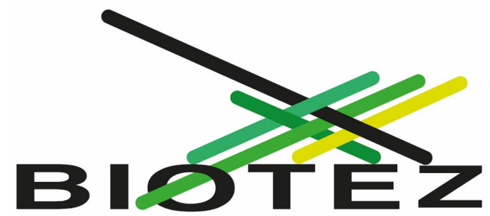
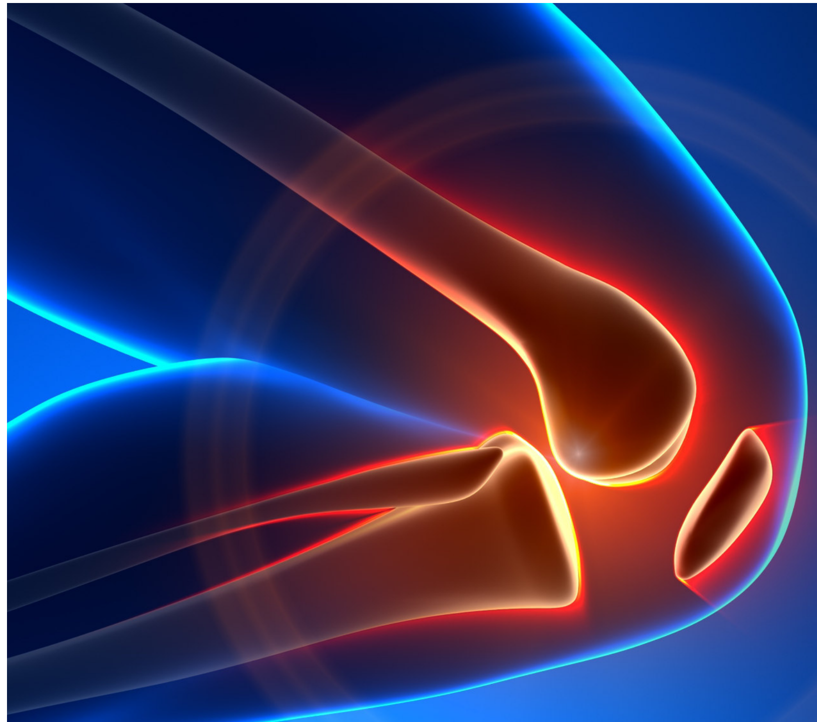


Universal Protease Activity ELISA

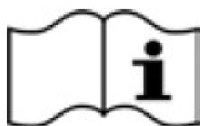
REF Cat. No.: 30516103



Quantification of protease activity
(based on HtrA1 as standard enzyme and ß-Casein as substrate)

For screening and evaluation of protease inhibitors

For research use only
Not for diagnostic use



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This manual is valid from November, 2015

Short Review: Proteases

Proteases play important roles in the control of multiple biological processes in all living organisms. They regulate the activities of many proteins, modulate protein-protein interactions, create new bioactive molecules, contribute to the processing of cellular information and generate, transduce and amplify molecular signals. As a direct result of these multiple actions, proteases influence DNA replication and transcription, cell proliferation and differentiation, tissue morphogenesis and remodelling, heat shock and unfolded protein responses, angiogenesis, neurogenesis, ovulation, fertilization, wound repair, stem cell mobilization, haemostasis, blood coagulation, inflammation, immunity, autophagy, senescence, necrosis and apoptosis. Consistent with these essential roles of proteases in cell behaviour, survival and death of all organisms, alterations in proteolytic systems underlie multiple pathological conditions such as cancer, neurodegenerative disorders, inflammatory and cardiovascular diseases.

Biotez Universal Protease Activity ELISA is based on a universal substrate for proteases, beta casein which is labeled with biotin and coupled on beads. The cleaved fragments of biotinylated beta casein are detected with an enzyme linked immuno assay (ELISA).

The combinations of bead technology and ELISA have a synergistic effect.

