

Aggrecanase Activity Assay

(Cat. No.: 30510111)



For quantitative determination of aggrecanase activity
For screening and evaluation of aggrecanase inhibitors

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Short Review: Aggrecan and Aggrecanases

Aggrecan is a large aggregating proteoglycan of articular cartilage [1]. It is found also in aorta tissue, discs, tendon [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 rod-like 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 sensitive determination of aggrecanase activity.

- 1) Knudsen, C.B. and Knudsen, W. (2001) *Seminars Cell & Developm. Biol.* **12**, 69-78; 2) Matthews, R.T. et al. (2002) *J. Neuroscience* **22**, 7536-7547; 3) Doege, K.J. et al. (1991) *J. Biol. Chem.* **266**, 894-902; 4) Hardingham, T.E. and Fosang, A.J. (1992) *FASEB J.* **6**, 861-870; 5) Tortorella, M.D. et al. (2000) *J. Biol. Chem.* **275**, 18566-18573; 6) Tortorella, M.D. et al. (2002) *Matrix Biology* **21**, 499-511; 7) Lohmander, L.S. et al. (1993) *Arthritis Rheumat.* **36**, 1214-1222; 8) Malfait, A.-M. et al. (2002) *J. Biol. Chem.* **277**, 22201-22208; 9) Westling, J. et al. (2002) *J. Biol. Chem.* **277**, 16059-16066; 10) Rodriguez-Manzaneque, J. C. et al. (2002) *Biochem. Biophys. Res. Commun.* **293**, 501-508; 11) Gao, G. et al. (2002) *J. Biol. Chem.* **277**, 11034-11041; 12) Hughes, C.E. et al. (1997) *J. Biol. Chem.* **272**, 20269-20274; 13) Miller, J.A. et al. (2003) *Analyt. Biochem.* **314**, 260-265.

Assay Description

The Aggrecanase Activity Assay measures activity of aggrecanases. It is used to screen and characterize aggrecanase inhibitors. It consists of two modules: Aggrecanase module and the ELISA module. In this Assay a recombinant fragment of human aggrecan interglobular domain (aggrecan-IGD) is first digested with aggrecanase (ADAMTS4). The proteolytic cleavage of the substrate releases an aggrecan peptide with the N-terminal sequence ARGSVIL (ARGSVIL-peptide). The ARGSVIL-peptide-s is then quantified with two monoclonal anti-peptide antibodies (Fig. 1).

Aggrecanase module: Proteolysis of aggrecan-IGD by Aggrecanase

Aggrecan-IGD (provided with this kit) is incubated with standard aggrecanase (ADAMTS4) and samples of unknown aggrecanase activity. In addition to aggrecanase, different concentrations of aggrecanase inhibitors can be added. The reaction is stopped by dilution with EDTA-containing buffer.

ELISA module: aggrecan peptide ELISA

ARGSVIL-peptide standard, proteolytic digested aggrecan-IGD with standard aggrecanase and test samples are incubated in microtiter wells pre-coated with anti-ARGSVIL-neoepitope antibody. ARGSVIL-peptide is bound to the coated antibody, while other components are removed by washing and aspiration. The bound ARGSVIL-peptide 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.

Test sample aggrecanase is evaluated by two methods:

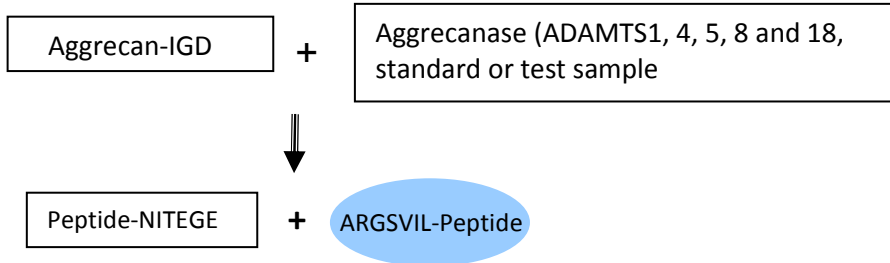
- 1.** The concentration of active aggrecanase in test samples is calculated from the standard curve obtained with purified standard aggrecanase.
- 2.** The amount of product ARGSVIL-peptide produced by aggrecanase is calculated from the standard curve of ARGSVIL-peptide.

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 and 3 standard curves of proteolytic reactions with purified aggrecanase.

