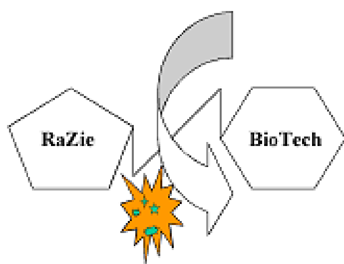

HEPARANASE ASSAY KIT, with positive control

Assay kit
for Screening of Heparanase inhibitors
and Quantification of Heparanase activity
in cells and other biological fluids

Quantity: 96 Tests **Cat.#:** Ra001-BE-K

August 2019

For Research Use Only.
Not for use in diagnostic or therapeutic procedures.

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PLEASE READ BEFORE USING ASSAY KIT

- **Avoid repeated freezing and thawing of the reagents and Enzyme preparations.**
- **Vials may contain small quantities of material, hence ensure that they are very briefly pulse centrifuged prior to opening**
- **Please read the whole manual as many solutions require preparation immediately before use i.e. preparation during the assay itself. The assay protocol indicates at which step certain solutions should be prepared.**

Intended use

The heparanase assay kit is an assay for quantitative detection of heparanase in cell culture supernatants, human plasma, biological fluids and tissue samples. The heparanase assay kit is for research use only. Not for use in diagnostic or therapeutic procedures.

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I. Background

Heparan sulphate proteoglycans (HSPG) are present in the extracellular matrix, in basement membranes and on cell surfaces. HSPG consist of a core protein containing heparan sulphate side chains. HSPG is known to play important structural and functional roles in linking the component proteins of the basement membrane, controlling the permeability properties of the membranes and signal transduction. This latter function is achieved by controlling the bioavailability of cytokines and growth factors to their cellular receptors. HSPG on the cell surface participates in signal transduction by facilitating the interaction between growth factors and their receptors[1,2].

Heparanase is an endoglycosidase that specifically cleaves heparan sulphate (HS) side chains of HSPG[3-6]. Due to the wide distribution and functions of HSPG, degrading activity of heparanase affects various cellular functions under normal and pathological conditions[7].

Cloning of human heparanase further intensified interest in this enzyme with particular emphasis in developing inhibitors or enhancers for this enzyme[8-11]. A handicap for this research has been a lack of a sensitive and more importantly specific test for human heparanase activity[12]. Furthermore unavailability of a purified enzyme or instability of the cloned enzyme limits assay design. To date the available tests have the above shortcomings and are time consuming, not useful for inhibitor screening or lack an appropriate positive control.
This heparanase assay has been developed to overcome the above problems. It is

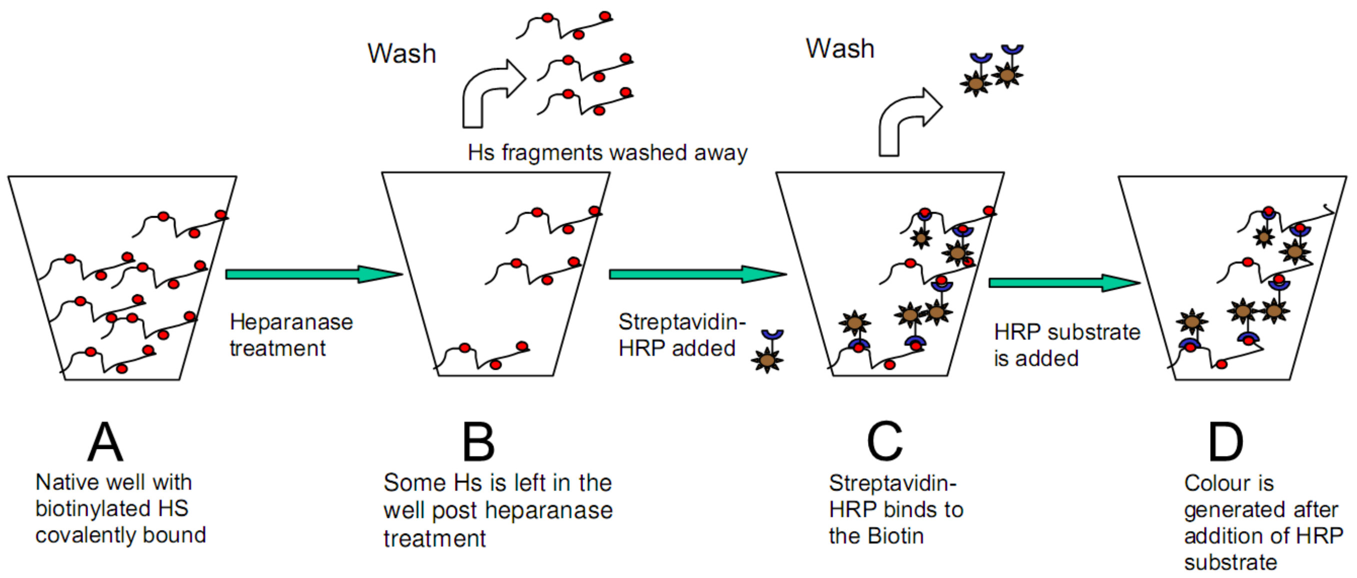
- Non-radioactive,
- Fast and easy to use,
- Sensitive and specific to heparanase,
- Uses a universal 96-well plate format ideal for inhibitor studies

