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A simple and rapid assay for heparanase activity using homogeneous time-resolved fluorescence

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Abstract

Degradation of heparan sulfate proteoglycan by heparanase is an important process in tissue invasion by malignant tumor cells and inflammatory cells. Several heparanase assays have been reported previously, but all of them are cumbersome and time-consuming for separating degradative products from uncleaved substrates and detecting these products. This paper describes the development of a simple and rapid assay for heparanase activity using homogeneous time-resolved fluorescence based on non-radiative energy transfer. In this assay, heparan sulfate proteoglycan labeled with biotin and europium cryptate (fluorescence donor) was used as a substrate for heparanase. Degradation of the substrate by incubation with murine melanoma cell extract was detected by measuring the time-resolved fluorescence after addition of XL665 (cross-linked allophycocyanin, fluorescence acceptor)-labeled streptavidin. As heparanase activity can be simply measured by successive addition of substrate, enzyme and detection reagent onto one assay plate, our heparanase assay allows rapid processing of large numbers of samples.

Keywords: Rare earth cryptate; Homogeneous time-resolved fluorescence; Heparanase; Heparan sulfate; Melanoma cells; Endoglycosidase assay

1. Introduction

Destruction of the endothelial basement membrane (EBM) and extracellular matrix (ECM) by malignant tumor cells and inflammatory cells is an important process in tumor invasion, tumor metastasis, angiogenesis and inflammation [1,2]. One of the major mechanisms degrading EBM and ECM proceeds through cleavage of heparan sulfate proteoglycan (HSPG) by heparanase [1–4]. HSPG consists of a core protein and heparan sulfate (HS) glycosaminoglycan with repeating disaccharide units consisting of *N*-acetylglucosamine and glucuronic acid. HS chains undergo N- and/or O-sulfation and isomerization to iduronic acid. Heparanase has been identified from a murine melanoma subline as an endoglucuronidase cleaving the linkage between glucuronic acid and *N*-acetylglucosamine on HS [4]. As heparanase cleaves HS at only a few specific sites, degraded HS has characteristic large molecular weight fragments composed of 10–20-sugar units [4]. In order to investigate the characteristic features of heparanase, various methods for detecting heparanase activity have been developed. They are polyacrylamide gel electrophoresis [3], high-speed gel permeation chromatography using radiolabeled or fluorescein-labeled HS [4–7], and sepharose bead-based separation assay utilizing the interaction of HS with chicken histidine-rich glycoprotein [8]. However, these heparanase assays are cumbersome and time-consuming for separating degradative products from uncleaved substrates and detecting these products. Moreover, some of them use radioisotopes, which make necessary measures to prevent contamination and radiation exposure. Due to these disadvantages, it has taken 20 years since the report of the existence of heparanase to purify the heparanase protein and isolate the heparanase gene [7,9,10]. To overcome these problems, we developed a simple, rapid, non-radioisotopic and microtiterplate-based heparanase assay using homogeneous time-resolved fluorescence (HTRF®).

HTRF technology is based on non-radiative energy transfer from europium cryptate to cross-linked allophycocyanin (XL665) [11–13]. When both fluorescent molecules are in proximity, XL665 emits long-lived fluorescence at 665 nm. This makes it possible to omit the non-specific short-lived fluorescence from other components and to detect the analyte without any separation step. Measurement of the ratio of fluorescence at 665 nm to that at 620 nm, which is emitted from europium

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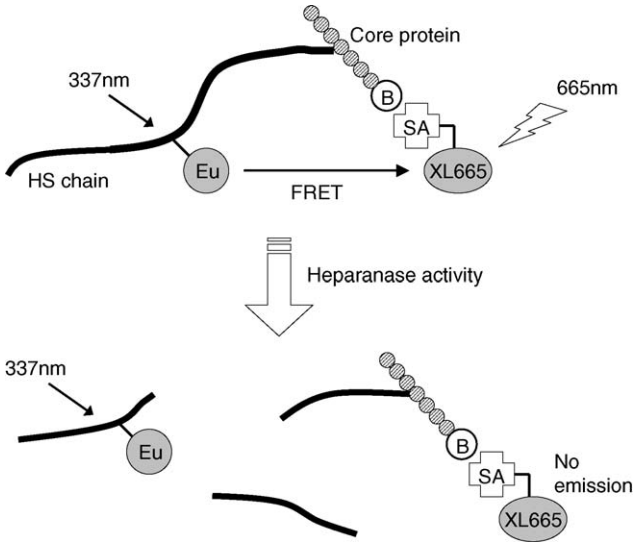

