash as in step 4.
9. Add 100 µL of Substrate Solution to each well. Incubate for 20 minutes **in an incubator at 25°C. Avoid placing the plate in direct light.**
10. Add 50 µL of Stop Solution to each well. Gently tap the plate to ensure thorough mixing.
11. Determine the optical density of each well immediately, 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.

12. 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.

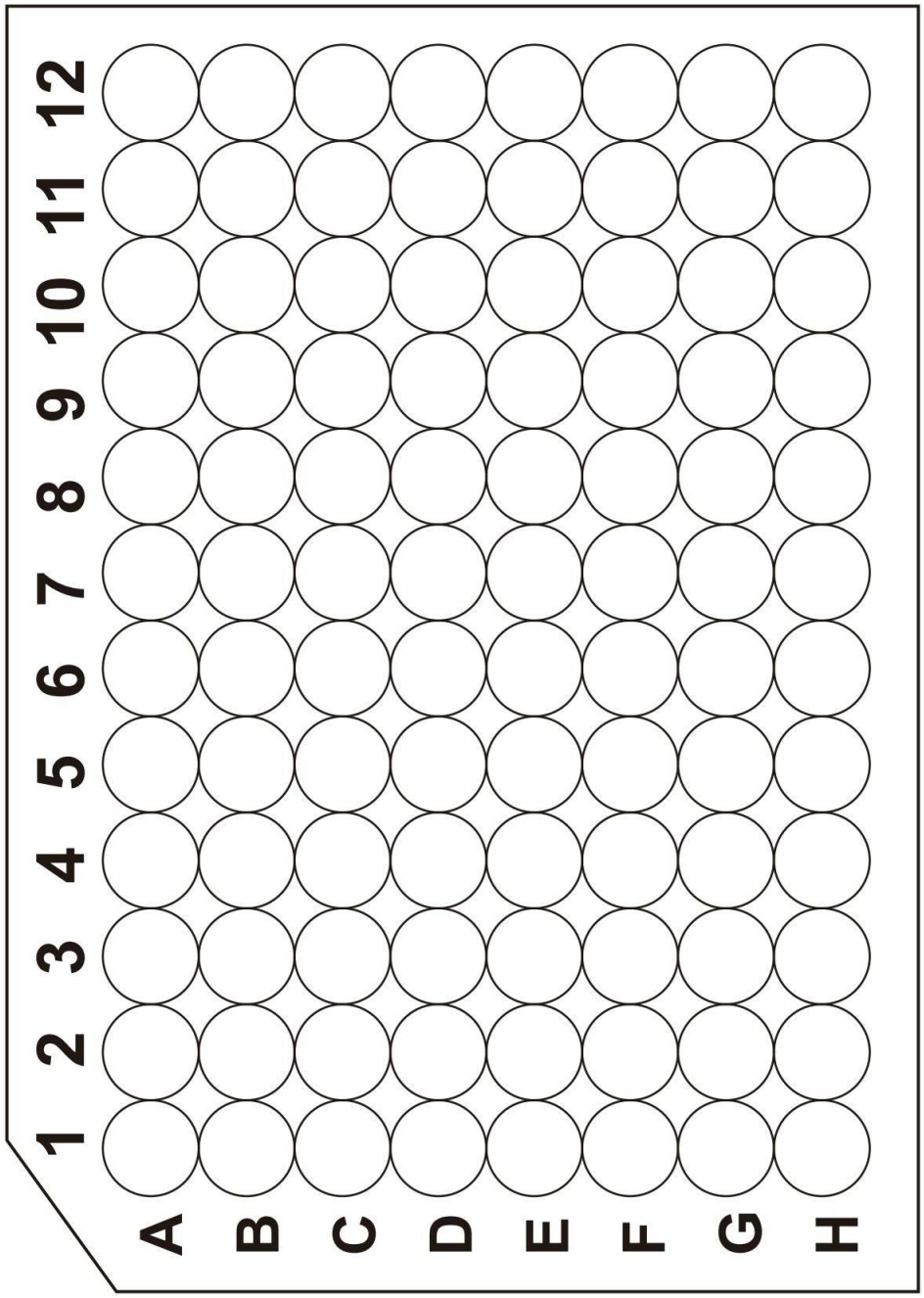
If samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