Two formats of the kit are available:

- Cat#Ra001-02-K (kit containing plate and reaction buffers but NO positive control).
- Cat# Ra001-BE-K (this kit, containing in addition a positive control Enzyme.

- Biotinylated heparan sulphate coated 96-well plate (Cat#RA1001-03-P) is also available for your own designed experiments.

II. Principles of the assay



The decrease in the OD of well D compared to well A is directly proportional to the heparanase activity.

III. Precautions and Limitations

For Research Use Only. Not for use in diagnostic or therapeutic procedures.

The physical, chemical, and toxicological properties of the chemicals and reagents contained in the Heparanase Assay Kit may not yet have been fully investigated. Therefore Razie Ltd recommends the following:

- All chemicals should be considered as potentially hazardous. We therefore recommend that this product is handled only by those persons who have been trained in laboratory techniques and that it is used in accordance with the principles of good laboratory practice. Wear suitable protective clothing such as laboratory overalls, safety glasses and gloves. Care should be taken to avoid contact with skin or eyes. In the case of contact with skin or eyes wash immediately with water. In case of any hypersensitivity reaction see a medical doctor.
- Do not mix or substitute reagents with those from other lots or other sources.
- Do not use positive control enzyme for any other test or procedure
- Do not use kit reagents beyond expiration date on label.
- Do not expose kit reagents to strong light during storage or incubation.
- Do not pipette by mouth.
- Do not eat or smoke in areas where kit reagents or samples are handled.
- Avoid contact of skin or mucous membranes with kit reagents or specimens.
- Rubber or disposable latex gloves should be worn while handling kit reagents or specimens.
- Reagents might contain strong preservatives and may be toxic if ingested.
- Avoid contact of substrate solutions with oxidizing agents and metal.
- Avoid splashing or generation of aerosols.
- In order to avoid microbial contamination or cross-contamination of reagents or specimens which may invalidate the test use disposable pipette tips and/or pipettes.
- Use clean, dedicated reagent trays for dispensing the substrate reagents.
- Exposure to acids will inactivate the signal generating reagents.
- Glass-distilled water or deionized water must be used for reagent preparation.
- Substrate solutions must be at room temperature prior to use.
- Decontaminate and dispose specimens and all potentially contaminated materials as if they could contain infectious agents. The preferred method of decontamination is autoclaving for a minimum of 1 hour at 121.5°C.
- Liquid wastes not containing acid and neutralized waste may be mixed with sodium hypochlorite in volumes such that the final mixture contains 1.0 % sodium hypochlorite. Allow 30 minutes for effective decontamination. Liquid waste containing acid must be neutralized prior to the addition of sodium hypochlorite.

