Heparan Degrading Enzyme Assay Kit

For quantitative determination of Heparan Degrading Enzyme

Cat No. 40-831-160016
For 1 Set

Table of Contents

Description.....................................................................................................2
Principle.........................................................................................................4
Kit Components.............................................................................................4
Storage..........................................................................................................4
Purpose of this kit..........................................................................................4
Protocol
  Preparation of the reagents.......................................................................6
  Sample preparation...................................................................................7
  Procedure..................................................................................................8
Performance...................................................................................................9
Basal data....................................................................................................10
Assay example.............................................................................................12
References...................................................................................................13
Heparan Degrading Enzyme Assay Kit

Description:

Heparan sulfate (also called heparatin sulfate) is a complex glycosaminoglycan consisting of polysaccharide chains composed of D-glucosamine, D-glucuronic acid, and L-iduronic acid, which are modified with N- and O-linked sulfate and N-linked acetyl groups. It is similar to heparin, but does not have anticoagulant activity. Also it has lower content of sulfate residue, L-iduronic acid and N-sulfoglucosamine than heparin, and is isolated as a mixture of the polysaccharides which have quite different molecular weight and ratio of composing sugars.

Heparan sulfate is a polysaccharide which is ubiquitously present in mammals, and exists in lung, liver, kidney, spleen, brain and aorta in the form of proteoglycan bound with protein as a component of cytoplasmic membrane. Heparan sulfate functions as anchor for lipoprotein lipase on the surface of capillary endothelial cells and also participates in mutual recognition, prevention of cell infiltration, intercellular adhesion and so on.

Heparan sulfate is very important in its defensive role against invasion of tumor cells. It has been reported that the activity of heparan sulfate degrading enzyme in high-invasive cancer cells was considerably higher than that in low-invasive cells. Heparan sulfate degrading enzyme, generally called as heparanase, is an endo-β-D-glucosidase that specifically cleaves β-D-glucuronosyl-N-acetylglucosaminyl bond in heparan sulfate and its activity is found in the liver, spleen, skin, placenta, and platelet of mammals. The activity of this enzyme is also found in tumor tissues such as melanoma, lymphoma, sarcoma, fibrosarcoma and colon cancer, and the correlation of heparan sulfate degrading enzyme activity with malignancy of cancer cells is attracting interest.

Heparitinase has been conventionally known as a Heparan sulfate degrading enzyme originated from bacteria (Flavobacterium heparinium), but it is distinguished from mammalian heparanase by the cleavage pattern of heparan sulfate.

In 1998, Freeman at Australia isolated this enzyme from human platelet and purified. In the next year of 1999, Freeman and other 3 groups succeeded in cloning of human, mouse and rat heparanase almost in the same time but individually, and then its whole amino acid sequence was resolved. Heparanase is generated as a precursor of 65kDa and is reported that its activity would raise by around 100-fold when becoming 50kDa molecule by processing between 157 and 158th amino acid residues from N-termini. Also it is reported that the heterodimer of the molecule from the N-termini to 157th and the 50kDa molecular is essential for the enzyme activity.

Some reports say that the heparanase activity in serum drastically increased in a rat transplanted with high-metastatic cancer, when comparing rats between transplanted with high-metastatic and non-metastasis cancer, as the time passed after transplantation. Also seen from the mRNA level, heparanase tends to overexpress in high-invasive and high-metastatic tumor cells. However, as the heparanase activity increases after processed, the correlation between mRNA amount and activity level does not always correspond each other.
Heparan Degrading Enzyme Assay Kit

The following methods have been applied to measure the activity of heparan degrading enzyme.

(1) After the reaction with $^{35}$S-labeled heparan sulfate as substrate, carry out the gel filtration with Sephacryl S-200, and then check the radioactivity of low molecular weight fraction.

(2) After the reaction with $^3$H-labeled heparan sulfate as substrate, measure the radioactivity originated from degraded heparan. This method is based on the property that undegraded heparan sulfate binds to HRG (histidine-rich glycoprotein) and but degraded ones do not.

Both methods have several disadvantages, for instance, these methods must use radioactivity and cannot process many samples simultaneously. Besides, the method (2) cannot use the supernatant of culture with serum as sample, due to the inhibition of bovine HRG.

Apart from the enzyme assay, 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.

When heparan sulfate is degraded by heparan sulfate degrading enzyme, it loses the binding activity to bFGF. So the enzyme activity in a sample can be determined through quantitative comparison of undegraded heparan sulfate bound to bFGF between two conditions with and without sample.

