Cat. #MK412

For Research Use

TaKaRa

Heparan Degrading Enzyme Assay Kit

Product Manual

v201708Da

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I. Description

Heparan sulfate (or heparatin sulfate) is a polysaccharide that is ubiquitously expressed in mammals and exists in proteoglycan form in the lung, liver, kidney, spleen, brain, and aorta, as a component of the cytoplasmic membrane. As a complex glycosaminoglycan, heparan sulfate may also contain polysaccharide chains containing D-glucosamine, D-glucuronic acid and L-iduronic acid, and these proteoglycans may be further modified with N- and O-linked sulfate and N-linked acetyl groups.[1)] Overall, heparan sulfate is a complex mixture of polysaccharides of varying molecular weights and sugar ratios.

Heparan sulfate is similar to heparin but does not have anticoagulant activity. It also has a fewer sulfate groups, L-iduronic acid, and N-sulfoglucosamine than heparin.

When located on the surface of capillary endothelial cells, the polysaccharide can function as an anchor for lipoprotein lipase; it also participates in cell-cell recognition, prevention of cell infiltration, and intercellular adhesion.

Heparan sulfate plays a key role in defense against tumor cell invasion; the activity of heparan sulfate degrading enzyme (i.e., heparanase) is significantly higher in invasive cancer cells compared to cancer cells that are not as invasive.[2)] Heparanase is an endo-beta-D-glucuronidase that specifically cleaves the beta-D-glucuronosyl-N-acetylglucosaminyl bond in heparan sulfate, and its activity may be found in mammalian liver, spleen, skin, placenta and platelets. Heparanase activity can also be found in tumor tissues, including those of melanoma, lymphoma, sarcoma, fibrosarcoma, and colon cancer. There has been increasing interest in the correlation of and significance between heparanase activity and cancer malignancy.[3, 4)]

It is reported that the heparanase activity in rat serum increases dramatically following transplantation with high-metastatic cancer versus non-metastatic cancer.[12)] Also, heparanase mRNA levels are overexpressed in highly invasive and metastatic tumor cells. However, because heparanase activity dramatically increases only after mRNA processing, the correlation between high heparanase mRNA levels and protein activity is not always direct.

The following methods are used to measure the activity of heparan degrading enzyme.

(1) Following reaction with the ^{35}S-labeled heparan sulfate substrate, Sephacryl S-200 gel filtration is performed and total radioactivity of the low molecular weight fraction is measured.[4)]
(2) Following reaction with ^{3}H-labeled heparan sulfate substrate, total radioactivity of the degraded heparan is measured. This assay is based on the premise that intact heparan sulfate binds HRG (histidine-rich glycoprotein) but degraded substrate does not.[13)]

Both of these methods have several disadvantages, including the use of radioactivity and inability to process many samples. Furthermore, method 2 cannot be used with cell culture supernatants containing FBS due to bovine HRG interference.

In addition to the above enzyme assays, it has been reported that the interaction between heparan sulfate or heparin-like molecule and bFGF (basic fibroblast growth factor) can be determined quantitatively based on the property that heparin-like molecule binds to bFGF.[14)] When heparan sulfate is degraded by heparan sulfate degrading enzyme, it loses the ability to bind to bFGF. As a result, enzymatic activity can be determined by quantitative comparison of undegraded heparan sulfate bound to bFGF in the presence and absence of sample.

Based on this principle, this kit was designed to measure the enzyme activity without using radioisotopes and to allow high-throughput screening. This kit adapts the DOC (Domain Oriented Capture) method using CBD-FGF, a fusion protein of the cell-binding domain of human fibronectin and human fibroblast growth factor.[15] CBD-FGF bound to a microtiterplate through interaction with an anti-fibronectin antibody[16] that recognizes the CBD region. The DOC method is a solid phase method that allows bFGF to retain its natural 3-dimentional structure. In addition, biotinylated heparan sulfate is used as a substrate of the enzyme in this kit. Since only undegraded substrate can bind to CBD-FGF, the detection of the remaining undegraded substrate by avidin-peroxidase allows highly sensitive measurement without radioactivity.

The heparan degrading enzyme assay can be completed in about 100 min, including a 45 min degradation reaction. Samples containing very low levels of enzymatic activity can be analyzed by extending the time of the assay. A correlation between heparan sulfate degrading enzyme activity and malignancy has been identified, and this kit can also be used to screen for inhibitors.