VIII. REFERENCES

1. Noble, P.W. et al. (2011) Physiol. Rev. 91:221.
2. Weigel, P.H. et al. (1997) J. Biol. Chem. 272:13997.
3. Bost, F. et al. (1998) Eur. J. Biochem. 252:339.
4. Stern, R. (2004) Eur. J. Cell Biol. 83:317.
5. Gaffney, J. et al. (2010) Mol. BioSyst. 6:437.
6. Karangelis, D.E. et al. (2010) Curr. Med. Chem. 17:4018.
7. Jackson, D.G. (2009) Immunol. Rev. 230:216.
8. Itano, N. and K. Kimata (2002) IUBMB Life 54:195.
9. Csoka, A.B. et al. (2001) Matrix Biol. 20:499.
10. Day, A.J. (2001) http://www.glycoforum.gr.jp/science/hyaluronan/HA16/HA16J.html.
11. Gariboldi, S. et al. (2008) J. Immunol. 181:2103.
12. Scheibner, K.A. et al. (2006) J. Immunol. 177:1272.
13. Powell, J.D. and M.R. Horton (2005) Immunol. Res. 32:207.
14. Bollyky, P.L. et al. (2009) J. Leukoc. Biol. 86:567.
15. Aytekin, M. et al. (2008) Am. J. Physiol. Lung Cell Mol. Physiol. 295:L789.
16. Mine, S. et al. (2006) Endocrine J. 53:761.
17. Ferguson, E.L. et al. (2001) Int. J. Pharm. 420:84.
18. Masuko, K. et al. (2009) Int. J. Gen. Med. 2:77.
19. Bollyky, P.L. et al. (2007) J. Immunol. 179:744.
20. Sironen, R.K. et al. (2011) Exp. Cell Res. 317:3

PLATE LAYOUT

Use this plate layout to record standards and samples assayed.



产品信息及操作手册

Hyaluronan Valukine™ ELISA 试剂盒

目录号：VAL152

适用于定量检测天然和重组 Hyaluronan 的浓度

科研专用，不可用于临床诊断

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目录

I. 背景 20

II. 概述 21

III. 优势 22

IV. 实验 24

V. 试剂盒组成及储存 25

VI. 实验前准备 27

VII. 操作步骤 29

VIII. 参考文献 30

I. 背景

透明质酸(HA)，也称为玻尿酸或透明质酸钠，是一种天然存在的高度水合线性聚合物，由重复的双糖D-葡萄糖醛酸-β-1，3-N-乙酰氨基葡萄糖-β-1,4组成(1-6)。它是一种广泛存在于所有脊椎动物细胞外基质中的糖胺聚糖(GAG)。与硫酸软骨素、硫酸皮肤素和硫酸乙酰肝素等其他GAG不同，HA缺乏硫酸基团，几乎从不与其他蛋白质共价结合(3, 6, 7)。哺乳动物HA由三种不同的跨膜HA合成酶HAS1、2和3中的一种合成(1-4, 7, 8)。在HA合成过程中，随着UDP糖底物的加入，生长的聚合物通过膜转移到细胞外表面(7)。三种HA合成酶的表达模式和合成速率不同，可能产生不同链长的HA聚合物(8)。通常，HA合成酶产生高分子量HA (HMW，>500 kDa)，而降解是低分子量HA片段(LMW，<500 kDa)的主要来源。HA在哺乳动物中通过一系列透明质酸酶的协同作用迅速转化，并被肝脏和肾脏清除(1, 3, 5, 7,9)。