Based on this principle, GenWay's Heparan Degrading Enzyme Assay Kit was designed to measure the enzyme activity without using radioisotope and to allow screening of many samples. One of the features of this kit is adapting the DOC (Domain Oriented Capture) method which employs uniquely prepared CBD-FGF. CBD-FGF is a fusion protein of cell-binding domain of human fibronectin and human fibroblast growth factor. This CBD-FGF is bound on a microtiterplate supplied in this kit, with captured by anti-fibronectin antibody having epitope in CBD region. DOC method is a solid phase method enabling to keep the most natural 3-dimensional structure when bFGF is bound to heparan sulfate. In addition, biotinylated heparan sulfate is used as a substrate of the enzyme in this kit. Since only undegraded substrate can combine to CBD-FGF, the detection of the remaining undegraded substrate by avidin-peroxidase realizes high-sensitive non-RI measurement.

The duration of a series of assay is around 100 minutes, including 45 min of enzyme degradation reaction. Tiny enzyme assay can be detected by extending the time of enzyme degradation reaction. As the correlation of heparan sulfate degrading enzyme activity with malignancy of cancer cells is attracting interest, this kit can also be utilized for screening of its inhibitor.
Heparan Degrading Enzyme Assay Kit

Principle:

A. CBD-FGF protein is immobilized on a 96-well microtiterplate mediated by anti-fibronectection antibody.

B. React the biotinylated heparan sulfate with sample in the reaction buffer*, in another 96-well plate.
   Transfer the reactant into well of CBD-FGF immobilized 96-well plate.

C. After washing, react remaining undegraded biotinylated heparin sulfate bound to CBD-FGF with avidin POD conjugate.

D. After washing, develop the color by POD substrate. Measure the activity by decrease of the absorbance.

* As the supplied reaction buffer contains protease inhibitor and inhibitors to glucuronidase, non-specific degradation is minimized. Also this buffer is designed to offer the optimized reaction condition.

Kit Components:

1. CBD-FGF immobilized microtiterplate
   (96 wells: 8 well x 12 strips) ......................................................1 plate

2. Biotinylated heparan sulfate in reaction buffer
   .................................................................................................1 vial (for 5.5 ml)

3. Reaction buffer for dilution ...........................................................1 vial (11 ml)

4. Extraction buffer ..........................................................................1 vial (11 ml)

5. Standard........................................................................................1 vial (for 250 µl)

6. Avidin POD conjugate.................................................................1 vial (for 11 ml)

7. POD substrate................................................................................1 vial (12 ml)

Storage: 4 °C

Purpose of this kit:

This kit allows the measurement of the activity of heparan sulfate degrading enzyme in cultured cells, tissues or blood by DOC method. As the principle of this kit is based on DOC method which utilizes binding reaction between bFGF and heparin-like substances, it can be applied to the research of interaction between bFGF and heparin-like substances, or other affinity substances. The 96-well plate allows to process many samples simultaneously. As this kit is to measure the enzyme activity, it can be used to any kinds of animal tissue, cell, or serum regardless of species of originated animals.
Heparan Degrading Enzyme Assay Kit

**Catalog Number:**

40-831-160016

**Heparan Degrading Enzyme Assay Kit**

1. **Biotinylated heparan sulfate** Sample
2. **Degradation reaction**
   - (37°C, 45 min)
   - Degrading reaction
   - Degraded products
3. **Transfer of reactant**
   - Anti-fibronectin antibody
   - CBD-FGF immobilized plate
4. **Binding of undegraded substrates**
   - (37°C, 15 min)
5. **Washing**
6. **Avidin POD conjugates**
   - (37°C, 30 min)
7. **POD substrate**
   - Washing
   - Color development
   - (room temperature 5-15 min)
8. **1 N H₂SO₄**
9. **Stop reaction**
10. **Measure by plate reader**
    - (450 nm)
Heparan Degrading Enzyme Assay Kit

Protocol:

1. Preparation of the reagents
   - CBD-FGF immobilized microtiterplate (1), Reaction buffer for dilution (3), Extraction buffer (4) and POD substrate (7) are used without further preparation.
   - Biotinylated heparan sulfate (labeling substrate) (2)
     Dissolve the whole content of one vial in 5.5 ml of distilled water. One vial corresponds to the amount for one 96-well microtiterplate. Use 50 ul per well. Dissolved solution can be stored for 3 weeks at -80°C.
   - Standard (5)
     Dissolve the whole content of one vial in 250 μl of distilled water. Dilute the dissolved solution by 2-fold with reaction buffer for dilution (3). Prepare the dilution series using the 2-fold diluted standard solution as the highest concentration.
     As the activity of the standard varies depending on a lot, establish a standard curve each time assaying. For the zero concentration, use the reaction buffer for dilution directly. The dissolved standard can be stored for 3 weeks at -80°C, however, the dilutions cannot be stored.