Assay Overview

1) PROTEOLYSIS OF AGGREGAN-IGD BY AGGREGANASE

a) Incubation of aggrecan-IGD with aggrecanase: (duration: ca. 15 min)

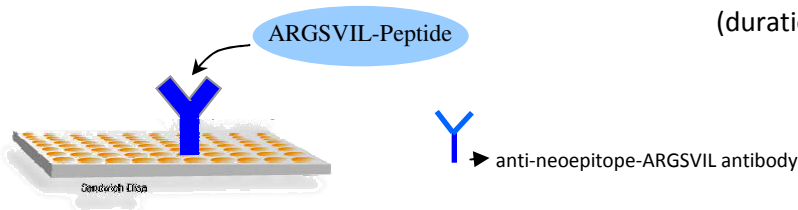


b) Addition of EDTA-dilution buffer

2) ELISA FOR ARGSVIL-PEPTIDE

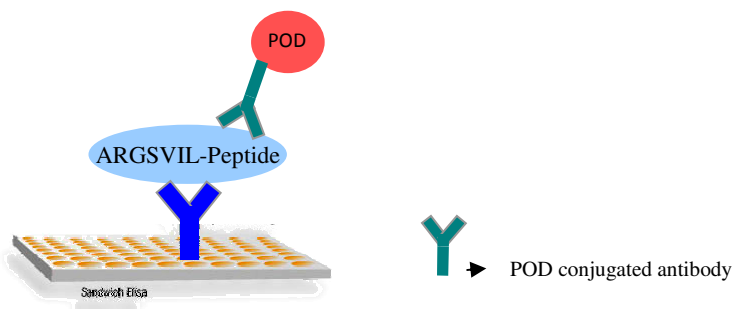
a) Binding of ARGSVIL-peptide to anti-neoepitope-ARGSVIL antibody:

(duration: ca. 90 min)



b) Binding of POD-conjugated anti-aggrecan antibody

(duration: ca. 90 min)



c) Addition of TMB and colour development

(duration: 30 min)

Fig. 1: Overview of Aggrecanase Activity Assay. POD: Peroxidase

Components of Assay System

AGGRECANASE MODULE

Aggrecan-IGD: Tris-buffered solution of 1 μM aggrecan-IGD with additives and preservative. Ready to use.

ADAMTS4-Standard: Solution of 0.3 μM recombinant truncated human aggrecanase 1 (ADAMTS4 amino acids F₂₁₃ - A₅₇₉ with C-terminal His-tag) with additives. Ready to use.

Inhibitor Solution: Inhibitor concentrated 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 μM 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 to 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 Standard: ARGSVIL-peptide at a concentration of 40 nM in EDTA-dilution buffer. Ready to use.

Antibody-Peroxidase Conjugate: Anti-aggrecan antibody coupled to horseradish peroxidase in stabilization 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 to use.

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

EQUIPMENTS REQUIRED BUT NOT PROVIDED

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

Safety Warning and 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 ELISA to reach 20°C - 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 and Storage

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. Attention should be given to:

- preequilibration and maintenance of proteolytic reactions at 37°C
- equilibration of all ELISA reagents to a temperature of 20°C - 25°C

AGGRECANASE MODULE

Aggrecan-IGD: Thaw and keep it on ice until use. Freeze remaining substrate again and store at - 20 °C.

ADAMTS4-Standard: Thaw for preparation of aggrecanase working standards. Freeze remaining stock solution in dry ice or liquid nitrogen and keep it at - 20 °C or -80 °C.

Inhibitor Solution: Thaw and keep it on ice until use. Freeze remaining solution again and store at - 20°C.

Reaction Buffer: Store at 4°C.

EDTA-Dilution Buffer: Store at 4°C.

ELISA MODULE

ELISA Buffer: Store at 4°C.

ARGSVIL-Peptide Standard: Thaw solution for preparation of ARGSVIL-peptide working standards. Freeze the remaining undiluted peptide standard and store at - 20°C.

Antibody Peroxidase Conjugate: Store undiluted antibody-peroxidase conjugate at 4°C. Immediately before use, dilute the required amount of peroxidase conjugate 100-fold with ELISA buffer pre equilibrated to 20°C.

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. Store diluted wash buffer at 4°C.