Fig. 1. Thematic scheme of the heparanase assay using HTRF. HSPG labeled with europium cryptate and biotin (EuK–HSPG–biotin) is detected by measuring time-resolved fluorescence after addition of XL665-labeled streptavidin (XL665-SA). Degradation of EuK–HSPG–biotin by heparanase decreases the HTRF signal. HS chain, heparan sulfate chain; Eu, europium cryptate; B, biotin; SA, streptavidin; FRET, fluorescence resonance energy transfer.

cryptate (internal reference), makes it possible to correct for media absorbency in real time. Because of these advantages, HTRF has been utilized for high-throughput screening of various biomolecular targets [14–21].

The strategy used for measuring heparanase activity using HTRF is shown in Fig. 1. The core protein of HSPG was biotinylated via primary amines generated by trypsin digestion, and then polysaccharides on HS were labeled with europium cryptate via formyl groups formed by $NaIO_4$ oxidation. Degradation of HSPG labeled with europium cryptate and biotin (EuK–HSPG–biotin) by heparanase was detected by measuring time-resolved fluorescence after addition of XL665-labeled streptavidin. In the present method, heparanase activity can be simply measured by successive addition of substrate, enzyme and detection reagent onto one assay plate. Thus, our heparanase assay makes it possible to rapidly determine the heparanase activity of a large number of samples.

2. Materials and methods

2.1. Materials

Europium cryptate diamine derivative and XL665-labeled streptavidin were obtained from CIS Bio International (Marcoule, France). EZ-Link™ sulfo-NHS-LC-biotin, M-PER® mammalian protein extraction reagent and 2-(4′-hydroxyazobenzene) benzoic acid (HABA) were acquired from Pierce (Rockford, IL, USA). EDTA-free complete protease inhibitor cocktail and Matrigel™ basement membrane matrix were obtained from Roche Diagnostics (Mannheim, Germany) and Becton Dickinson (Franklin Lakes, NJ, USA), respectively. The Bradford protein assay kit and rapid assay kit for chondroitin sulfate were acquired from Biocolor Ltd. (Newtownabbey, Northern Ireland), and Hokudo (Sapporo, Japan), respectively. Blyscan™ sulfated glycosaminoglycan assay kit and D-saccharic acid 1,4-lactone, a potent exo-β-glucuronidase inhibitor, were from Nacalai tesque (Kyoto, Japan). Heparin from porcine mucosa was purchased from ICN Biomedicals Inc. (Aurora, OH, USA). Heparitinase from *Flavobacterum heparinum*, chondroitin-4-sulfate (C4S) from sturgeon notochord, chondroitin-6-sulfate (C6S) from shark cartilage, dermatan sulfate (DS) from pigskin, keratan sulfate (KS) from bovine cornea, HS from bovine kidney and hyaluronic acid (HA) from human umbilical cord were acquired from Seikagaku Corporation (Tokyo, Japan). Trypsin from bovine pancreas and fluoresceinisothiocyanate (FITC)-labeled dextran were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All chemicals were of analytical grade, unless otherwise specified.

2.2. Mouse melanoma cell extract

The highly invasive and lung metastatic murine B16 melanoma subline, B16-BL6 cells [4] were provided by Dr. I.J. Fidler (M.D. Anderson Cancer Center, Houston, TX). B16-BL6 cells (1×10^7 cells) were pelleted and suspended in 10 ml of M-PER® mammalian protein extraction reagent containing EDTA-free complete protease inhibitor cocktail. After incubation at room temperature for 20 min, the supernatant was collected as a cell extract by centrifugation at 13,000 rpm for 15 min. Protein content in the cell extract was determined using the Bradford protein assay kit. The cell extract was stored at −80 °C.

