

Sensitive Aggrecanase Activity ELISA

Catalog Number M046009

For the quantitative determination of aggrecanase activity.

For research use only.

This product insert must be read in its entirety before using this product.



SUMMARY AND EXPLANATION

Aggrecan is a large aggregating proteoglycan of articular cartilage [1]. It is found also in aorta tissue, discs, tendons [1] and in the perineuronal net [2]. The aggrecan core protein consists of 2317 amino acids [3]. Up to 130 glucosaminoglycan chains are attached to the core protein and the total molecular mass can reach $2.2 - 3.0 \times 10^6$ Daltons [4].

Within the aggrecan molecule 3 globular domains G1, G2 and G3 can be distinguished. Domains G1 and G2 are connected by a rod-shaped polypeptide called interglobular domain (IGD), while the sequence between domains G2 and G3 contains attachment regions for keratan sulfate and chondroitin sulfate chains. Aggrecan interacts via the G1 domain with hyaluronan and link protein to form large aggregates. Such aggregates can contain up to 50 -100 aggrecan monomers noncovalently bound to a single hyaluronan chain through 2 link proteins [1,4].

Aggrecan degradation is catalyzed by proteinases of the matrix metalloproteinase and ADAMTS (a disintegrin and metalloproteinase with thrombospondin motif) families. Aggrecan cleaving ADAMTS4 and ADAMTS5, named also aggrecanase 1 and aggrecanase 2, hydrolyze aggrecan at five different sites *in vitro* and *in vivo* [5,6,7,8]. Four cleavage sites are located in the chondroitin sulfate-rich region between aggrecan globular domains G2 and G3 (sites E₁₆₆₇ - G₁₆₆₈, E₁₄₈₀ - G₁₄₈₁, E₁₇₇₁ - A₁₇₇₂, E₁₈₇₁ - L₁₈₇₂), while one site is placed in the rodlike polypeptide between globular domains G1 and G2 (E₃₇₃ - A₃₇₄). In experiments with isolated proteins additional cleavage sites in aggrecan were identified both for ADAMTS4 [9] and ADAMTS5 [6]. A third proteinase of the ADAMTS family, ADAMTS1, also hydrolyses aggrecan at multiple sites including the unique site in the aggrecan interglobular domain [10]. The enzymatic activity of aggrecanases has been analyzed with isolated aggrecan preparations [11], recombinant aggrecan fragments [12] and a 41-residue peptide immobilized onto streptavidin-coated microplates [13]. The Aggrecanase Activity Assay provides an improved and ready-to-use method for quantitative determination of aggrecanase activity. Activities of pM concentrations of aggrecanase can best be measured with the Sensitive Aggrecanase Activity ELISA.

PRINCIPLE OF THE ASSAY

The Sensitive Aggrecanase Activity Assay measures activities of aggrecanases in the pM concentration range. This high sensitivity is achieved with an engineered aggrecanase substrate derived from aggrecan interglobular domain. It is used to measure aggrecanase activity in serum-free cell culture supernates. This assay consists of two modules, the Aggrecanase Module and the ELISA Module. The modified aggrecan interglobular domain (aggrecan-IGD-s) is first digested with aggrecanase. Proteolytic cleavage of the substrate releases an aggrecan peptide with the N-terminal sequence ARGSVIL (ARGSVIL-peptide-s). The ARGSVIL-peptide-s is then quantified with two monoclonal anti-peptide antibodies.

Aggrecanase Module: Proteolysis of aggrecan-IGD-s by aggrecanase

Aggrecan-IGD-s is incubated with standard aggrecanase and samples of unknown aggrecanase activity. In addition to aggrecanase, different concentrations of aggrecanase inhibitors can be added. The reaction is then stopped by dilution with EDTA-containing buffer.

ELISA module: Aggrecan peptide ELISA

ARGSVIL-peptide-s standard, proteolytic digestions of aggrecan-IGD-s with standard aggrecanase and samples are incubated in microtiter wells precoated with anti-ARGSVIL-neoepitope antibody. ARGSVIL-peptide-s is bound to the coated antibody, while other components are removed by washing and aspiration. The bound ARGSVIL-peptide-s is detected with a second peroxidase-labeled antibody. Any excess of the conjugate is removed by washing and aspiration. The amounts of peroxidase bound to different wells are determined in reactions with peroxidase substrate TMB. The reactions are stopped by addition of sulfuric acid solution and absorbance is read at 450 nm in a microtiter plate spectrophotometer.