NOTE: Please do not fail to refer to "Basal data" at page 10 for the definition of activity and for the content of standard.
   - Avidin POD conjugate (6)
     Dissolve the whole content of one vial in 11 ml of distilled water. When you do not use up the whole solution, store it at -20°C. The dissolved solution can be stored for 3 weeks at -20°C. Avoid repeated freeze-thaw cycles.
   - POD substrate (7)
     Bring back POD substrate to room temperature before use, and use it without any preparation. Make sure that the substrate has never changed its color to deep blue before use.
     Take care not to contact the substrate with metal ion, since it may develop color when it contacts with metal ions. When you use it in several times, take the required quantity for your immediate use and store the rest until next use.

- Required reagents and instruments not supplied in the kit:
  1) Stop solution: 1 N sulfuric acid, 11 ml for one kit
  2) Washing solution: PBS containing 0.1% Tween 20
  3) 37°C Incubator
  4) Microplate reader (available at 450 nm)
  5) Distilled water
  6) Micropipette
  7) 96-well plate or microtube for enzyme reaction (A plate used for culturing cells can be re-used after washing.)
Heparan Degrading Enzyme Assay Kit

2. Sample preparation

- Suspension-cultured cells
  Wash 1-5 x 10^6 cells with PBS, and collect the cells as pellet. Add 1 ml of extraction buffer(4) to this pellet and gently suspend with a vortex. Centrifuge at 14,500 rpm (10,000 x g) at 4°C for 5 min, and take the supernatant as sample.

- Adhesive cultured cells
  Remove the culture supernatant from the cells (1-5 x 10^6) on 9 cm culture dish, and wash the adhered cells with PBS once. Add 1 ml of extraction buffer(4) and collect the cells into a microtube using a rubber scraper. Centrifuge at 14,500 rpm (10,000 x g) at 4°C for 5 min, and take the supernatant as sample.

- Cultured cells on 96-well microtiterplate
  After removing the culture supernatant, add 50-100 μl of extraction buffer(4) into the wells and peel the cells with pipetting. When possible, centrifuge at 14,500 rpm. When handling many samples simultaneously and it is impossible to centrifuge, the suspension can be used directly as sample.

- Platelet
  Collect the platelet as precipitate by centrifugation, and suspend it in an appropriate amount of extraction buffer(4). Centrifuge at 14,500 rpm (10,000 x g) at 4°C for 5 min., and use the supernatant as sample. In case of using plasma including platelet, mix it with the same amount of extraction buffer and mix to suspend. If there are any insoluble substances, collect it by centrifugation and remove.

Prepare the 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 a kind of sample. In case that an assay cannot be done right after the sample preparation, store it at -80°C. However, even if it is stored at -80°C, the sample must be applied for assay within 2 weeks after preparation.

NOTE: If a sample is contaminated with a substance which inhibits the binding of FGF and heparan sulfate, it may be difficult to distinguish the enzyme activity and the binding inhibition.
Heparan Degrading Enzyme Assay Kit

3. Procedure

1) Add each 50 µl of the dilution series of the standard and the prepared samples into each well of a different 96-well plate or microtube. (The samples have been already diluted by 2-fold. If necessary, dilute it in further.)

2) Heparan sulfate degrading reaction
Add 50 µl of biotinylated heparan sulfate (labeling substrate (2), which has been already dissolved in 5.5 ml distilled water ) into each well of the 96-well plate or microtubes which are added with standard and samples. Incubate at 37 °C for 45 min. ( The concentration of the standard is designed for the reaction of 45 min. If the activity of assay samples is supposed to be weak, the reaction time can be extended to several hours for the qualitative detection.)

3) Binding reaction of undegraded heparan sulfate
Transfer each 90 µl of the mixture of biotinylated heparan sulfate and standard or samples, which were already incubated at step 2), into each well of CBD-FGF immobilized microtiterplate supplied in the kit. (When there are shortage in the liquid volume due to several transfer, change the volume of all standard and samples to make all wells have equal volume.) Incubate at 37 °C 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% Tween20). Add 100 µl of dissolved Avidin POD conjugate (6) into each well and incubate at 37 °C for 30 min.

5) POD color development
Discard the reaction solution and wash each well three times with washing buffer (PBS containing 0.1% Tween20). Add 100 µl of POD substrate (7) into each well and perform color development at room temperature for 5-15 min. As the reaction is influenced by the room temperature, adjust the reaction time by observing the color and avoid over-development.

6) In the same order as adding the substrate, add 100 µl of stop solution (1N sulfuric acid) into each well and mix well.