The sensitivity and specificity via antibodies is a feature of ELISA and the beads have no scale limit and can be used in variable volumes.

Assay Description

The Universal Protease Activity ELISA measures activity of proteases like matrix metalloproteases (MMPs), **A** **D**isintegrin **A**nd **M**etalloproteinase with **T**hrombo**s**pondin Motifs (ADAMTS), especially High temperature requirement factor A (HtrA). It consists of two modules: Protease module and the ELISA module. In this assay, a biotinylated ß-Casein coupled beads is first digested with protease(s)/HtrA1. The proteolytic cleavage of the substrate releases a biotinylated ß-Casein fragment, without beads, that will bind on streptavidin-coated plate and can be further quantified with one specific monoclonal anti-ß-Casein antibodies and with a Peroxidase-labeled antibody (Fig. 1). The kit uses HtrA1 as standard protease and biotinylated ß-Casein as substrate.

Protease module: Proteolysis of Biotinylated-ß-Casein by Protease

Biotinylated ß-Casein coupled beads (provided with this kit) are incubated with standard protease (HtrA1) and samples of unknown protease activity. The reaction is stopped by dilution with corresponding buffer.

ELISA module: Biotinylated-ß-Casein Fragment ELISA

Biotinylated ß-casein-fragment-standard, proteolytic digested biotinylated ß-casein coupled beads with standard HtrA1 and test samples are incubated in microtiter wells precoated with streptavidin. Biotinylated ß-casein-fragment is bound to the coated plate, while other components are removed by washing and aspiration. The bound biotinylated ß-casein-fragment is detected first with a ß-casein specific antibody and with a secondary 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 stop solution and absorbance is read at 450 nm in a microtiter plate spectrophotometer.

Test sample protease is evaluated by two methods:

1. The concentration of active protease(s) in test samples is calculated from the calibration curve obtained with purified enzyme-standard HtrA1.

2. The amount of biotinylated ß-casein-fragment produced by protease(s) is calculated from the standard curve of fragment standard.

The protease module provides reagents for 35 proteolytic reactions, while the ELISA module contains reagents for 96 determinations including reagents for 4 standard curves of fragment standard and 3 standard curves of proteolytic reactions with purified HtrA1-enzyme-standard.