Sample aggrecanase is evaluated by two methods:

1. The concentration of active aggrecanase in samples is calculated from the standard curve obtained with purified standard aggrecanase.
2. The amount of product ARGSVIL-peptide-s produced by aggrecanase is calculated from the standard curve of ARGSVIL-peptide-s.

The Aggrecanase Module provides chemicals for 50 proteolytic reactions, while the ELISA Module contains reagents for 96 determinations including reagents for 2 standard curves of ARGSVIL-peptide-s and 3 standard curves of proteolytic reactions with purified aggrecanase.

KIT COMPONENTS

Aggrecanase Module:

Aggrecan-IGD-s - Tris-buffered solution of 1 μ M aggrecan-IGD-s with additives and preservative. Ready to use.

ADAMTS4-Standard - Solution of 20 nM recombinant truncated human aggrecanase 1 (ADAMTS4 amino acids F₂₁₃-A₅₇₉ with C-terminal His-tag) with additives. Ready to use.

Inhibitor Solution - Inhibitor concentrate with 4 mM Pefabloc[®], 0.01 mM pepstatin, 0.01 mM leupeptin in 10 mM MES buffer, pH 6.0. Ready to use.

Reaction Buffer - Solution of 0.05 M Tris-HCl buffer pH 7.5, 0.15 M NaCl, 5 mM CaCl₂, 10 μ M ZnCl₂, 0.05 % Brij 35 with preservative. Ready to use.

EDTA Dilution Buffer - Solution of 0.01 M EDTA, 10 mg/ml bovine serum albumin, 0.15 M NaCl, 0.02 M Na-phosphate pH 7.2 with preservative. Ready to use.

ELISA Module:

Microtiter Plate - The plate contains 6 x 16 well strips coated with anti-neoepitope ARGSVIL-antibody. Ready for use.

ELISA Buffer - Solution of 10 mg/ml bovine serum albumin, 0.15 M NaCl, 0.02 M Na-phosphate pH 7.2 with preservative. Ready to use.

ARGSVIL-Peptide-s Standard - ARGSVIL-peptide-s at a concentration of 14 nM in EDTA-dilution buffer. Ready to use.

Antibody-Peroxidase Conjugate - Anti-aggrecan antibody coupled to horseradish peroxidase in stabilizer solution. The conjugate has to be diluted 100-fold prior to use.

Wash Buffer - Bottle contains 25 ml phosphate buffer concentrate which when diluted gives 0.02 M Na-phosphate buffer pH 7.2, 0.15 M NaCl, 0.05 % Tween 20.

Detection Solution TMB - Bottle contains 3, 3',5, 5'-tetramethylbenzidine (TMB)/hydrogen peroxide. Ready for use.

Sulfuric Acid - Bottle contains 0.25 M sulfuric acid. Ready to use.

Plate Sealer - 2 Adhesive Strips.

STORAGE

ELISA Module	Store at 2 - 8° C. Do not use past the kit expiration date.	
Aggrecanase Module	Store at -20° C. Do not use past the kit expiration date.	
Opened Reagents	Aggrecan-IGD-s	Store unused substrate at -20° C.
	ADAMTS4 Standard	Freeze remaining stock solution in liquid nitrogen to maintain temperatures below -20° C.
	Inhibitor Solution	Store unused inhibitor solution at -20° C.
	Reaction Buffer	Store remaining buffers at 2 - 8° C.
	EDTA-Dilution Buffer	
	ELISA Buffer	
	ARGSVIL Peptide-s Standard	Store undiluted standard at -20° C.
	Antibody Peroxidase Conjugate	Store undiluted conjugate at 2 - 8° C.
	Wash Buffer	Store at 2 - 8° C

SUPPLIES REQUIRED BUT NOT PROVIDED

- Water bath or thermoshaker at 37° C
- Spectrophotometer plate reader with 450 nm optic filter
- Pipettes or pipetting equipment with disposable polypropylene tips (10 µL, 100 µL, 1 mL)
- Disposable polypropylene test tubes
- Glass measuring cylinders
- Distilled or deionised water

PRECAUTIONS

Warning: Inhibitor solution contains 0.4 mM Pefabloc . The assay protocol requires the use of 0.25 M sulfuric acid. Wear eye, hand, face, and clothing protection when using these materials. All chemicals should be considered as being potentially hazardous. This product should be handled therefore only 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 case of contact with skin or eyes, wash immediately with water.