II. Principle

A. CBD-FGF protein is immobilized on a 96 well microtiterplate via interaction with an anti-fibronection antibody.

B. In a separate 96 well plate, biotinylated heparan sulfate is incubated with the sample in reaction buffer.*
The reactant is transferred to the wells of CBD-FGF immobilized 96 well plate.

C. After washing, the remaining undegraded biotinylated heparin sulfate can bind to CBD-FGF with the avidin POD conjugate.

D. After washing, POD substrate is added. The activity is inversely correlated with absorbance.

* The supplied reaction buffer contains protease inhibitor and glucuronidase inhibitors to minimize non-specific degradation. This buffer is optimized for the reaction conditions.

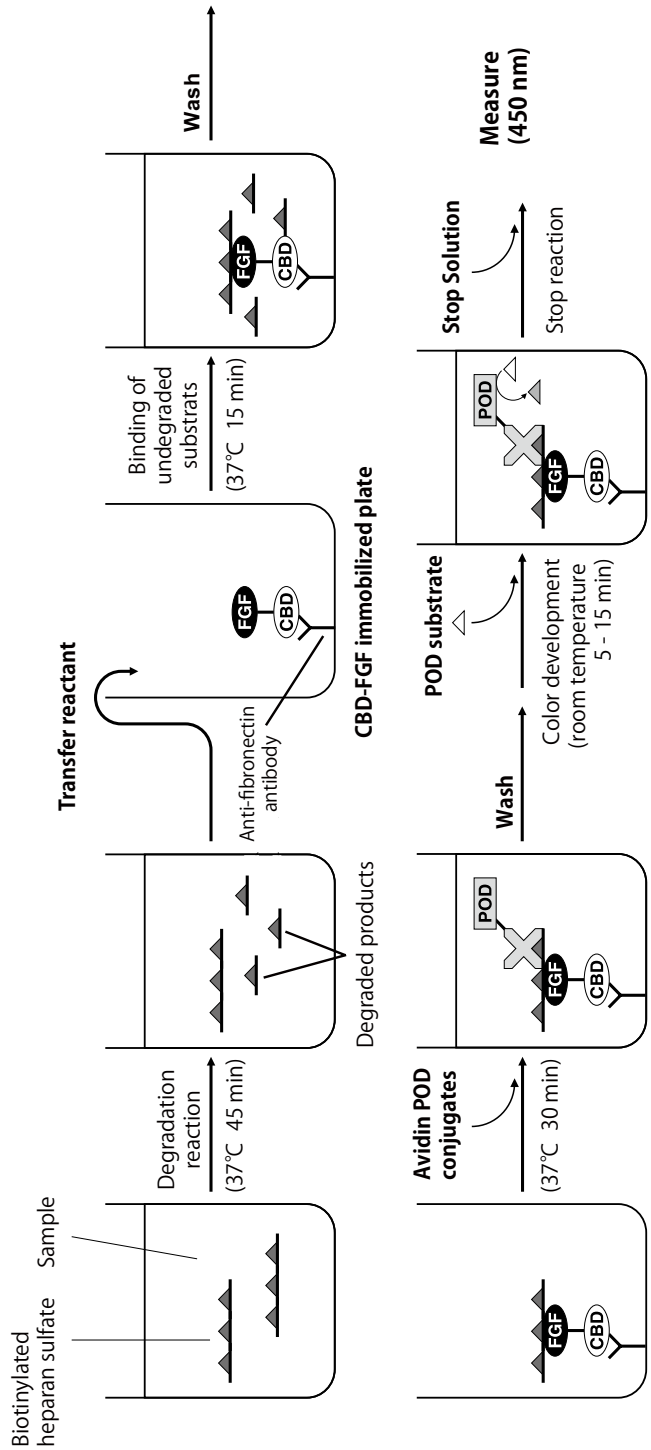


Figure.1. Assay overview.

III. Components

(1)	CBD-FGF immobilized microtiterplate (96 well: 8-well x 12 strips)	1 plate
(2)	Biotinylated heparan sulfate in reaction buffer (lyophilized)	for 5.5 ml
(3)	Reaction buffer for dilution	11 ml
(4)	Extraction buffer	11 ml
(5)	Standard	for 250 μl
(6)	Avidin POD conjugate	for 11 ml
(7)	POD substrate	12 ml