IV. Reagents Provided

Catalog #	Component	Quantity	Storage Temperature
FB001-01-6K	Base Buffer	6ml	-20°C
FB001-02-30	Additive 1	30 µl	4°C
FB001-03-30	Additive 2	30 µl	4°C
FB001-04-5K	4X HEPARANASE Buffer	5ml	-20°C
FB001-05-20	Strep-HRP	10 µl	-20°C
FB001-06-10K	HRP Substrate	10ml	4°C
FB001-01P-HE	HS-plate	1	-20°C
FB001-BE-50	Positive Control Enzyme	100 µl	-20°C
FB001-BE-PB200	Buffer for Positive Control	200 µl	4°C

.

V. Materials Required But Not Supplied

- PBS, pH 7.4
- Tween 20
- Glass-distilled or deionized water (preferentially filtered through 0.45µm filter)
- 0.2 N HCl or H_2SO_4 for stopping the peroxidase reaction.
- Phenylmethyl Sulfonyl Fluoride (PMSF) and other protease inhibitors (optional, to protect tissue/cell extract)
- 10 µl to 1,000 µl adjustable single channel micropipettes with disposable tips
- 50 µl to 300 µl adjustable multichannel micropipette with disposable tips
- Multichannel micropipette reservoir
- Centrifuge tubes, beakers, flasks (necessary for preparation of reagents or for dilution of enzyme/buffers).
- Glass Homogenizer (optional, for tissue extract assays)
- Device for delivery of wash solution such as multichannel wash bottle or automatic wash system (optional)
- 37°C incubator
- 96-well plate reader capable of reading at 450 nm (650 nm as optional reference wavelength).
- Inhibitors or cells/tissue.

VI. Reagent Preparation

Please note this is how the reagents are prepared. Please refer to the actual protocol for when to prepare the reagents. Bring all reagents to the room temperature.

1. **Reaction buffer**

   Note:

   The Reaction Buffer should be prepared immediately before use and used within one hour of preparation. A solid precipitate might form in additives **1 & 2** due to refrigeration. Bring the solution to the room temperature and vortex the tube vigorously to dissolve the precipitate. Briefly centrifuge the tubes prior to opening.

   Mix 6ml of the Base Buffer (Cat# FB001-01-6K) with an equal volume (6ml) of filtered distilled water in a clean tube and vortex.

   Add **12µl Additive 1** solution (Cat# FB 001-02-30) to the above diluted base buffer and vortex.

   Add **12µl Additive 2** solution (Cat# FB 001-03-30) to the above solution and vortex.

   The reaction buffer is now ready. Keep at room temperature.

2. **1X HEPARANASE Buffer**

   Dilute the **4X** HEPARANASE Buffer (Cat# FB001-04-5K) **1:4** with filtered dH_2O. The **1X** HEPARANASE Buffer is used to

   - Dilute the test samples and any inhibitors to be tested
   - Prepare cell / tissue extracts.

3. **Positive control Enzyme** (Optional)

   At the start of setting up the plate, add 50µl of the **enzyme (FB001-BE-50)** to a well followed by 50µl **enzyme buffer (FB001-BE-PB200)** to the same well. Mix the solution very gently by pipetting up and down. Repeat the same for the second designated positive control well. **There is no need for addition of any other buffer or additives** to these wells. Please note that the positive control will not be used in calculating results.

4. **Strep-HRP**

   Immediately before use, dilute 8µl of the supplied Strep-HRP (Cat# FB001-05-20) in 12 ml **PBS**. Use the diluted Strep-HRP within 30 min. A total volume of 100 µl/well of diluted Strep-HRP is required in the assay.

5. **HRP substrate**

   The supplied Peroxidase substrate solution (Cat# FB001-06-10K) is ready for use. Bring to room temperature prior to use.

6. **Controls**

   i. **<u>Negative control (must be included in each test)</u>:** A negative control without heparanase, test sample or cell extract should be included. This will provide the baseline reading (i.e. no enzyme present, hence strongest colour is produced in these wells.)

   Add a further 50µl of the reaction buffer to four wells (ie 100µl of the reaction buffer is present in each well)

   ii. **<u>Positive control (optional)</u>:** see section VI:3 above. **There is no need to add any other buffer or additives** to these two wells.