Assay Overview

1) PROTEOLYSIS OF BIOTINYLATED ß-CASEIN COUPLED BEADS BY HtrA1

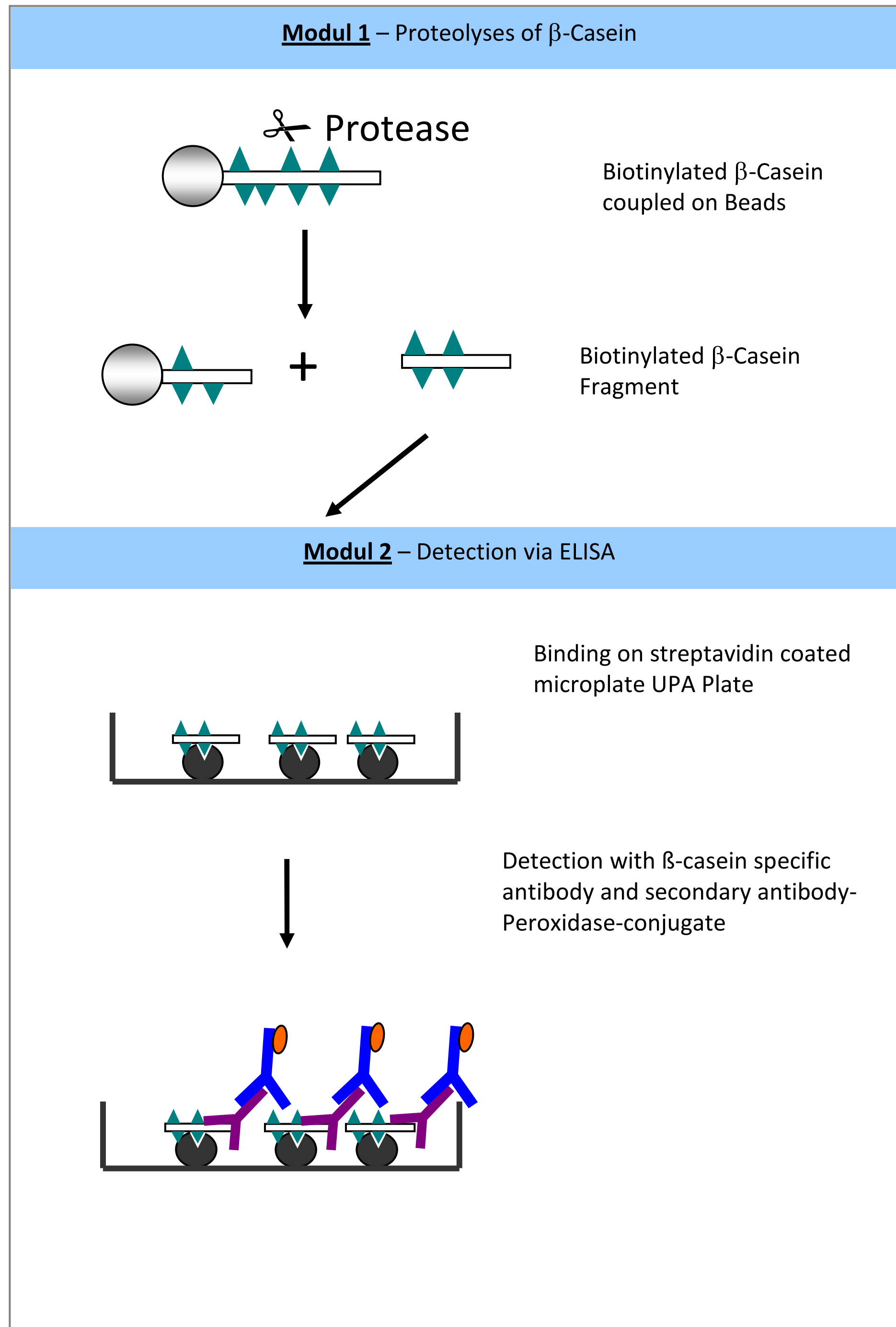


Fig. 1: Overview of Universal Protease Activity ELISA.
POD: Peroxidase

Components of Assay System

	quantity (tubes)	Vol. /tube	Concentration	Ready to use (Yes/No)	Dilution	Storage temperature
BA-ß-casein-beads*	35	90 µl	0.2 µM	Yes	-	- 20°C
HtrA1-Enzyme Standard	3	2 µl	4 µM	Yes	-	- 20°C
Buffer for HtrA1-Enzym-Standard	1	1 ml	-	Yes	-	- 20°C
Detection Buffer	2	25 ml	-	Yes	-	2-8°C
UPA Plate	1	6 x 16 well strips	-	Yes	-	2-8°C
TW-Buffer	1	12 ml	-	Yes	-	2-8°C
Fragment-Standard**	1	1 ml	5 nM	Yes	-	- 20°C
Antibody Solution HH1	1	150 µl	-	No	100x	2-8°C
Antibody Solution AM	1	150 µl	-	No	100x	2-8°C
Wash Buffer	1	25 ml	-	No	20x	15-30°C
TMB Substrate	1	12 ml	-	Yes	-	2-8°C
Stop solution	1	12 ml	-	Yes	-	15-30°C

EQUIPMENTS REQUIRED BUT NOT PROVIDED

- 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 1 ml)
- Disposable polypropylene test tubes
- Glass measuring cylinders
- Distilled or deionised water

Safety Warning and Precautions

Warning: 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.