IV. Storage 4℃

V. Materials Required but not Provided

1) Wash and Stop Solution for ELISA without Sulfuric Acid (Cat. #MK021)
   Contains wash solution components (10X PBS, 50 ml x 5; Tween 20, 3 ml) and reaction stop solution (60 ml).
   NOTE: This product is a peroxidase reaction stop solution that does not contain 1N sulfuric acid. 1N sulfuric acid can also be used as the stop solution. Please handle 1N sulfuric acid with care.
2) 37℃ Incubator
3) Microplate reader (450 nm)
4) Distilled water
5) Micropipette
6) 96 well plate or microtubes for enzyme reaction

VI. Purpose

This kit measures heparan sulfate degrading enzyme activity in cultured cells, tissues, or blood using the DOC method. This kit is based on binding between bFGF and heparin-like substances and can be used to research the interaction between bFGF and heparin-like substances or other high affinity substances. The 96 well plate format allows processing of many samples simultaneously. As this kit is used to measure enzyme activity, it can be used for any kind of animal tissue, cell, or serum regardless of species.

VII. Protocol

1. Preparation of the reagents

- The CBD-FGF immobilized microtiterplate (1), Reaction buffer for dilution (3), Extraction buffer (4), and POD substrate (7) can be used directly.

- Biotinylated heparan sulfate (labeling substrate)
  (2)Dissolve the entire contents of one vial in 5.5 ml of distilled water. One vial is sufficient for one 96 well microtiterplate (50 μl per well). The dissolved solution can be stored for 3 weeks at -80℃.

- Standard (5)
  Dissolve the entire contents of one vial in 250 μl of distilled water. Dilute the dissolved solution 2 - fold with Reaction buffer for dilution (3). Prepare a dilution series using the 2 - fold diluted standard solution as the highest concentration.
  As the activity of the standard varies depending on the lot, establish a standard curve for each experiment. For the zero concentration, use the Reaction buffer for dilution directly. The dissolved standard can be stored for 3 weeks at -80℃, however, the dilutions cannot be stored.
  NOTE: Refer to "**IX. Basal Data**" for the definition of activity and for the contents of standard.

- Avidin POD conjugate (6)
  Dissolve the entire contents of one vial in 11 ml of distilled water. When you do not use all of the solution, store at -20℃ for up to 3 weeks. Avoid repeated freezing and thawing cycles.

- POD substrate (7)
  Bring POD substrate to room temperature before use, and use directly. Make sure that the substrate has not changed color to deep blue before use.
  Avoid contact with metal ions, as this may cause color development. When you use it in several times, take the required quantity for your immediate use and store the rest until next use.

- Stop Solution
  The stop solution included in Wash and Stop Solution for ELISA without Sulfuric Acid (Cat. #MK021) is ready to use. This product is a peroxidase stop solution that does not contain sulfuric acid. Because the stop solution is highly viscous, mix well after adding with a plate mixer or similar device.

- PBS with 0.1% Tween 20 for Washing
  Dilute one bottle (50 ml) of 10X PBS from the Wash and Stop Solution for ELISA without Sulfuric Acid (Cat. #MK021) with 450 ml of distilled water and add 500 μl of Tween 20. After mixing well, use this as the wash buffer (0.1% Tween 20/PBS).

2. Sample Preparation

< Suspension-cultured cells >

Wash 1 - 5 x 10^6 cells with PBS, and pellet the cells. Add 1 ml of Extraction buffer (4) to this pellet and gently suspend with a vortex. Centrifuge at 14,500 rpm (10,000*g*) at 4℃ for 5 min, and use the supernatant as the sample.

< Adherent cultured cells >

Remove the culture supernatant from the cells (1 - 5 x 10^6) in a 9 cm culture dish, and wash the cells once with PBS. Add 1 ml of Extraction buffer (4) and remove the cells from the dish using a rubber scraper. Transfer the cells to a microtube Centrifuge at 14,500 rpm (10,000*g*) at 4℃ for 5 min, and use the supernatant as the sample.

< Cultured cells in a 96 well microtiterplate >

After removing the culture supernatant, add 50 - 100 μl of Extraction buffer (4) to the wells and dissociate the cells by pipetting. When possible, centrifuge at 14,500 rpm. If it is not possible to centrifuge, the suspension can be used directly as the sample.

< Platelets >

Collect platelets by centrifugation, and suspend in an appropriate amount of Extraction buffer (4). Centrifuge at 14,500 rpm (10,000*g*) at 4℃ for 5 min, and use the supernatant as the sample. If using plasma that includes platelets, add an equal volume of Extraction buffer and mix to suspend. If there are any insoluble substances, remove by centrifugation.