7) After zero adjustment of microplate reader using distilled water as zero control, measure the absorbance of each well at 450 nm. The developed color is stable for around 1 hour after stopping the reaction.

8) When establishing a standard curve using the standard, plot the each enzyme activity at horizontal axis, and the corresponding absorbance at vertical axis. Utilizing this standard curve, calculate the enzyme assay corresponding to each sample absorbance.
Heparan Degrading Enzyme Assay Kit

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 each time of assay.

\[
y = \frac{(A - D)}{\left(1 + \left(\frac{x}{C}\right)^B\right)} + D
\]

\[
A = 2.77 \quad B = 0.965 \quad C = 0.827 \quad D = 0.0241
\]

<table>
<thead>
<tr>
<th>Activity (U/ml)</th>
<th>OD_{450}</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.20</td>
<td>0.542</td>
</tr>
<tr>
<td>2.10</td>
<td>0.748</td>
</tr>
<tr>
<td>1.05</td>
<td>1.242</td>
</tr>
<tr>
<td>0.525</td>
<td>1.702</td>
</tr>
<tr>
<td>0.262</td>
<td>2.173</td>
</tr>
<tr>
<td>0.131</td>
<td>2.360</td>
</tr>
<tr>
<td>0.066</td>
<td>2.459</td>
</tr>
<tr>
<td>0.00</td>
<td>2.822</td>
</tr>
</tbody>
</table>

3. Assay precision

Intra-assay precision (n=8)  Assay was carried out with 3 kinds of cell extraction samples.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Average (U/ml)</th>
<th>SD</th>
<th>CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample A</td>
<td>0.764</td>
<td>0.044</td>
<td>5.8</td>
</tr>
<tr>
<td>Sample B</td>
<td>1.158</td>
<td>0.041</td>
<td>3.5</td>
</tr>
<tr>
<td>Sample C</td>
<td>1.708</td>
<td>0.052</td>
<td>3.1</td>
</tr>
</tbody>
</table>
Heparan Degrading Enzyme Assay Kit

Basal data:

1. CBD-FGF immobilized microtiterplate (DOC method)
   CBD-FGF is bound on a microtiterplate with CBD part supported monoclonal antibody against CBD and with FGF part free.
   As FGF part is left free, this kit can achieve more sensitive capture and detection of undegraded heparan sulfate, than binding FGF 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 roughly extracted from cells should be used as the assay standard. The activity of heparan sulfate degrading enzyme is too unstable to stand for long time 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 which is obtained when performing degradation reaction at 37°C for 45 min using the activity fraction of heparan sulfate degrading enzyme originating from platelet. When you perform assay in several times on different days and compare those assay results using the supplied standard as an index, please perform reaction at 37°C for 45 min. One unit is defined as the activity which can degrade 0.063 ng of biotinylated heparan sulfate when reacted at pH5.8, at 37°C for one min.
Heparan Degrading Enzyme Assay Kit

4. Influence by co-existence

This kit adapts the assay principle utilizing the binding affinity between CBD-FGF and heparan sulfate with DOC method. So no color development would be detected if there might be any substances which has strong affinity to FGF in the reaction system and thus undegraded biotinylated heparan sulfate cannot bind.

Therefore, confirmation that no inhibition would occur is necessary, when you need any additional substances in the system eg. for inhibitor screening. The method of this confirmation is;
- incubate biotinylated heparan sulfate only with additional substance instead of assay sample,
- transfer it into CBD-FGF immobilized microtiterplate
- check if the result is the same as the one obtained from the reaction without any addition.

On the other hand, this kit can also be used for screening of affinity substances to FGF and the research of its interaction, by utilizing this inhibition. The following lists the influence by co-existing substances which Takara has studied using this kit.

<table>
<thead>
<tr>
<th>Interact with FGF</th>
<th>Not interact with FGF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dextran sulfate</td>
<td>Chondroitin sulfate A</td>
</tr>
<tr>
<td>Chondroitin sulfate B</td>
<td>Chondroitin sulfate C</td>
</tr>
<tr>
<td>Carrageenan Iota, κ, λ</td>
<td>Chondroitin sulfate D</td>
</tr>
<tr>
<td></td>
<td>Keratan sulfate</td>
</tr>
<tr>
<td></td>
<td>Pullulan</td>
</tr>
<tr>
<td></td>
<td>Galactan</td>
</tr>
<tr>
<td></td>
<td>Suramin</td>
</tr>
</tbody>
</table>
Heparan Degrading Enzyme Assay Kit

Assay example:

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

![Graph showing enzyme activity in cell strains]

- **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
Heparan Degrading Enzyme Assay Kit

References:


NOTE: For Food and Environmental Testing use only.
Not for use in human and animal diagnostic or therapeautic.