* BA-ß-casein-beads = Biotinylated- ß-casein-beads
** Fragment standard = Biotinylated- ß-casein-Fragment Standard

Critical Parameters

- Precise temperature control is important: Store reagents at recommended temperatures.
- Keep enzyme dilutions on ice before starting proteolytic reactions.
- 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.

PROTEASE MODULE

Biotinylated-ß-casein-beads: Store at -20°C. Thaw only the tubes that should be used and keep on ice until use. Do not freeze the remaining substrate again.
HtrA1-Enzyme-Standard: Store at -20°C. Thaw one tube on ice and centrifuged for one minute. Do not freeze the remaining stock solution after preparation of HtrA1 working standards. Another two separate tubes are available for another two standards preparation.
Buffer for HtrA1-Enzym Standard: Store at -20°C. Thaw the buffer and vortex in order to solve the precipitated salts.
Detection Buffer: Store at 4°C.

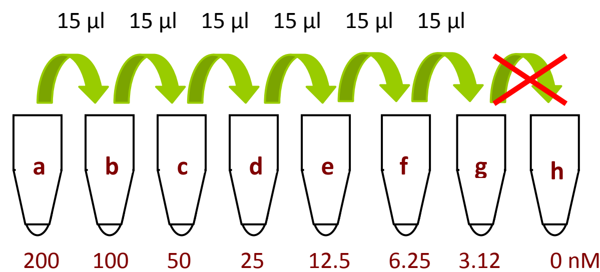
ELISA MODULE

UPA Plate: Store at 4°C; pre equilibrate at 20-25 °C (room temperature), before use.
Detection Buffer: Store at 4°C; pre equilibrate at 20-25 °C, before use.
TW-Buffer: Store at 4°C; pre equilibrate at 20-25 °C, before use.
Fragment-Standard: Thaw and keep it on ice until use. Freeze the remaining fragment-standard again and store at -20°C.
Antibody Solution HH1: Store undiluted antibody at 4°C. Immediately before use, dilute the required amount of peroxidase conjugate 100-fold with Detection Buffer pre equilibrated to room temperature.
Antibody Solution AM: Store undiluted antibody at 4°C. Immediately before use, dilute the required amount of peroxidase conjugate 100-fold with Detection Buffer pre equilibrated to room temperature.
Wash Buffer 20x*:* Store undiluted Wash Buffer at room temperature. Immediately before use, dilute the required Wash Buffer 20-fold with distilled water. Store the diluted wash buffer at 4°C.
TMB Substrate: Store at 4°C. Before use, should be pre equilibrated to room temperature.
Stop Solution: Store at 20-25°C (room temperature).

Assay Procedure Part 1: HtrA1 Reaction

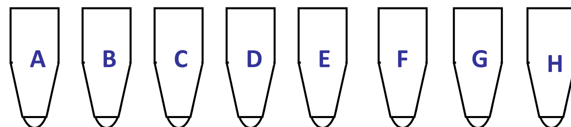
Preparation of HtrA1 working standards

1. Pipette 38 µl "Buffer for HtrA1-Enzym-Standard" into the 2 µl HtrA1-Enzyme-Standard tube. Label first tube with 200 nM or " a". Keep on ice.
2. Label the next 7 polypropylene tubes 100; 50; 25; 12.5; 6.25; 3.12 and 0* nM or label with b – h.
3. Pipette 15 µl of "Buffer for HtrA1-Enzym Standard" into all these 7 tubes (100 nM to 0 nM). Place tubes on ice.
4. Make a serial dilution by pipetting 15 µl from the 200 nM tube into the 100 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 HtrA1 ranging from 200 nM to 0 nM.