2.3. Preparation of HSPG

HSPG was purified from Matrigel™ basement membrane matrix extracted from Engelbreth-Holm-Swarm mouse tumor as described previously [22,23]. Briefly, Matrigel was denatured with 4 M urea at 4 °C followed by loading onto a DEAE-Sepharose Fast Flow column (Amersham Pharmacia Biotech, Uppsala, Sweden). HSPG was eluted with a linear gradient of 0–0.75 M NaCl in Tris–HCl (pH 6.8; 50 mM) containing 4 M urea. After removing urea by dialysis, the core protein of HSPG was thoroughly digested with excess trypsin. Digestion of the core protein was confirmed by SDS-PAGE, and then trypsin-digested HSPG was purified using DEAE-Sepharose column again. HSPG at each step was monitored using the Bradford protein assay kit and the rapid assay kit for chondroitin sulfate. Trypsin-digested HSPG was air-dried and stored at 4 °C after precipitation with 4 volumes of ethanol–potassium acetate (0.13 M) (95:5, v/v) at −20 °C.

2.4. Preparation of EuK–HSPG–biotin

To 186 μg of trypsin-digested HSPG dissolved in 0.2 ml of PBS was added 100 μg of EZ-Link™ sulfo-NHS-LC-biotin. After incubation at 4 °C for 16 h, 0.6 ml of 10 mM $NaIO_4$ was added. After incubation for 30 min at room temperature, 20 μl of ethylene glycol was added to the reaction mixture, which was further incubated for 10 min. After dialysis against sodium acetate (pH 5.5; 0.1 M), 30 μg of europium cryptate diamine

derivative was added to HSPG solution and the pH was adjusted to 9.0 by addition of 0.1 M K_2CO_3. Forty-five microliters of 1 M $NaBH_3CN$ was added to the reaction mixture, which was incubated at 4 °C for 16 h. After dialysis against Tris-buffered saline (TBS), HSPG solution was applied to Immunopure® immobilized monomeric avidin column (Pierce), and the column was extensively washed with PBS. After washing, HSPG binding to the column was eluted using 20 mM D-biotin and subjected to ethanol precipitation as described above. The precipitate was dissolved in TBS and desalted by gel filtration using PD-10 column (Amersham Pharmacia Biotech). The concentration of EuK–HSPG–biotin was quantified using Blyscan™ sulfated glycosaminoglycan assay kit. The amount of europium cryptate incorporated into HS was determined using the molecular extinction coefficient of europium cryptate at 305 nm (30,000 M^{-1} cm^{-1}). The number of biotin attached to HSPG was determined using HABA reagent.

2.5. Heparanase assay using HTRF

A 10 μl portion of 3 μg/ml EuK–HSPG–biotin diluted in enzyme buffer (sodium acetate (pH 5.0; 0.1 M), 0.1% BSA, 0.2% Triton X-100, 20 mM D-saccharic acid 1,4-lactone) was added to each well of a 384-well assay plate (black color with flat bottom; Corning Incorporated, Acton, MA, USA). The concentration of EuK–HSPG–biotin was determined as the fluorescence at 620 nm came to 20,000 cps. A 10-μl portion of enzyme buffer or glycosaminoglycans (GAGs) diluted in the enzyme buffer was then added to each well. Finally, 10 μl of B16-BL6 cell extract diluted in the enzyme buffer was added to each well, and the plate was incubated at 37 °C for an appropriate period. After the incubation, 30 μl of 5000 ng/ml XL665-labeled streptavidin in HTRF buffer (phosphate buffer (pH 8.0; 0.1 M), 0.8 M potassium fluoride and 0.1% BSA) was added to the well. After incubation for 60 min at room temperature, the HTRF value ([ratio of fluorescence at 665 nm to that at 620 nm] × 10,000) in each well was measured using a Victor²™ multilabel counter (Wallac Oy and Perkin Elmer, Inc., Turku, Finland).

2.6. High-speed gel permeation chromatography

Enzyme reactions prepared in the same manner as the HTRF assay were stopped by addition of 1/10 volume of Tris–HCl (pH 9.0; 1 M) and then a 20-μl aliquot of each reaction was analyzed by HPLC system (Shimadzu, Kyoto, Japan) equipped with a TSK-gel super SW3000 column (4.6 mm × 30 cm, Tosoh, Tokyo, Japan). Chromatographic elution was performed with TBS at a flow rate of 0.3 ml/min and 0.5-min fractions were collected. A 50-μl portion of each fraction and a 10-μl portion of TBS containing 2.4 M KF and 1% BSA were added to each well of a 384-well assay plate, and then the fluorescence at 620 nm of each well was measured using a Victor²™ multilabel counter.