HA在细胞外基质的水化、细胞粘附和迁移的组织中非常重要(1, 6, 7)。它与大量透明质酸结合蛋白或透明质粘着蛋白相互作用。在细胞外基质中，HA能结合链接蛋白，如软骨中的aggrecan和HAPLN1，平滑肌中的versican，大脑中的brevican，以及神经组织中的neurocan (1, 6, 7, 10)。在循环中，它结合血浆结合蛋白，如ITIH1/2、TSG-6和HABP1/C1qBP (1, 3, 5, 7, 10)。HA结合许多细胞表面受体，如广泛表达的CD44、血管内皮细胞和其他细胞上的RHAMM、淋巴管内皮细胞上的LYVE-1，以及内皮窦（如淋巴结和肝脏）中的内吞受体Stabilin-2 (5-7, 10)。髓细胞TLR2和TLR4也结合LMW-HA片段 (11-13)。

通过HA受体激活的细胞信号通路部分依赖于HA的大小(4, 6, 8, 10-13)。虽然HMW-HA被认为具有抗血管生成、抗炎和免疫抑制作用，但LMW-HA具有高度的血管生成和促炎症作用，并促进树突状细胞成熟(1, 4-6, 12, 14)。HA主要由成纤维细胞产生，有助于成纤维细胞产生炎症介质并向纤维化区域迁移(1, 6)。在老化的动脉壁中，主动脉平滑肌和成纤维细胞的生成增加(6, 15)。HA促进胚胎干细胞向中胚层和心脏的分化，以及体外造血干细胞向造血细胞的分化(1)。血浆HMW-HA主要来自淋巴循环(7)。在血管病、肝纤维化、自身免疫性疾病如晚期硬皮病、类风湿性关节炎和狼疮性肾炎、终末期肾功能衰竭、特发性肺动脉高压、成人呼吸窘迫综合征以及血糖控制不佳的糖尿病患者中升高(1, 15, 16)。HA作为药物载体用于治疗，并注射到病变关节中以增加水合作用和抑制炎症(5, 17, 18)。它通过清除活性氧来保护组织；然而，活性氧也会导致HA碎裂，导致炎症(1)。HMW-HA与CD44结合促进调节性T细胞的活性，从而控制炎症(14, 19)。HA促进上皮细胞向间充质细胞的转化，无论是在发育过程中还是在致癌转化过程中(1, 7, 20)。癌细胞经常产生透明质酸酶降解HA，促进血管生成和炎症(4, 20)。

II. 概述

A. 检测原理

本实验采用双抗体夹心ELISA法。抗Hyaluronan抗体包被于微孔板上，样品和标准品中的Hyaluronan会与固定在板上的抗体结合，游离的成分被洗去；接着加入生物素化的抗Hyaluronan检测抗体进行孵育，洗涤去除未结合的物质后，加入链霉亲和素标记的辣根过氧化物酶（streptavidin-HRP）孵育。洗涤去除未结合的试剂后，加入底物溶液（显色剂）。溶液颜色与结合的目标蛋白成正比；加入终止液；用酶标仪测定吸光度。

B. 检测局限

- **仅供科研使用，不可用于体外诊断；**
- 该试剂盒适用于细胞培养上清样本和血清样本；
- 请在试剂盒有效期内使用；
- 不同试剂盒及不同批号试剂盒的组分不能混用；
- 样本值若大于标准曲线的最高值，应将样本用试剂稀释液（1×）稀释后重新检测；
- 检测结果的不同可由多种因素引起，包括实验人员的操作、移液器的使用方式、洗板技术、反应时间或温度、试剂盒的储存等。

III. 优势

A. 精确度

板内精确度（同一板内不同孔间的精确度）

已知浓度的三个样本，在同一板内分别检测20次，以确定板内精确度。

板间精确度（不同板之间的精确度）

已知浓度的三个样本，在不同板中分别检测28次，以确定板间精确度。

	板内精确度			板间精确度		
样本	1	2	3	1	2	3
平均值 (ng/mL)	1.2	4.3	17.9	1.2	4.5	18.4
标准差	0.1	0.3	1.1	0.1	0.4	1.5
CV%	7.3	6.1	6.2	7.2	8.0	8.1