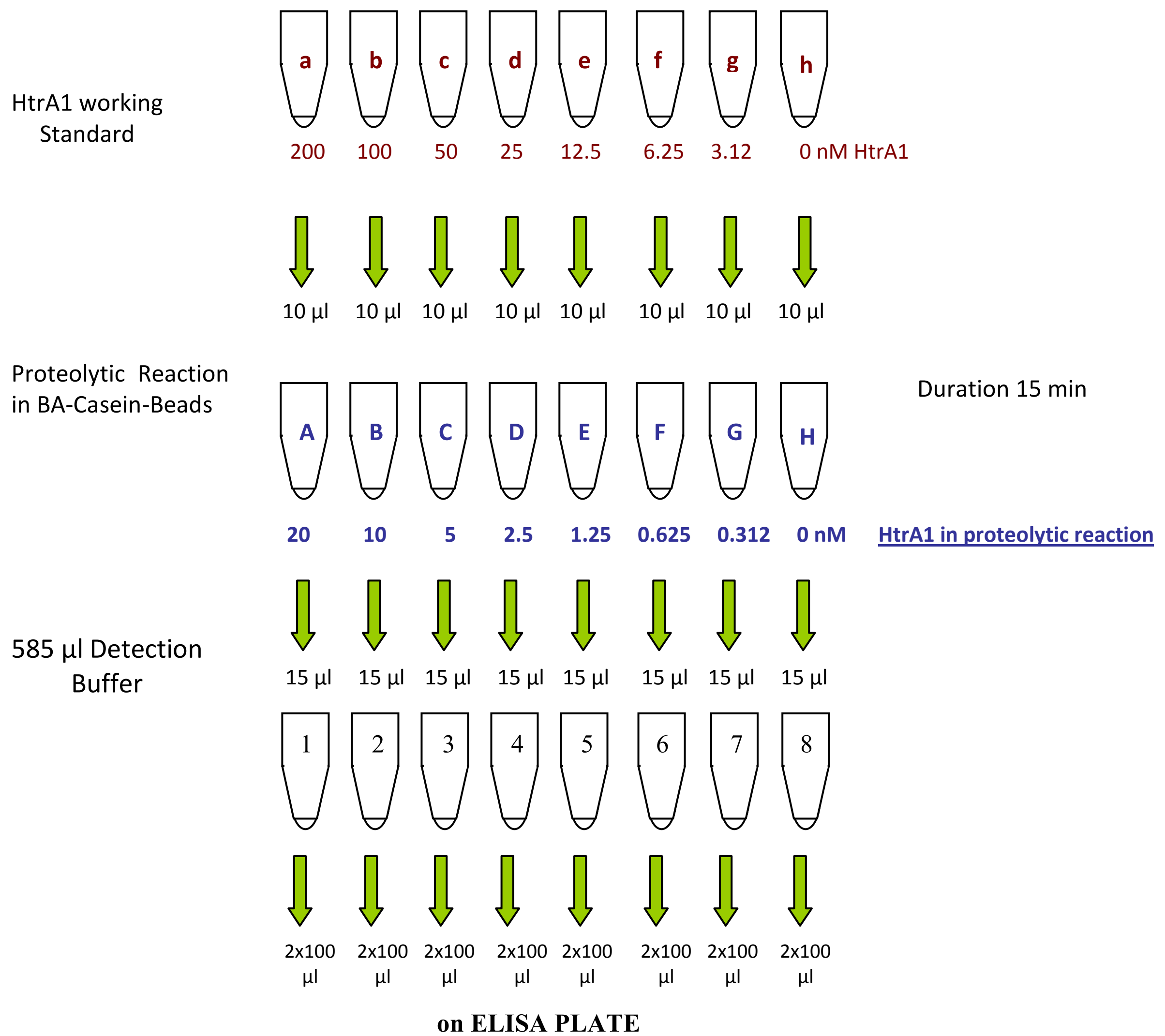
Proteolysis of BA-ß-Casein Beads ** as standard and test sample

1. The BA-ß-Casein-Beads** are ready to use for the proteolysis reaction.
2. Thaw on ice 8 tubes with BA-ß-Casein-Beads that must be used for 8 proteolytic reactions for HtrA1-Enzyme-Standard dilution and a defined number for tubes BA-ß-Casein-Beads that will be used for reactions with the test samples.
3. Label the tubes for 8 proteolytic reactions for HtrA1-Enzyme-calibration curve with A-H.