CRITICAL PARAMETERS

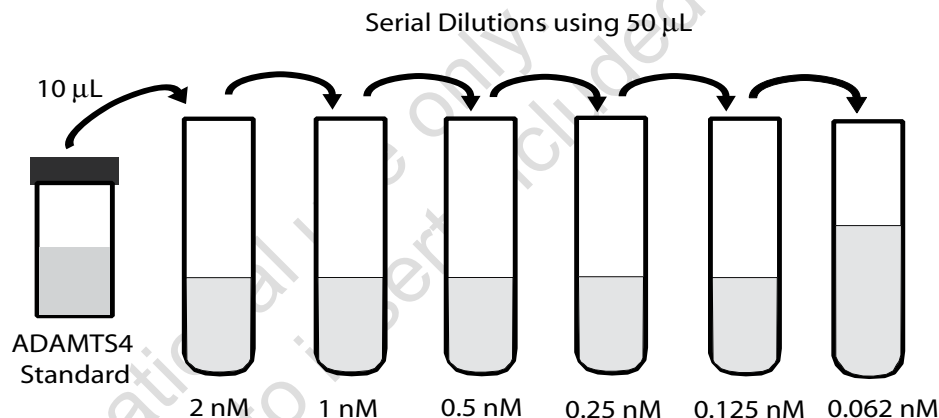
- **Precise temperature control is important:** Store reagents at recommended temperatures.
- **Keep enzyme dilutions on ice before starting proteolytic reactions.** Allow all reagents and samples for ARGSVIL-peptide-s ELISA to reach room temperature (22 - 25° C) prior to performing the assay.
- Follow suggested incubation times. Dispense indicated reagent volumes exactly.
- Mix samples and all reagents thoroughly before use.
- Avoid foaming of solutions.
- Wash all wells of the ELISA plate thoroughly and uniformly.
- Avoid touching the tops of ELISA plate wells before and after filling.
- Keep microtiter plate covered with foil except when adding reagents and reading.
- Pipette and measure standards and samples in duplicates.
- Avoid long dispensing times in ELISA steps.

REAGENT PREPARATION

Note: All reagents should be stored at the recommended temperatures. **Before starting the assay, frozen reagents should be thawed and kept on ice until use. Proteolytic reactions and the ELISA procedure should be carried out at the indicated temperatures.** Bring all other reagents to room temperature (22 - 25 °C) before use. Reagents can easily be trapped in the caps of small microfuge tubes. It is recommended to briefly centrifuge these reagents before use.

ADAMST4- Standard (For use in the aggrecanase activity module)

1. Label 6 polypropylene tube 2, 1, 0.5, 0.25, 0.125, and 0.062 nM.
2. Pipette 90 μ L of Reaction Buffer into the first 2 nM tube and 50 μ L of Reaction Buffer into all other tubes. **Place tubes on ice.**
3. Thaw ADAMTS4 Standard and add 10 μ L of standard to 90 μ L Reaction Buffer in the first 2 nM tube. Vortex mix.
4. Pipette 50 μ L from the 2 nM tube into the 1 nM tube. Vortex mix.
5. Pipette 50 μ L from the 1 nM tube into the 0.5 nM tube. Vortex mix.
6. Repeat the two-fold dilution step with the remaining tubes.
7. Aliquots of the serial dilutions give rise to 6 working standard concentrations of aggrecanase ranging from 2 nM to 0.062 nM.
8. A tube labeled "0" contains no aggrecanase and serves as control