B. 回收率

在细胞培养基样本中掺入检测范围内不同水平的Hyaluronan，测定其回收率。回收率范围在84-106.9%，平均回收率在95.0%。

在人血清样本中掺入检测范围内不同水平的Hyaluronan，测定其回收率。回收率范围在88.1-118.8%，平均回收率在102.7%。

C. 灵敏度

Hyaluronan的最低可测剂量（MDD）一般小于0.081ng/mL。

MDD是根据20个重复的零标准品孔的吸光度值的平均值加两倍标准差计算得到的相对应浓度。

D. 校正

该免疫测定法根据R&D Systems®的高纯度透明质酸（Catalog #GLR004）进行校准。

E. 线性

不同的样本中含有或掺入高浓度的Hyaluronan，然后用试剂稀释液（1×）将样本稀释到检测范围内，测定其线性。

稀释倍数	平均值(%)	范围 (%)
1:2	100.1	90.3-110.3
1:4	105.2	86.1-114.9
1:8	107.2	106.2-120.9
1:16	100.8	87.9-115.1

F. 样本预值

细胞上清样本–TF-1人血白血病细胞（1×10^5细胞/mL）和SNU-1人胃癌细胞（2×10^5细胞/mL）培养于含有10%胎牛血清、2mM L-谷氨酰胺、100U/mL青霉素和100μg/mL硫酸链霉素的RPMI1640培养基中3天；MCF-7人乳腺癌细胞（4×10^5细胞/mL）培养于含有10%胎牛血清、2mM L-谷氨酰胺、100U/mL青霉素和100μg/mL硫酸链霉素的DMEM培养基中3天。取细胞培养上清液，测定人细胞培养上清样本中人Hyaluronan的水平，检测值在35.8-44.4ng/mL之间，平均值为38.8ng/mL。

血清样本 - 使用本试剂盒检测了6份人血清样本中Hyaluronan的水平。6份样本的检测值范围为44-126ng/mL，平均值为77ng/mL。

G. 特异性

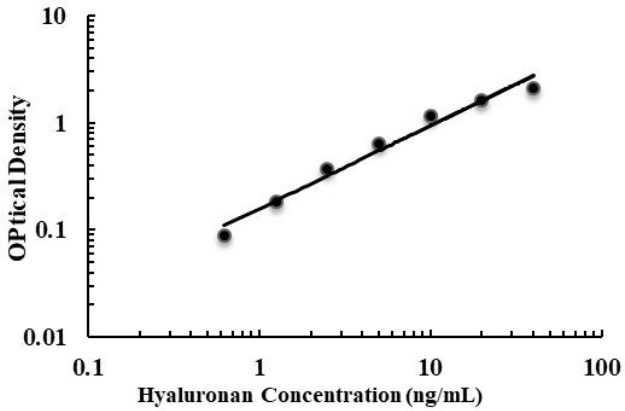
分别在10、50和1000μg/mL浓度下检测胆红素、血红蛋白和肝素，，结果显示在本试验中无交叉反应或干扰。

低分子量（15-40 kDa）、中分子量（75-350 kDa）和高分子量（>950 kDa）的透明质酸均可在本试验中检测到。

IV. 实验

标准曲线实例

该标准曲线数据仅供参考，每次实验应绘制其对应的标准曲线。



ng/mL	OD	Average	Corrected
0	0.052 0.052	0.052	-
0.625	0.136 0.145	0.141	0.089
1.25	0.235 0.236	0.236	0.184
2.5	0.411 0.426	0.419	0.367
5	0.651 0.733	0.692	0.640
10	1.111 1.283	1.197	1.145
20	1.665 1.705	1.685	1.633
40	2.163 2.168	2.166	2.114