Prepare a 2 - fold dilution by mixing the extracted sample with the Reaction buffer (3) at the ratio of 1 : 1, and perform assay. Samples must be assayed immediately after extraction, regardless of the sample type. If the assay cannot be performed immediately, store at -80℃. Even stored at -80℃, the sample must be assayed within 2 weeks after preparation.

NOTE: If a sample is contaminated with a substance that inhibits binding of FGF and heparan sulfate, it may be difficult to distinguish enzyme activity and binding inhibition.

3. Procedure

1. Add each 50 μl of the dilution series of the standard and the prepared samples* to the wells of a 96 well plate or microtubes.
   * The samples have been already diluted 2 - fold. If necessary, dilute further.

2. Heparan sulfate degrading reaction
   Add 50 μl of Biotinylated heparan sulfate (labeling substrate (2), which has been dissolved in 5.5 ml distilled water) to each well of the 96 well plate or microtubes that contain the standards and samples. Incubate at 37℃ for 45 min.
   The concentration of the standard is designed for a reaction time of 45 min. If the activity of assay samples is suspected to be low, the reaction time can be extended to several hours for qualitative detection.

3. Binding reaction of undegraded heparan sulfate
   Transfer each 90 μl of the mixture of biotinylated heparan sulfate and standard or samples to the wells of the CBD-FGF immobilized microtiterplate supplied in the kit. If necessary, adjust the volume to ensure that all wells have an equal volume. Incubate at 37℃ for 15 min.

4. Biotin-Avidin binding reaction
   Discard the reaction solution and wash each well three times with washing buffer (PBS containing 0.1% Tween 20). Add 100 μl of dissolved Avidin POD conjugate (6) to each well and incubate at 37℃ for 30 min.

5. POD color development
   Discard the reaction solution and wash each well three times with washing buffer (PBS containing 0.1% Tween 20). Add 100 μl of POD substrate (7) to each well and perform color development at room temperature for 5 - 15 min. As the reaction is influenced by temperature, adjust the reaction time by observing the color to avoid over-development.

6. Add 100 μl of stop solution into each well in the same order as the substrate was added. Tap the plate gently to mix well.

7. After zeroing the microplate reader using distilled water, measure the absorbance of each well at 450 nm. The color reaction is stable for -1 hour after stopping the reaction.

8. When establishing a standard curve using the standards, plot enzyme activity on the x-axis and the corresponding absorbance on the y-axis. Using this standard curve, calculate the enzyme activity corresponding to each sample's measured absorbance.

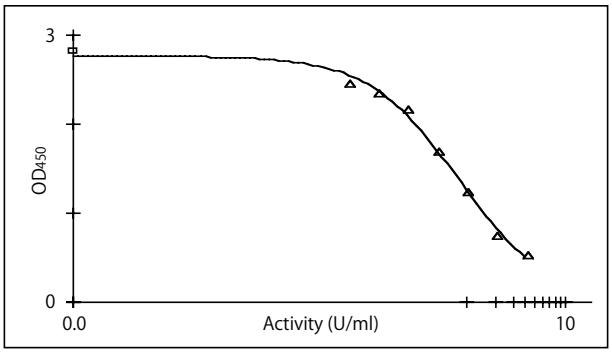
VIII. Performance

1. Detection sensitivity > 0.1 U/ml

2. Range of assay

The following shows a typical standard curve.
A standard curve must be prepared for each experiment.

Curve Fit : 4-Parameter Corr. Coeff : 0.997
$y = (A - D) / (1 + (x/C)^B) + D$
A = 2.77 B = 0.965 C = 0.827 D = 0.0241



Activity (U/ml)	4.20	2.10	1.05	0.525	0.262	0.131	0.065	0.00
OD_{450}	0.542	0.748	1.242	1.702	2.173	2.360	2.459	2.822

3. Assay precision

Intra-assay precision (n=8)
Assay was performed with 3 cell extraction samples.

	Average (U/ml)	SD	CV (%)
Sample A	0.764	0.044	5.8
Sample B	1.158	0.041	3.5
Sample C	1.708	0.052	3.1

IX. Basal Data

1. CBD-FGF immobilized microtiterplate (DOC method)
   CBD-FGF is bound on a microtiterplate by interaction of CBD with a monoclonal antibody against CBD. The FGF portion is entirely free.
   As FGF is unbound, this kit can achieve more sensitive capture and detection of undegraded heparan sulfate than FGF bound directly on a plate.

2. Biotinylated heparan sulfate
   Heparan sulfate is highly purified from bovine kidney and then biotinylated with N-hydroxysuccinimidobiotin. It is used as substrate of heparan sulfate degrading enzyme. The optimized amount for reaction in the reaction buffer is lyophilized and supplied in this kit.