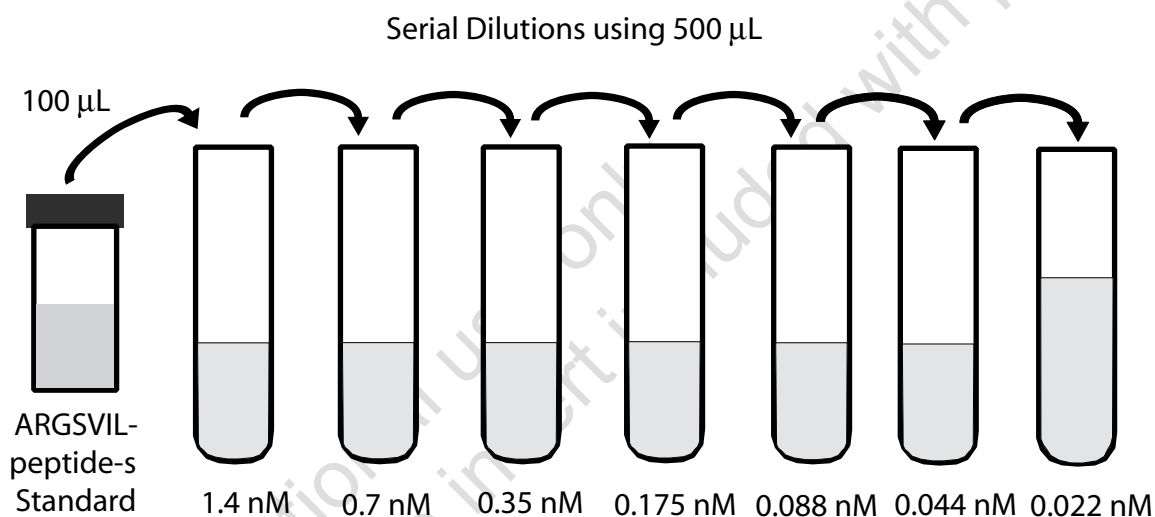
Substrate-Inhibitor mixture In proteolytic reactions 5 μ L of standard aggrecanase or sample are added to 95 μ L of substrate-inhibitor mixture in Reaction Buffer. The volume of the mixture prepared in advance depends on the number of proteolytic reactions to be carried out. For example, substrate-inhibitor mixtures for 10 and 50 proteolytic reactions are pipetted as follows:

Substrate-Inhibitor Mixture		
Reagents	10 reactions	50 reactions
Aggrecan-IGD-s	100 μ L	500 μ L
Inhibitor Solution	100 μ L	500 μ L
Reaction Buffer	750 μ L	3.75 mL

Mix substrate-inhibitor mixture thoroughly.

ARGSVIL-Peptide-s Standard (For use in the ELISA module)

1. Label 7 polypropylene tubes 1.4, 0.7, 0.35, 0.175, 0.088, 0.044 and 0.022 nM.
2. Pipette 900 μ L of ELISA Buffer into the first 1.4 nM tube and 500 μ L of ELISA Buffer into all other tubes. **Keep tubes at room temperature.**
3. Thaw ARGSVIL-Peptide Standard and add 100 μ L of standard to 900 μ L ELISA Buffer in the first 1.4 nM tube. Vortex mix.
4. Pipette 500 μ L from the 1.4 nM tube into the 0.7 nM tube. Vortex mix.
5. Pipette 500 μ L from the 0.7 nM tube into the 0.35 nM tube. Vortex mix.
6. Repeat the two-fold dilution step with the remaining tubes.
7. Aliquots from each serial dilution give rise to 7 working standard concentrations of aggrecan peptide ranging from 1.4 to 0.022 nM.
8. A tube labeled "0" contains no ARGSVIL-peptide-s and serves as a control.



Antibody Peroxidase Conjugate - Immediately before use, dilute the required amount of peroxidase conjugate 100-fold with ELISA buffer pre-equilibrated to room temperature.

Wash Buffer - Transfer the contents of the bottle to a 500 mL cylinder by repeated washing with distilled water. Adjust the final volume to 500 mL with distilled water and mix thoroughly.