V. 试剂盒组成及储存

A. 试剂盒组成

组成	描述	规格
Hyaluronan Microplate	包被人重组聚集蛋白聚糖的 96 孔聚苯乙烯板，8 孔×12 条	1 块板
Hyaluronan Standard	标准品（冻干粉），参考瓶身标签进行重溶	2 瓶
Hyaluronan Detection Antibody	生物素化的人聚集蛋白聚糖，冻干粉，参考瓶身标签进行重溶	1 瓶
Streptavidin-HRP B (40×)	40×浓缩的链霉亲和素标记的 HRP	1 瓶
Reagent Diluent (5×)	浓缩的试剂稀释液（5×），用于稀释标准品、样品、检测抗体和 HRP。	1 瓶
PBS (10×)	浓缩的 PBS（10×）	1 瓶
Wash Buffer Concentrate (25×)	浓缩洗涤缓冲液（25×）	1 瓶
Color Reagent A	显色液 A	1 瓶
Color Reagent B	显色液 B	1 瓶
Stop Solution	终止液	1 瓶
Plate Sealers	封板膜	3 张

B. 试剂盒储存

<table>
<tr><td>未开封试剂盒</td><td colspan="2">2-8℃储存；请在试剂盒有效期内使用</td></tr>
<tr><td rowspan="8">已打开，稀释或重溶的试剂</td><td>链霉亲和素-HRP B</td><td rowspan="6">2-8℃储存，最多 30 天*</td></tr>
<tr><td>洗涤缓冲液（1×）</td></tr>
<tr><td>显色液 A</td></tr>
<tr><td>显色液 B</td></tr>
<tr><td>PBS（10×）</td></tr>
<tr><td>终止液</td></tr>
<tr><td>标准品</td><td>使用时新鲜配制*</td></tr>
<tr><td>检测抗体</td><td>分装， -20℃储存，最多 30 天*</td></tr>
<tr><td>试剂稀释液（5×）</td><td>2-8℃储存，最多 30 天*
请每次使用新鲜配制的 1×试剂稀释液</td></tr>
<tr><td>包被的微孔板条</td><td>将未用的板条放回带有干燥剂的铝箔袋内，密封：2-8℃储存，最多 30 天*</td></tr>
</table>

*必须在试剂盒有效期内

C. 实验所需自备试验器材

- 酶标仪（可测量450nm检测波长的吸收值及540nm或570nm校正波长的吸收值）
- 高精度加液器及一次性吸头
- 蒸馏水或去离子水
- 洗瓶（喷瓶）、多通道洗板器或自动洗板机
- 500mL量筒
- 25℃恒温箱

D. 注意事项

- 试剂盒中的一些组分含有防腐剂，可能引起皮肤过敏反应，避免吸入。
- Color Reagent B可能引起皮肤、眼睛和呼吸道刺激，避免吸入。
- 试剂盒中的终止液是酸性溶液，使用时请做好眼睛、手、面部及衣服的防护。使用后请彻底洗手。

VI. 实验前准备

A. 样品收集及储存

细胞培养上清液：颗粒物应离心去除；立刻检测样本。样本收集后若不及时检测，需按一次使用量分装，冻存于-20℃冰箱内，避免反复冻融。样本可能需要用试剂稀释液（1×）稀释。

血清样本: 用血清分离管(SST)分离血清。使血样室温凝集30分钟，然后1000 x g离心15分钟。吸取血清样本之后即刻用于检测，或者分装，-20℃贮存备用。避免反复冻融。

B. 样本准备工作

细胞上清样本需要用试剂稀释液（1×）10倍稀释后进行检测，例如：20μL样本+180μL试剂稀释液（1×）。

血清样本需要用试剂稀释液（1×）20倍稀释后进行检测，例如：10μL样本+190μL试剂稀释液（1×）。

C. 检测前准备工作

使用前请将所有试剂放置于室温

洗涤液：从冰箱中取出的浓缩洗涤液可能有结晶，属于正常现象；放置室温，轻摇混匀，待结晶完全溶解后再配制洗涤液。可将20mL浓缩洗涤液用去离子水或蒸馏水稀释配制成500mL工作浓度的洗涤液。