VII. HEPARANASE Activity in biological samples

A. Preparation of Cell culture samples

i. Adherent cells:

Before analysis it is important to be able to transfer your cell culture to a **Serum-Free Media (SFM)** as follows:

1. Grow your cell culture to confluence.
2. Discard the culture media containing serum.
3. Wash the cell culture 4X by adding 1X PBS to the culture vessel, gently shaking the culture vessel and discarding the PBS.
4. Add SFM to the culture vessel and culture overnight in the incubator. The culture time in SFM will depend on the nature of the cells and should be determined independently.
5. Collect the SFM from the culture vessel. Use aliquots of this SFM for the enzyme assay. Follow steps in enzyme assay as described (section VIII)

ii. Non-adherent cells:

1. Centrifuge 2 x 10^6 to 1 x 10^7 non-adherent cells grown in a suitable SFM to form a pellet at 1500g for 3 min (3000 RPM in an ordinary bench top centrifuge).
2. Centrifuge the supernatant (at 1500g for 2min) to pellet any cellular contaminant.
3. Treat this final supernatant as your test sample and carry out the heparanase assay as described in section VIII.

B. Tissue Extract

The following procedure must be carried out in a safety hood / cabinet. Appropriate eye protection and surgical mask should be used.

1. Wash the tissue thoroughly using cold PBS. The tissue must be clean and as far as possible free from contaminants such as blood or blood clots.
2. Cut a small piece of the tissue and place it inside a clean glass mortar. Add a volume of 1x Heparanase Buffer (at least 1ml of buffer per every mg tissue)
3. Grind and homogenize the tissue.
4. Transfer the homogenate into a test tube or microfuge tube and centrifuge at around 2000g for 2min.
5. Use 50µl of the clear supernatant and carry out the enzyme assay as described (section VIII). It might be necessary to repeat centrifugation to obtain a supernatant free from tissue debris. **Avoid repeated cycles of freeze / thaw of this supernatant.**

C. Biological Fluids

There is no particular preparation for biological fluids, as long as it is clear and free from any contaminants such as particulate material. This can be achieved as described for tissue extracts by a simple centrifugation step. Biological fluids should be kept ice cold and used immediately or frozen (preferably at -80°C) until use. **Avoid repeated cycles of freeze/thaw as this will lead to inactivation of the enzyme**.

For blood, it is advisable to use only plasma and not serum or whole blood to avoid contamination by activated platelets products.

VIII. HEPARANASE Assay Protocol

Bring the reaction buffer and HRP substrate to the room temperature.

A. Enzyme reaction

1. Rehydrate the microwell plate by adding 100µl of PBS to each well of the plate. Incubate at room temperature for a minimum of 30 min or overnight at 4ºC. Cover the microwell plate with the lid or a Plate Cover.
2. Remove the PBS from the microwells. Take care not to scratch the surface of the microwells. **Wash the wells once, by adding and removing 200** µl **Distilled Water to the wells.** Ensure that all the liquid is removed by tapping plate onto towels to remove excess liquid. Use the microwell strips immediately or place upside down on a wet absorbent paper for no longer than 15 minutes. Do not allow wells to dry.
3. Add 50µl of the reaction buffer to all wells, except the two for positive control (see section VI.3 above)
4. Add extra 50 µl of the reaction buffer to four wells as the negative control.
5. Add 50µl of your test samples to duplicate wells. (you can allocate four or more wells to your test samples)
6. Add 100µl of the positive control enzyme (See 6-ii above) to two wells for positive control.
7. Incubate the microwell plate (ideally at 37º C) for one hour on a plate shaker.
8. Wash the microwell strips four times with approximately 200 µl per well 1X PBS containing 0.1% (v/v) Tween20 and then twice with approximately 200 µl Distilled Water per well with thorough aspiration of microwell contents between washes. Take care not to scratch the surface of the microwells. After each wash, empty wells and tap microwell plate on paper towels to remove excess liquid. Use the microwell strips immediately after washing or place upside down on a wet absorbent paper. Do not allow wells to dry.