ASSAY PROTOCOL

Read the entire protocol before beginning the assay. It is recommended that all standards and samples be assayed in duplicate. Reagents can easily be trapped in the caps of small microfuge tubes. It is recommended to briefly centrifuge these reagents before use. *Note: Reagents and samples may require specific handling temperatures and need preparation prior to the assay. See the Reagent and Sample Preparation sections before proceeding.*

Aggrecanase Reaction	<ol style="list-style-type: none"> 1. Label polypropylene tubes for 7 proteolytic reactions with standard aggrecanase and a defined number of reactions with samples. 2. Dispense 95 µL of substrate-inhibitor mixture into each labeled polypropylene tube. 3. Preincubate tubes for 5 minutes at 37° C in a water bath or thermoshaker. <p>Proteolytic Reaction</p> <ol style="list-style-type: none"> 4. Start proteolytic reactions by adding 5 µL of ADAMTS4 working standard or sample to the substrate-inhibitor mixture (<i>Final concentrations of standard aggrecanase in proteolytic reactions are 100, 50, 25, 12.5, 6.25, and 3.12 pM</i>). Additions should be made according to a strict time schedule every 30 seconds or every 1 minute. 5. Incubate proteolytic reactions for 15 min at 37° C. <p>Stop Reaction</p> <ol style="list-style-type: none"> 6. Stop proteolytic reactions after 15 min by pipetting 200 µL of EDTA Dilution Buffer into each tube. Buffer additions to different tubes should be done in the same order and according to the same time schedule as in step 4 to assure exactly 15 min duration for each proteolytic reaction. 7. Proceed directly to ELISA for ARGSVIL-peptide-s or store tubes at - 20° C.
ELISA for ARGSVIL-peptide	<ol style="list-style-type: none"> 1. Set up the microtiter plate with sufficient wells for standards and samples as required. Recommended positions of standard ARGSVIL-peptide-s (0 - 1.4 nM) are microtiter plate rows 1 and 2. Recommended positions for standard aggrecanase reactions (0 - 100 pM) are plate rows 3 and 4. All other wells can be used for sample reactions. <p>Standard/Sample Incubation</p> <ol style="list-style-type: none"> 2. Pipette 100 µL of each ARGSVIL-peptide-s working standard, standard aggrecanase reactions, and sample reactions into the appropriate wells. 3. Cover plate with foil provided and incubate for 90 min at 20° - 25° C on a microplate shaker. <p>Wash</p> <ol style="list-style-type: none"> 4. Aspirate and wash all wells 3 times with Wash Buffer. Make sure that wells are completely filled and emptied at each wash. Blot the plate on tissue paper to remove any residual liquid. <p>Antibody Incubation</p> <ol style="list-style-type: none"> 5. Pipette 100 µL of 100-fold diluted Antibody-Peroxidase Conjugate into all wells. 6. Cover the plate with foil and incubate for 90 min at 20 - 25° C on a microplate shaker. <p>Wash</p> <ol style="list-style-type: none"> 7. Aspirate and wash all wells 5 times with Wash Buffer. Make sure that wells are completely filled and emptied at each wash. Blot the plate on tissue paper to remove any residual liquid. <p>Substrate Incubation</p> <ol style="list-style-type: none"> 8. Immediately dispense 100 µL of Detection Solution TMB into all wells. 9. Cover the plate with foil and let stand for 30 minutes at room temperature in the dark. <p>Stop</p> <ol style="list-style-type: none"> 10. Stop peroxidase reactions by the addition of 100 µL of Sulfuric Acid to all wells and read the absorbance at 450 nm within 30 min. Use a reference filter of ≥ 620 nm.

SUMMARY

Part 1: AggreCanase Reaction

Prepare reagents of the AggreCanase module, the ADAMTS4 working standards and the substrate-inhibitor mixture.



Carry out proteolytic reactions (15 min) with the ADAMTS4 standards and samples.
Stop reactions with EDTA-Dilution Buffer.



Part 2: ELISA

Prepare the ELISA reagents and ARGSVIL-peptide-s working standards.



Pipette 100 μ L of standards and stopped proteolytic reactions in duplicates into wells of the microtiter plate. Incubate 90 min. at room temperature (22 - 25 $^{\circ}$ C) on a shaker.