Assay Procedure Part 1: Aggrecanase Reaction

Preparation of ADAMTS4 working standards

1. Label 8 polypropylene tubes 30, 15, 7.5, 3.75, 1.88, 0.94, 0.47 and 0* nM.
2. Pipette 90 μ l Reaction Buffer into the 30 nM tube and 50 μ l of Reaction Buffer into all other tubes. Place tubes on ice.
3. Thaw and add 10 μ l of ADAMTS4 Standard to the 90 μ l Reaction Buffer (30 nM tube). Vortex.
4. Make a serial dilution pipetting 50 μ l from the 30 nM tube into the 15 nM tube. Continue the two-fold dilution with the remaining tubes except with tube 0. Vortex in between. Aliquots of the serial dilutions give rise to 8 working standard concentrations of aggrecanase ranging from 30 nM to 0.00 nM.

Proteolysis of aggrecan-IGD by standard and test sample

1. Prepare the substrate-inhibitor mixture as below:

Reagents	For 10 reactions	For 50 reactions
Aggrecan-IGD (1 μ M)	100 μ l	500 μ l
Inhibitor Solution	100 μ l	500 ml
Reaction Buffer	750 μ l	3.75 ml

5. Mix substrate-inhibitor mixture thoroughly and pre-incubate tubes for 5 minutes at 37°C in a water bath or thermoshaker.
6. Label polypropylene tubes for 8 proteolytic reactions for standard aggrecanase and a defined number for reactions with the test samples.
7. Dispense 95 μ l of substrate-inhibitor mixture into each labeled tube.
8. Start proteolytic reactions by adding 5 μ l of ADAMTS4 working standard or test sample to the substrate-inhibitor mixture. Additions should be done according to a strict time schedule, i.e. every 30 seconds or every 1 minute.
9. Incubate proteolytic reactions for 15 min at 37°C.
10. Stop proteolytic reactions after 15 min by pipetting 150 μ l of EDTA Dilution Buffer into each tube. Buffer additions to different tubes should be done in the same succession and according to the same time schedule as in step 4 to assure exactly 15 min duration for each proteolytic reaction.
11. End concentrations of standard Aggrecanase (ADAMTS4) in proteolytic reactions are going to be 1.5, 0.75, 0.375, 0.188, 0.094, 0.047, 0.024 and 0* nM
12. Proceed directly to ELISA for ARGSVIL-peptide or store tubes at - 20°C.

* Tube "0" contains no aggrecanase and serves as blank control.

Assay Procedure Part 2: ELISA

Preparation of ARGSVIL-peptide working standards for standard curve

1. Label 8 polypropylene tubes with 4, 2, 1, 0.5, 0.25, 0.125, 0.062 and 0* nM.
2. Pipette **900 µl of ELISA Buffer into the first 4 nM tube and 500 µl of ELISA Buffer into all other tubes**. Keep tubes at room temperature.
3. Thaw and add 100 µl ARGSVIL-Peptide Standard to the 900 µl ELISA Buffer in the first 4 nM tube. Vortex.
4. Pipette 500 µl from the 4 nM tube into the 2 nM tube. Continue two-fold dilution step with the remaining tubes as done with previous one.

* Tube "0" contains no ARGSVIL-peptide and serves as control.

ELISA for ARGSVIL-peptide

1. Dilute Wash Buffer as described in page 7.
2. Set up the microtiter plate with sufficient wells for standards and test samples as required. Recommended positions of standard ARGSVIL-peptide (0 - 4 nM) are microtiter plate rows 1 and 2. Recommended positions for standard aggrecanase reactions (0 - 1.5 nM) are plate rows 3 and 4. All other wells can be used for test sample reactions.
3. Pipette 100 µl of each ARGSVIL-peptide working standard into the appropriate wells.
4. Pipette 100 µl of each standard aggrecanase reaction into the appropriate wells.
5. Pipette 100 µl of test sample reactions into remaining wells.
6. Cover plate with foil provided and incubate it for 90 min at 20°C - 25°C on a microplate shaker.
7. Aspirate and wash all wells 3 times with Wash Buffer. It is absolutely important that all wells are washed thoroughly and uniformly. When washing is done by hand, ensure that all wells are completely filled and emptied at each step by discarding the contain of the plate with forceful motion and drying by tapping the inverted plate on dry absorbent surface (see technical advice on the following link: <http://www.youtube.com/watch?v=FZirnCas17Y>)
8. Pipette 100 µl of 100-fold diluted Antibody-Peroxidase Conjugate into all wells.
9. Cover the plate with foil and incubate for 90 min at 20°C - 25°C on a microplate shaker.
10. Aspirate and wash all wells 5 times with Wash Buffer (see point 7).
11. Immediately dispense 100 µl of room temperature equilibrated Detection Solution TMB into all wells.
12. Cover the plate with foil and let it 5-30 minutes at room temperature in the dark.
13. 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.