3. Results

In order to develop our heparanase assay using HTRF technology, the core protein of HSPG was digested with trypsin and biotinylated, and then europium cryptate was incorporated into HS by polysaccharide oxidation. The amounts of europium cryptate and biotin incorporated were 23.7 and 220 pmol per 1 μg of HSPG, respectively (1 molecule of europium cryptate and 10 molecules of biotin per 100 disaccharide units). To examine whether EuK–HSPG–biotin could be degraded by heparanase, the degradation of EuK–HSPG–biotin was analyzed by high-speed gel permeation chromatography. We utilized an extract prepared from highly invasive murine melanoma B16-BL6 cells as a source of heparanase [4]. EuK–HSPG–biotin was treated with 30 μg/ml of B16-BL6 cell extract at 37 °C for 4 h or 24 h and fractionated by TSK-gel super SW3000 column. Next, the fluorescence at 620 nm of europium cryptate in each fraction was measured to determine the EuK–HSPG–biotin content. The chromatographic profiles are shown in Fig. 2. The main peak of untreated EuK–HSPG–biotin was at an elution time of 7.5 min. The average molecular weight of intact EuK–HSPG–biotin estimated from chromatogram was around 70 kDa. After incubation with B16-BL6 cell extract, the original peak of EuK–HSPG–biotin decreased, while fragments of molecular weight ranges between 4 and 20 kDa increased in time-dependent manner. Tetra- or di-saccharides produced by bacterial heparitinase were eluted at around 15 min (data not shown). These results reflect the characteristic endoglycosidic degradation of EuK–HSPG–biotin by heparanase. The degradation rates of EuK–HSPG–biotin estimated from the decrease in the forward half area of the intact EuK–HSPG–biotin peak (shown in Fig. 2) were 47.0% after 4 h and 73.9% after 24 h.

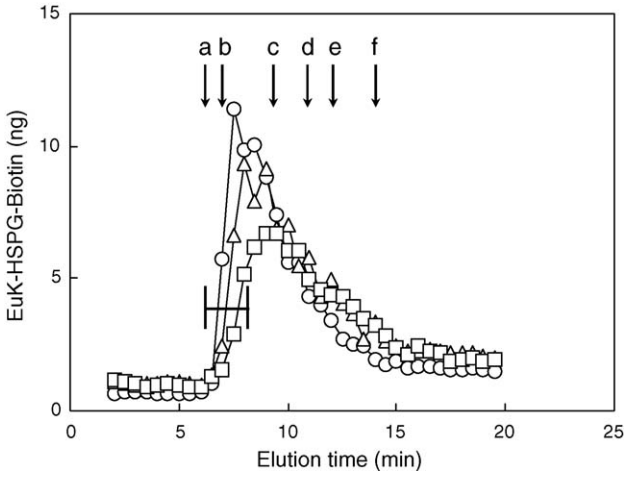

Fig. 2. Analysis of EuK–HSPG–biotin degradation by high-speed gel permeation chromatography. EuK–HSPG–biotin was incubated with 30 μg/ml of B16-BL6 cell extract for 0 h (◯), 4 h (△) and 24 h (□) at 37 °C. EuK–HSPG–biotin was fractionated using a TSK-gel super SW3000 column. The fluorescence intensity at 620 nm of each fraction was measured to determine the concentration of EuK–HSPG–biotin. Arrows (a–f) indicate the elution positions of FITC-labeled dextran: (a) $M_r \sim 250{,}000$; (b) $M_r \sim 70{,}000$; (c) $M_r \sim 40{,}000$; (d) $M_r \sim 20{,}000$; (e) $M_r \sim 10{,}000$ and (f) $M_r \sim 4000$. Bracket indicates 'forward half area' used for estimation of degradation rates.