PBS（1×）：使用去离子水或蒸馏水稀释制备PBS（1×）。

试剂稀释液（1×）：使用PBS（1×）稀释试剂稀释液（5×），制备试剂稀释液（1×）。

检测抗体：参考检测抗体瓶标签指示，用试剂稀释液（1×）将冻干粉进行重溶。再用试剂稀释液（1×）稀释至工作浓度400ng/mL，至少在使用前15分钟准备。

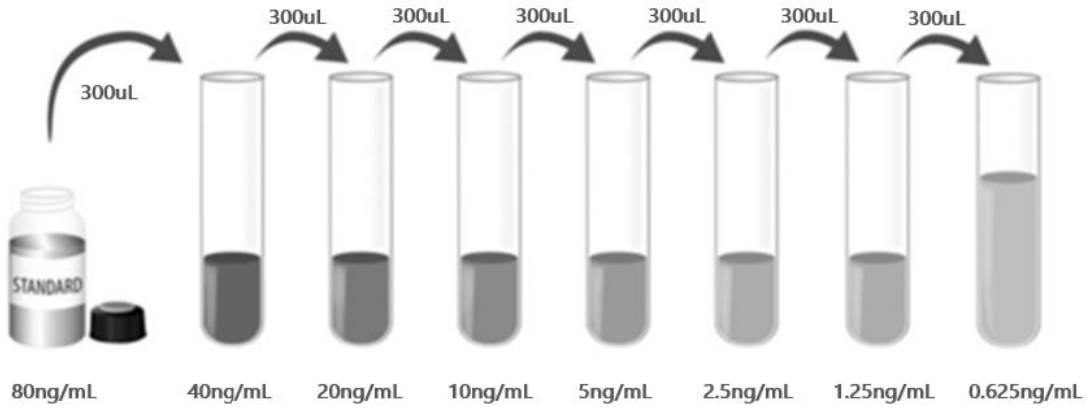
链霉亲和素- HRP B：用试剂稀释液（1×）将链霉亲和素- HRP B（40×）稀释至工作浓度链霉亲和素- HRP B（1×）。

显色剂：按试验所需用量（100μL/孔）将显色液A和显色液B等体积混合，避光保存，现用现配，须在15分钟内使用。

Hyaluronan标准品：冻干标准品的重溶体积请参考瓶身标签，得到浓度为80ng/mL标准品母液。轻轻震摇至少15分钟，使其充分溶解。

*如有疑问，请咨询我们的技术支持。

向各稀释管中加入300μL试剂稀释液（1×）。将标准品母液参照下图做系列稀释，每管须充分混匀后再移液到下一管。40ng/mL管作标准曲线最高点，试剂稀释液（1×）可用作标准品零点（0ng/mL）。



D. 技术小提示

- 当混合或重溶蛋白液时，尽量避免起沫；
- 为了避免交叉污染，配制不同浓度标准品、上样、加不同试剂都需要更换枪头。另外不同试剂请分别使用不同的移液槽；
- 建议15分钟内完成一块板的上样；
- 每次孵育时，正确使用封板胶纸可保证结果的准确性；
- 混合后的显色底物在上板前应为无色，请避光保存；加入微孔板后，将由无色变成不同深度的蓝色；
- 终止液上板顺序应同显色底物上板顺序一致；加入终止液后，孔内颜色由蓝变黄；若孔内有绿色，则表明孔内液体未混匀，请充分混合。