Assay Protocol 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 ADAMTS4 working standards and test samples. Stop reactions with EDTA-Dilution Buffer



Part 2: ELISA

Prepare reagents of the ELISA Module and ARGSVIL-peptide 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 on a shaker



Aspirate and wash 3 times



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



Aspirate and wash 5 times



Add 100 μ l of Detection Solution TMB to each well. Incubate for 5-30 min at room temperature **in the dark**



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

Data Processing

For the evaluation of test sample aggrecanase activity, absorbance values of proteolytic reactions catalyzed by test 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 formed during aggrecanase-catalyzed reactions. The calculations are illustrated using representative data.

Estimation of the concentration of active test sample aggrecanase

1) Calculate the average absorbance for each standard ADAMTS4 reaction. Absorbance data for reactions with standard ADAMTS4 should be similar to that shown in Table 1.

ADAMTS4 (nM) in standard reactions	OD Range (450 nm)	Average
0	0.040 - 0.035	0.038
0.024	0.066 - 0.067	0.067
0.047	0.097 - 0.097	0.097
0.094	0.175 - 0.157	0.166
0.188	0.297 - 0.303	0.300
0.375	0.605 - 0.570	0.588
0.75	1.159 - 1.183	1.171
1.5	2.155 - 2.055	2.105

Table 1: Typical assay data for standard aggrecanase reactions

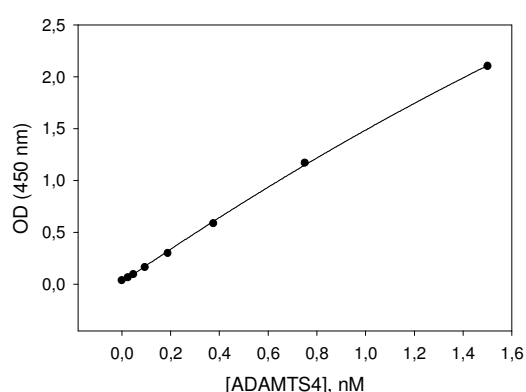


Fig. 2: Typical ADAMTS4 standard curve

2) Plot the mean absorbance against the concentration of ADAMTS4 (nM). The curve shape should be similar to fig. 2. The concentration of active test 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 (in case you have diluted your test sample before) to obtain the effective aggrecanase concentration in the original test sample.

Calculation of ARGSVIL-peptide concentration formed in proteolytic reactions

1) Calculate the average absorbance for each set of ARGSVIL-peptide standard wells. The data should be similar to that shown in Table 3.

2) Plot the mean absorbance against the concentration of ARGSVIL-peptide standard. The curve shape should be similar to fig. 3. The concentrations of ARGSVIL-peptide corresponding to absorbencies of aggrecanase reactions (Table 1, fig. 2) can be read directly from the graph or can be calculated using appropriate computer software.

3) Multiply the concentration values for ARGSVIL-peptide by the dilution factor 2.5 to obtain the concentration of ARGSVIL-peptide formed in aggrecanase-catalyzed reactions.

ARGSVIL-peptide (nM)	OD (450 nm)	Average
0	0.037- 0.042	0.040
0.062	0.070 - 0.071	0.071
0.125	0.103 - 0.118	0.111
0.25	0.171 - 0.196	0.184
0.5	0.284 - 0.312	0.298
1	0.580 - 0.639	0.610
2	1.118 - 1.252	1.185
4	1.915 - 2.231	2.073

Table 2: Typical data for ARGSVIL-peptide standard

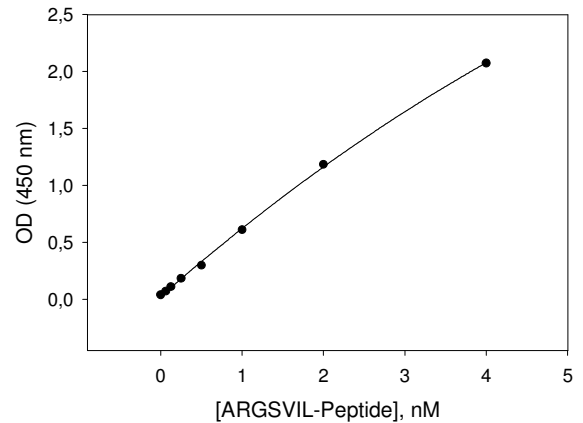


Fig. 3: Typical standard curve for ARGSVIL-peptide.