Aspirate and wash 3 times.



Add 100 μ L of the diluted Antibody-Peroxidase Conjugate to each well. Incubate for 90 minutes at room temperature on a shaker.



Aspirate and wash 5 times.



Add 100 μ L of Detection Solution TMB to each well. Incubate for 30 minutes at room temperature in the dark.



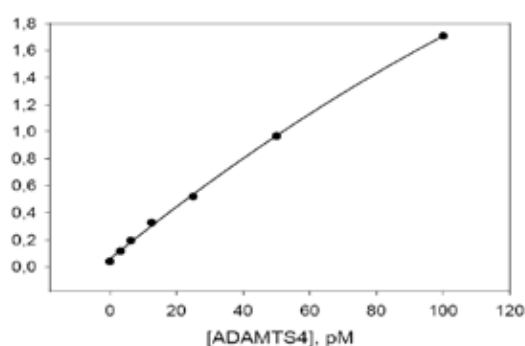
Add 100 μ L of Sulfuric Acid to each well. Read the absorbance within 30 min at 450 nm.

CALCULATION OF RESULTS

For the evaluation of sample aggrecanase activity, absorbance values of proteolytic reactions catalyzed by samples are compared with absorbance values of ADAMTS4 standard reactions. The specific activities of standard and sample aggrecanase are calculated from concentrations of product ARGSVIL-peptide-s formed during aggrecanase catalyzed reactions. The calculations are illustrated using representative data.

Estimation of the concentration of active aggrecanase in samples

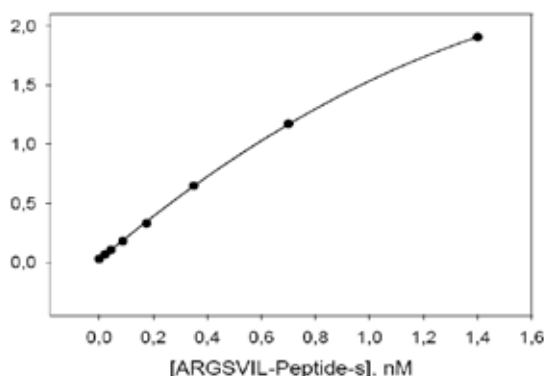
1. Calculate the average absorbance for each standard ADAMTS4 reaction. Absorbance data for reactions with standard ADAMTS4 should be similar to that shown below.
2. Plot the mean absorbance against the concentration of ADAMTS4 (pM). The curve shape should be similar to the curve shown below. (*This standard curve is provided for demonstration purposes only. A standard curve should be generated with each set of samples assayed*). The concentration of active sample aggrecanase equivalent to the activity of standard ADAMTS4 in proteolytic reactions can be read directly from the graph or calculated by regression analysis.
3. Multiply the concentration value by dilution factors to obtain the effective aggrecanase concentration in the original sample.



ADAMTS4 (pM)	O.D.		Average
0	0.042	0.037	0.040
3.125	0.120	0.112	0.116
6.25	0.190	0.200	0.195
12.5	0.328	0.327	0.328
25	0.498	0.543	0.520
50	0.966	0.969	0.968
100	1.677	1.739	1.708