Next, we tried to detect degradation of EuK–HSPG–biotin by B16-BL6 cell extract using HTRF. When EuK–HSPG–biotin was incubated with various amounts of B16-BL6 cell extract for up to 24 h, the HTRF values decreased from 6000 to around 3000

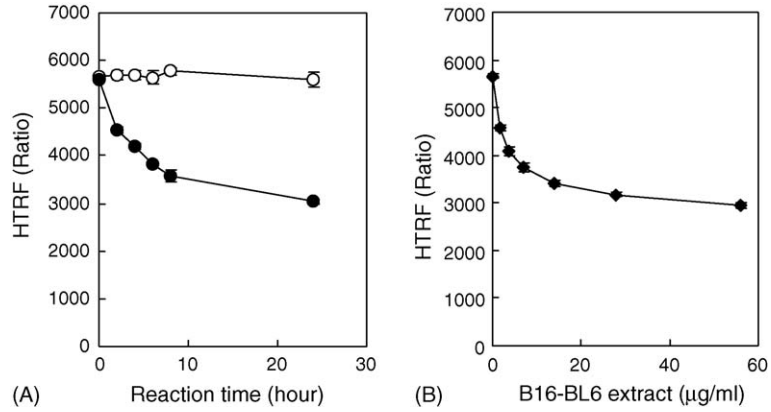

Fig. 3. Detection of heparanase activity by HTRF. (A) Time course of degradation. EuK–HSPG–biotin was incubated with (●) or without (◯) 30 μg/ml of B16-BL6 cell extract in a 384-well plate at 37 °C for indicated times. After addition of XL665-labeled streptavidin, the HTRF value of each well was measured. (B) Dose dependence of degradation. EuK–HSPG–biotin was incubated with different amounts of B16-BL6 cell extract at 37 °C for 24 h, and then the HTRF value of each well was measured. Each point represents a mean ± S.D. of three determinations.

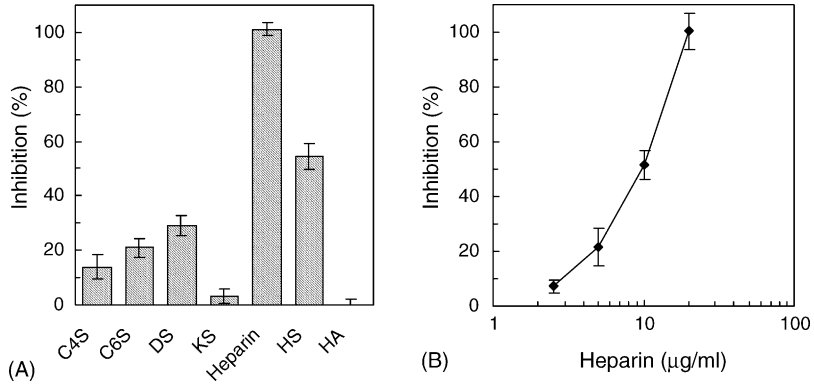

Fig. 4. Effect of GAGs on HTRF heparanase assay. EuK–HSPG–biotin was pre-mixed with 20 μg/ml of each GAG (A) or serial diluted heparin (B), and then incubated with 30 μg/ml of B16-BL6 cell extract at 37 °C for 24 h. After incubation, XL665-labeled streptavidin was added and the HTRF value of each well was measured. Each point represents a mean ± S.D. of three determinations.

in a time- and dose-dependent manner (Fig. 3). At 30 μg/ml of B16-BL6 cell extract, the reduction rates of HTRF values estimated from the results of Fig. 3 were 26.3% after 4 h and 45.7% after 24 h. In order to confirm the specificity of our HTRF assay, the inhibitory activities of various GAGs were examined. EuK–HSPG–biotin was mixed with 20 μg/ml of various GAGs followed by incubation with B16-BL6 cell extract for 24 h at 37 °C and then the HTRF of each well was measured. Heparin and HS showed strong inhibitory activities (100 and 60% inhibition, respectively). C4S, C6S and DS showed weak inhibitory activity (14.1–29.0%). KS and HA showed almost no inhibitory activities (Fig. 4A). The addition of heparin prevented reduction of the HTRF value by the B16-BL6 cell extract in a dose-dependent manner. The IC_{50} value was 8.8 μg/ml (Fig. 4B). These findings demonstrated that heparanase in the B16-BL6 cell extract caused signal reduction in our HTRF assay.

Finally, we validated the HTRF assay for detection of heparanase activity. In the case of 24 h reaction using 5.3 μg/ml B16-BL6 cell extract (160 ng cell protein per well), the Z' factor as an indicator of assay quality [24] was 0.69 ($n = 8$). When more than 5.3 μg/ml of the enzyme source was used, the Z' factor continually exceeded 0.50. Therefore, the lower detection limit of enzyme activity was estimated to be 160 ng as the protein content of the B16-BL6 cell extract. Compared with previous methods that required microgram order of cell protein [3–7], the amount required for our HTRF assay was much smaller. In an intra-plate assay, the average of the HTRF values and variation (CV) for negative controls ($n = 8$) in the absence of enzyme source were 6214 and 1.8%, respectively, and those for positive controls ($n = 8$) in the presence of 5.3 μg/ml of B16-BL6 cell extract were 4543 and 1.3%, respectively. The inter-plate variations (CVs) of negative controls and positive controls were 0.6 and 1.5%, respectively ($n = 3$).