Calculation of specific activity of aggrecanase

The concentration of ARGSVIL-peptide formed in aggrecanase-catalyzed reactions is plotted against aggrecanase concentration (nM). For standard ADAMTS4 the relation should be linear in the initial part of the reaction (10 - 15 % cleavage of aggrecan-IGD) and similar to that shown in Fig. 4. The slope of the linear dependence gives the specific activity of truncated ADAMTS4. From Fig. 4 a value of 0.45 nM ARGSVIL-peptide/ min · nM ADAMTS4 is calculated. When related to mg enzyme the value is 11.28 nmoles ARGSVIL-peptide/ min · mg ADAMTS4.

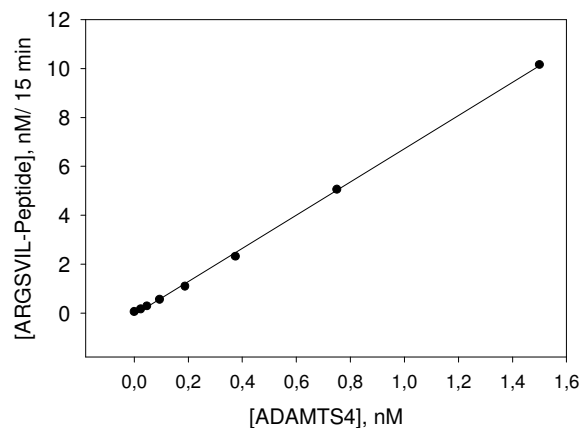


Fig. 4: Activity of recombinant truncated human ADAMTS4

Additional Information

Specificity - The Aggrecanase Activity Assay is specific for proteinases releasing peptides with the N-terminal sequence ARGSVIL from aggrecan-IGD. 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 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 0.012 nM ADAMTS4. The sensitivity of the detection of ARGSVIL-peptide was determined as 0.025 nM.

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

Within assay precision - The within assay precision was measured by assaying three control samples 16 times on one plate.

Control	Mean (nM)	Standard deviation	CV %	N
A	0.161	0.016	9.9	16
B	0.341	0.026	7.5	16
C	0.737	0.040	5.4	16

Table 3: Within assay precision for standard aggrecanase

Between assay precision - The between assay precision was assessed by repeated measurements of three control samples in successive assays.

Control	Mean (nM)	Standard deviation	CV %	N
A	0.200	0.026	13.0	18
B	0.385	0.035	9.1	18
C	0.814	0.079	9.7	18

Table 4: Between assay precision for standard aggrecanase

Precision profile

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

ADAMTS4 (nM) in standard reactions	OD (450 nm) Mean \pm SD	% CV	N
0	0.036 \pm 0.003	8.3	12
0.024	0.067 \pm 0.004	6.0	12
0.047	0.099 \pm 0.012	12.1	12
0.094	0.169 \pm 0.013	7.7	12
0.188	0.303 \pm 0.022	7.3	12
0.375	0.584 \pm 0.056	9.6	12
0.75	1.083 \pm 0.075	6.9	12
1.5	2.008 \pm 0.156	7.8	12

Table 5: Precision profile

Troubleshooting

Problem	Recommendation
Low absorbance	<ul style="list-style-type: none">• Check reagents for proper storage Control expiration date.• Check preparation of reagents• Control incubation times and temperature• Equilibrate ELISA reagents to room temperature• Check reader wavelength
High absorbance/ high zero standard value	<ul style="list-style-type: none">• Check preparation of reagents• Control incubation times and temperature• Equilibrate ELISA reagents to room temperature• Ensure that every well of the ELISA plate is completely filled and emptied at every wash step• Check that plates are blotted on tissue paper after washing
Flat curve/ poor reproducibility	<ul style="list-style-type: none">• Check reagents for proper storage Control expiration date• Check preparation of working standards• Check incubation times and temperature• Use separate reservoirs for pipetting different solutions with multichannel pipettes. Always use new pipette tips• Check pipette calibration• Ensure sufficient washing procedure

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