3. Standard
   In principle, a purified enzyme or an enzyme extracted from cells should be used as the assay standard. The activity of heparan sulfate degrading enzyme is too unstable to withstand long term storage. Therefore, this kit includes an unlabeled heparan sulfate as a standard substitute. This standard is designed to establish almost the equal standard curve to the enzyme activity that is obtained when performing degradation reaction at 37℃ for 45 min using the activity fraction of heparan sulfate degrading enzyme originating from platelets.
   If you perform the assay several times on different days and compare those assay results using the supplied standard, perform the reaction at 37℃ for 45 min. One unit is defined as the activity which can degrade 0.063 ng of biotinylated heparan sulfate when reacted at pH 5.8, at 37℃ for 1 min.

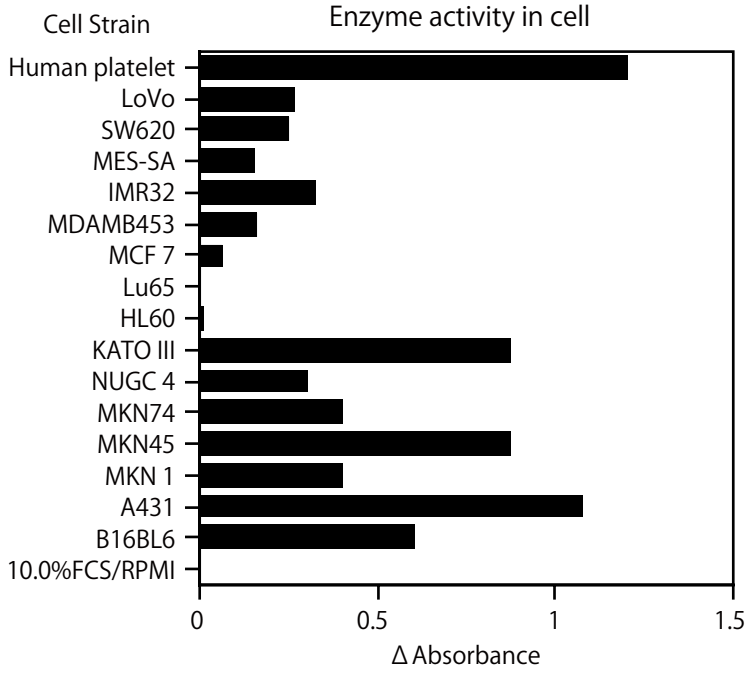
4. Influence of co-existing substances
   This kit uses the binding affinity between CBD-FGF and heparan sulfate with DOC method. No color development would be detected if there are any substances that have strong affinity for FGF in the reaction system and thus preventing undegraded biotinylated heparan sulfate from binding.
   Confirmation that no inhibition is occurring is necessary when any additional substances are added in the system (e.g., for inhibitor screening).
   The method of this confirmation is;
   - incubate biotinylated heparan sulfate only with additional substance instead of assay sample,
   - transfer to the CBD-FGF immobilized microtiterplate
   - check if the result is the same as the one obtained from the reaction without addition.

   This kit can also be used for screening the affinity of substances with FGF by utilizing this inhibition. The following table lists the influence of co-existing substances that Takara bio has studied with this kit.

Interacts with FGF	Does not interact with FGF
Dextran sulfate Chondroitin sulfate B Carrageenan Iota, κ, λ	Chondroitin sulfate A Chondroitin sulfate C Chondroitin sulfate D Keratan sulfate Pullulan Galactan Suramin[17)]

X. Assay Example

The activity of heparan sulfate degrading enzyme in various cultured cell extracts were measured with this kit. Each sample (5 x 10^6 cells) was processed with 1 ml of extraction buffer. Each extract was diluted with reaction buffer and used for assay. The activity is shown as a decrease in absorbance.



LoVo:	human colon adenocarcinoma
SW620:	human colon adenocarcinoma
MES-SA:	human uterus sarcoma
IMR32:	human neuroblastoma
MDAMB453:	human breast cancer
MCF7:	human breast cancer
Lu65:	human lung carcinoma
HL60:	human promyelocytic leukemia
KATOIII:	human gastric carcinoma
NUGC4:	human gastric carcinoma
MKN74:	human gastric carcinoma
MKN45:	human gastric carcinoma
MKN1:	human gastric carcinoma
A431:	human epidermoid
B16BL6:	mouse melanoma

XI. References

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