4. Discussion

In order to enable measurement of heparanase activity more easily and rapidly than previous methods, we developed a heparanase assay using HTRF technology. At first, we prepared

HSPG, which was labeled with europium cryptate through primary amines generated by trypsin digestion and biotinylated through formyl groups formed by polysaccharide oxidation. However, we could not obtain stable HTRF values when it was reacted with XL665-labeled streptavidin (data not shown). In this type of substrate, several molecules of biotin seem to have been incorporated into one HS chain. As a result of each HS chain interacting with several streptavidin molecules, it was supposed that intermolecular multimetric complexes were formed. This complicated multimetric interaction may have caused the unstable HTRF value. Our solution was to label HSPG with europium cryptate and biotin using formyl groups formed by polysaccharide oxidation and primary amines generated by trypsin digestion, respectively. Mixing this substrate with XL665-labeled streptavidin led to a stable HTRF signal based on non-radiative energy transfer.

Periodate oxidation of HSPG might damage the recognition site of heparanase. However, high-speed gel permeation chromatography revealed that incubation with B16-BL6 cell extract cleaved EuK–HSPG–biotin into fragments characteristic of heparanase digestion. Incubation of EuK–HSPG–biotin with the B16-BL6 cell extract decreased the HTRF values in a time- and dose-dependent manner. Moreover, addition of heparin prevented reduction of the HTRF signal by the B16-BL6 cell extract in a dose-dependent manner. HTRF signals were completely recovered in the presence of 20 μg/ml heparin. The addition of HS impeded HTRF signal reduction, but its inhibitory effect was lower than heparin. Other GAGs (HA, KS, C4S, C6S and DS) had little or no effect on the HTRF assay. The results of these inhibition studies nearly correspond to those reported by Nakajima et al. [4]. These findings show that EuK–HSPG–biotin works well as a substrate for heparanase and our HTRF assay can specifically measure heparanase activity but not other enzymes such as exoglycosidase or protease present in the B16-BL6 cell extract.

In our HTRF assay, signal reduction rates did not correspond to the degradation rates of EuK–HSPG–biotin measured by high-speed gel permeation chromatography. Perhaps HTRF signals from cleaved substrates that have both europium cryptate and biotin may be in part responsible for the observed discordance between the two methods. The results from chromatography showed that the EuK–HSPG–biotin contained relatively lower molecular weight HSPG which might not be cleaved after the enzyme treatment. Therefore, HTRF signals from these lower molecules might also cause the inconsistency. If only relatively higher molecular weight HSPG (elution time at 7–8 min) is purified and used for HTRF assay, the HTRF values may decrease more sensitively in relation to the substrate degradation. These possibilities are under investigation. In the present method, since there is no linearity between reduction of HTRF signal and degradation of substrate, it is difficult to determine heparanase activity as units or any other kinetic parameters. However, our HTRF assay should be valuable for high-throughput detection of heparanase activity.

The validation study showed that the detection limit of heparanase activity was 160 ng as total cell protein at 24-h reaction. At the detection limit, although the HTRF values decreased by only 26%, the precision of the assay was satisfactory for identification of heparanase activity or screening of heparanase inhibitors. The HTRF assay was performed at pH 5.0, which was optimal for HSPG degradation. Under this condition, some cellular proteins precipitate [4]. The HTRF method enables minimization of signal variation related to light diffraction caused by insoluble particles. In addition, the signals measured by the HTRF assay were very stable for several days (data not shown).

In conclusion, we developed a simple and rapid heparanase assay using HTRF technology. Our HTRF assay uses only one 384-well assay plate in which both enzyme reaction and HTRF detection can be done, successively. Our heparanase assay is non-radioactive, simple and robust, and therefore makes possible the testing of large numbers of samples and application to robotic automation for screening heparanase inhibitors and discovering anti-cancer or immunosuppressive drugs.

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