VII.操作步骤

使用前请将所有试剂和样本放置于室温，建议所有的实验样本和标准品做复孔检测

1. 按照上一节的说明，准备好所有需要的试剂和标准品；
2. 从已平衡至室温的密封袋中取出微孔板，未用的板条请放回铝箔袋内，重新封口；
3. 分别将不同浓度标准品，实验样本或者质控品加入相应孔中，每孔100μL。用封板膜封住反应孔，**25℃恒温箱孵育2小时。**说明书提供了一张96孔模板图，可用于记录标准品和试验样本的板内位置；（样本需要稀释，详情参见样本制备部分。）
4. 将板内液体吸去，使用洗瓶、多通道洗板器或自动洗板机洗板。每孔加洗涤液400μL，然后将板内洗涤液吸去。重复操作3次，共洗4次。每次洗板尽量吸去残留液体会有助于得到好的实验结果。最后一次洗板结束，请将板内所有液体吸干或将板倒置，在吸水纸拍干所有残留液体；
5. 在每个微孔内加入100μL配制好的检测抗体。用封板胶纸封住反应孔，**25℃恒温箱孵育2小时；**
6. 重复第4步洗板操作；
7. 在每个微孔内加入100μL稀释好的链霉亲和素- HRP B工作液。用封板膜封住反应孔，**25℃恒温箱孵育20分钟，注意避光；**
8. 重复第4步洗板操作；
9. 在每个微孔内加入100μL显色剂，**25℃恒温箱孵育20分钟，注意避光**；
10. 在每个微孔内加入50μL终止液，请轻拍微孔板，使溶液混合均匀。孔内溶液颜色会从蓝色变为黄色；
11. 加入终止液后30分钟内，使用酶标仪测量450nm的吸光度值，设定540nm或570nm作为校正波长。如果没有使用双波长校正，结果准确度可能会受影响；
12. **计算结果：**将每个标准品和样品的校正吸光度值（OD450-OD540/OD570），复孔读数取平均值，然后减去平均零标准品OD值。使用计算机软件作四参数逻辑（4-PL）曲线拟合创建标准曲线。另一种方法是，可以通过绘制标准品浓度做对数与相应OD值对数生成曲线，并通过回归分析确定最佳拟合线。通过样本的OD值，可从标准曲线上得到样本中Hyaluronan的浓度。如果样品被稀释，从标准曲线读取的浓度必须乘以稀释倍数。

VIII. 参考文献

1. Noble, P.W. et al. (2011) Physiol. Rev. 91:221.
2. Weigel, P.H. et al. (1997) J. Biol. Chem. 272:13997.
3. Bost, F. et al. (1998) Eur. J. Biochem. 252:339.
4. Stern, R. (2004) Eur. J. Cell Biol. 83:317.
5. Gaffney, J. et al. (2010) Mol. BioSyst. 6:437.
6. Karangelis, D.E. et al. (2010) Curr. Med. Chem. 17:4018.
7. Jackson, D.G. (2009) Immunol. Rev. 230:216.
8. Itano, N. and K. Kimata (2002) IUBMB Life 54:195.
9. Csoka, A.B. et al. (2001) Matrix Biol. 20:499.
10. Day, A.J. (2001) http://www.glycoforum.gr.jp/science/hyaluronan/HA16/HA16J.html.
11. Gariboldi, S. et al. (2008) J. Immunol. 181:2103.
12. Scheibner, K.A. et al. (2006) J. Immunol. 177:1272.
13. Powell, J.D. and M.R. Horton (2005) Immunol. Res. 32:207.
14. Bollyky, P.L. et al. (2009) J. Leukoc. Biol. 86:567.
15. Aytekin, M. et al. (2008) Am. J. Physiol. Lung Cell Mol. Physiol. 295:L789.
16. Mine, S. et al. (2006) Endocrine J. 53:761.
17. Ferguson, E.L. et al. (2001) Int. J. Pharm. 420:84.
18. Masuko, K. et al. (2009) Int. J. Gen. Med. 2:77.
19. Bollyky, P.L. et al. (2007) J. Immunol. 179:744.
20. Sironen, R.K. et al. (2011) Exp. Cell Res. 317:3

96 孔模板图

请使用 96 孔模板图来记录标准品及样本在板内的位置