Calculation of ARGSVIL-peptide-s concentration formed in proteolytic reactions

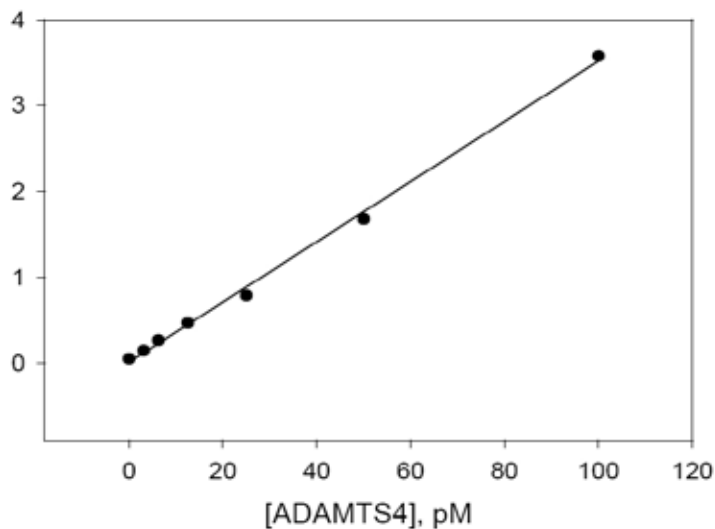
1. Calculate the average absorbance for each set of ARGSVIL-peptide-s standard wells. The data should be similar to that shown below.
2. Plot the mean absorbance against the concentration of ARGSVIL-peptide standard. The curve shape should be similar to the curve shown below. (*This standard curve is provided for demonstration purposes only. A standard curve should be generated with each set of samples assayed*). The concentrations of ARGSVIL-peptide-s corresponding to absorbances of aggrecanase reactions (from data above) can be read directly from the graph or can be calculated using appropriate computer software.
3. Multiply the concentration values for ARGSVIL-peptide-s by the dilution factor 3 to obtain the concentration of ARGSVIL-peptide-s formed in aggrecanase-catalyzed reactions.



ARGSVIL-Peptide-s (nM)	O.D.		Average
0	0.026	0.030	0.028
0.062	0.067	0.062	0.066
0.125	0.105	0.103	0.104
0.25	0.180	0.178	0.179
0.5	0.331	0.326	0.329
1	0.655	0.639	0.647
2	1.213	1.132	1.173
4	1.927	1.887	1.907

Calculation of specific activity of aggrecanase

The concentration of ARGSVIL-peptide-s formed in aggrecanase-catalyzed reactions is plotted against aggrecanase concentration (pM). The slope of the linear dependence gives the specific activity of truncated ADAMTS4 with aggrecan-IGD-s as a substrate. From the graph below, a value of 2.4 nM ARGSVIL-peptide-s/ min • nM ADAMTS4 is calculated. When related to mg enzyme the value is 60 nmoles ARGSVIL-peptide-s/ min • mg ADAMTS4.



PERFORMANCE CHARACTERISTICS

Specificity

The Sensitive Aggrecanase Activity Assay is specific for proteinases releasing peptides with the N-terminal sequence ARGSVIL from aggrecan-IGD-s. The aggrecanase standard provided in the assay is recombinant truncated human ADAMTS4. However, activities of human ADAMTS1 and ADAMTS5 can also be measured. Proteinases cleaving aggrecan-IGD-s at sites other than the aggrecanase site are not detected. For example, peptides produced by MMP 13 or MMP 14 catalytic domain do not give absorbance values above control levels in the ELISA.

Sensitivity

The sensitivity of the detection of recombinant truncated ADAMTS4, defined as two standard deviations above the mean of calculated concentrations of 40 blank replicates, was determined as 2 pM ADAMTS4.

Reproducibility

Reproducibility has been evaluated both for ELISA of ARGSVIL-peptide standard and for proteolytic reactions of standard aggrecanase. In the following only data for reproducibility of aggrecanase standards are given.

Intra-assay Precision (Precision within an assay) - The intra-assay precision was measured by assaying three control samples 16 times on one plate.

Inter-assay Precision (Precision between assays) - The inter-assay precision was assessed by repeated measurements of three control samples in successive assays.

Control	Intra-assay Precision			Inter-assay Precision		
	A	B	C	A	B	C
n	12	12	12	12	12	12
Mean (pM)	11.33	23.92	49.69	10.77	23.58	48.93
Standard Deviation	1.30	1.70	3.36	1.39	2.54	4.13
CV (%)	11.5	7.1	6.8	12.9	4.2	8.4

Precision profile - The precision profile was calculated as % CV from the mean and standard deviation of absorbance for each aggrecanase standard reaction.

ADAMTS4 (pM) in standard reactions	O.D. Mean \pm SD	% CV	n
0	0.067 \pm 0.013	19.4	12
3.12	0.140 \pm 0.028	20.0	12
6.25	0.255 \pm 0.056	22.0	12
12.5	0.370 \pm 0.044	11.9	12
25	0.638 \pm 0.057	8.9	12
50	1.060 \pm 0.052	4.9	12
100	1.763 \pm 0.096	5.4	12

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