You dont have to have a plate washer to use this kit. Washing stage for all sections of the manual can be carried out as described below:

a- Prepare the washing solution PBS/Tween20 as described. Pour enough of this solution into a flat bottom dish (around 30X15 cm) The solution should be around 5-6 cm deep.

b-Empty the plate at the end of the incubation period.

c- Submerge the plate into this wash solution. Make sure all wells are filled up with the wash solution. Shake the plate inside the solution.

d- Rapidly shake empty the plate into a sink. Tap the plate dry on a pad of kitchen towels. This is one wash cycle. Repeat the above procedure for each wash cycle needed.

Tap the plate dry on a pad of kitchen towels! Use the microwell strips immediately after washing or place upside down on a wet absorbent paper. Do not allow wells to dry.

Detection

Blank the plate reader according to the manufacturer's instructions against the air.

1. Add 100 µl of diluted Strep-HRP at room temperature (prepared in section VI.4) per well. Incubate at **37º C for** 50 minutes on a plate shaker.
2. Wash the microwell plate five times with approximately 200 µl 1X PBS / well with thorough aspiration of microwell contents between washes. Take care not to scratch the surface of the microwells. After each wash, empty wells and tap microwell plate on paper towels to remove excess liquid. Use the microwell strips immediately after washing or place upside down on a wet absorbent paper for not longer than 15 minutes. Do not allow wells to dry.
3. This section is carried out at room temperature. Add 100 µl of **room temperature** peroxidase substrate (see step VI.5) per well. Shake the plate by **Gently Tapping** the side of the plate to get a uniform colour. The colour of the liquid should start to turn blue. Please see the video clip on the AMSBIO site which shows an example of development stage. The colour generated in this clip are an example only and your test might show different colour intensity. In this clip, wells, 1A&B negative control and 1C&D are positive controls.

4. Making sure that there no bubbles on the surface of the liquid, read absorbance on of each microwell on a spectrophotometer using 650 nm as the reference wavelength, at intervals of 1 to 2 minutes. This step should be carried at room temperature. Please note, the rate of colour generation is very dependent on the ambient temperature. The higher the temperature the faster colour develops. The colour could develop very fast within minutes.
5. The substrate reaction should be stopped as soon as an OD of 0.6 – 0.7 is reached for the blank wells. Stop the enzyme reaction by quickly pipetting 100 µl of 0.12M HCl into each well, including the blank wells. It is important that the Stop Solution is spread quickly and uniformly throughout the microwells to completely inactivate the enzyme. The colour of the liquid in wells should have turned yellow at this stage.
6. If you found colour development too fast, **use FB001-06-10K, HRP substrate, straight out of the fridge at 4 degrees or lower and dont let it warm up to room temperature.** Doing this could slow the colour development.

(**Make sure there are not any bubbles at the surface of liquid**)
Determine the absorbance of all wells, at 450nm, within 5 min post stopping. The colour post stopping is relatively stable for one hour.

IX. HEPARANASE Inhibitor Screening Assay Protocol

It is important to make sure that the inhibitor preparation is as pure and homogeneous as possible, so that the any effect seen, is representative of the inhibitor and not other compounds. The inhibitor preparation should be checked in the absence of any enzyme in this assay, to ensure that it is has no effect on the mechanism of the assay. The wells should generate full colour with or without the inhibitor.
Similarly, for your targeted enzyme, it is recommended to run a pilot study to determine the enzyme's activity behavior, (e.g. check dilution response), using Razie's assay prior to the inhibition studies.

Potential inhibitors can be assayed using two different procedures

A. Concurrent inhibition

The inhibitor can be added to the enzyme at the time of preparing the reaction mixture.

1. Prepare dilutions of the inhibitor in the heparanase buffer (Cat# FB001-04-5K)
2. Prepare a dilution of the enzyme from your source in the heparanase buffer (prepared in step VI.2)
3. Follow procedure as VIII. A.1-5 above.
4. Add appropriate volumes of enzyme preparation and inhibitor together to one well. Make sure the final volume in the well is 100µl. (The base 50µl reaction buffer, step VIII. A.3, can be adjusted to achieve this volume).
5. Follow the procedure as for heparanase assay (Steps VIII. A.6, 7).
6. Detection: Follow VIII B1-5

B. Pre reaction inhibition

In this procedure the inhibitor is added prior to the reaction.

1. Mix aliquots of the enzyme and the inhibitor as required (For example a ratio of 1:1 v/v i.e. 100µl of inhibitor to100µl enzyme or 2:1, 3:1 and so on), mix.
2. Incubate at 4ºC for the desired period of time. This time may vary between a few minutes to overnight. Please note that the longer the incubation period the more chance there is of natural enzyme degeneration.
3. Use 50µl of the above enzyme/inhibitor mixture as your test sample and follow the normal assay procedure as in VIII.A 1-7
4. Detection: Follow VIII B1-5

X. Data Interpretation.

Calculate the activity of your sample (in ng HS removed per minute) using the following formulae

First calculate R: **R= (OD / Max OD) x 500**

MaxOD= maximum reading possible from your plate reader from the negative control wells (section VI, 6i and VIII),A4).

OD= reading obtained from your test well

Then substitute R in the following formulae to obtain the activity

Activity* = (500- R) / 60 (ng HS removed per minute)

Specific activity is then calculated by dividing the activity by protein concentration present in your sample to give: nanograms of HS removed per minute per mg protein.

* This figure is based on the amount of HS loaded onto the plate and assumes that all have been immobilized by the active sites in the well.

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XII. Troubleshooting

PROBLEM	CAUSE	SOLUTION
No colour reaction is seen.	Strep-HRP has not been added. Not enough incubation time	Make sure step VIII, B.1 is carried out. Prolong the incubation of strep-HRP.
	Cold HRP substrate has been used, or reaction is carried out in cold environment	Make sure HRP substrate is at room temperature Repeat, carrying out the HRP reaction at **37° C**
No detectable signal is generated.	Insufficient amount of the heparanase enzyme is present	Increase the concentration of protein in your sample enzyme
		Omit the addition of additive 2, Step VI.1 (this will increase the likelihood of false positive results).
		Use extra sensitive precoated plates (Cat# FB001-02P-HES)
		Increase incubation time to 1.5 hours.
		Increase the volume and/or concentration of cell extract added to each well.
Colour generated is above the working range of the plate reader.	Long incubation of HRP substrate prior to stopping the reaction	Stop the reaction much earlier (Step VIII, B 4,5)
		Increase the dilution of Strep-HRP (Step VI, 4) (i.e. 6µl in 12ml)

XIII. Related Products Available From Razie Biotech Ltd

Catalog #	Description	Quantity
FB001-01P- HE	HS coated plate	1 plate

XIV. Appendix

Reagent composition:

I. **1X PBS (pH 7.4) (not supplied):** 7.5 mM Na_2HPO_4, 2.5 mM NaH_2PO_4, 145 mM NaCl.

II. **2X Base Buffer:** Proprietary buffer solution containing active ions.

III. **500X Strep-HRP** Streptavidin Peroxidase tagged proprietary solution.

IV. **4X HEPARANASE Buffer:** Proprietary buffer solution.

V. **HRP Substrate:** 3,3’,5,5’-Tetramethyllbenzidine (TMB).

XV. ORDERING INFORMATION

Please address your orders to:

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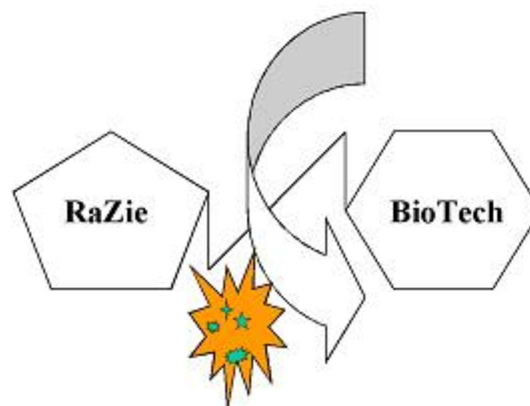

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