4. The additions to different tubes should be done in the same succession for each proteolytic reaction.
5. Start proteolytic reactions by adding 10 µl of HtrA1 working standard (a-h) or test sample into the BA-ß-Casein-Beads sample (A-H). Additions should be done ONLY on ice.

* Tube "0" contains no HtrA1 and serves as blank control.
** BA-ß-Casein-Beads: Biotinylated-ß-Casein-Beads



6. Incubate proteolytic reactions for 15 min at 37 °C, 1000 rpm in a thermomixer.
7. End concentrations of standard HtrA1 in proteolytic reactions are going to be 20, 10, 5, 2.5, 1.25, 0.625, 0.312 and 0* nM.
8. In this time, prepare another 8 polypropylene tubes with 585 µl Detection Buffer and labeled them from 1 to 8.
9. Stop proteolytic reactions after 15 min by placing the tubes immediately on ice and pipetting 15 µl of the supernatant from proteolysis reaction into each prepared tube (1 to 8). The dilution factor in the end will be 40.
10. Proceed directly to ELISA for Fragment-Standard (2 x 100 µl) .

* Tube "0" contains no HtrA1 and serves as blank control.

Assay Procedure Part 2: ELISA

Preparation of Fragment working standards for calibration curve

1. Label 8 polypropylene tubes with 2.5, 1.25, 0.625, 0.312, 0.156, 0.078, 0.039 and 0* nM.
2. Pipette 250 µl of TW-Buffer into all tubes. Keep tubes at 4°C.
3. Thaw and pipette 250 µl Fragment-Standard to the first 2.5 nM tube. Vortex.
4. Pipette 250 µl from the 2.5 nM tube into the 1.25 nM tube. Continue two-fold dilution step with the remaining tubes as done with previous one.

* Tube "0" contains no Fragment-Standard and serves as control.
** Fragment standard = Biotinylated- ß-casein-Fragment Standard

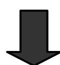
Assay protocol

1. Set up the UPA microtiter plate with sufficient wells for standards and test samples as required for a double estimation. Recommended positions of Fragment-standard (0 – 2.5 nM) are microtiter plate rows 1 and 2. Recommended positions for standard HtrA1 reactions (0 – 20 nM) are plate rows 3 and 4. All other wells can be used for test sample reactions.
2. Pipette 100 µl of each Fragment working standard into the appropriate wells.
3. Pipette 100 µl of each Standard HtrA1 reaction into the appropriate wells.
4. Pipette 100 µl of test sample reactions into remaining wells.
5. Seal the plate with the provided foil and incubate on a shaker at room temperature for 120 minutes.
6. Dilute Wash Buffer as described in page 7.
7. Aspirate and wash the wells 4 times with Wash Buffer. Take care that all wells are completely filled and emptied at each wash.
8. Blot the plate on paper towels to remove residual fluid from the plate.
9. Add 100 µl of diluted Antibody Solution HH1 into each well.
10. Seal the plate and incubate on a shaker at room temperature for 90 minutes.
11. Aspirate and wash the wells 4 times with Wash Buffer. Take care that all wells are completely filled and emptied at each wash.
12. Blot the plate on paper towels to remove residual fluid from the plate.
13. Add 100 µl of diluted Antibody Solution AM into each well.
14. Seal the plate with the provided foil and incubate on a shaker at room temperature for 30 minutes.
15. Aspirate and wash the wells 6 times with Wash Buffer. Take care that all wells are completely filled and emptied at each wash.
16. Blot the plate on paper towels to remove residual fluid from the plate.
17. Add 100 µl of TMB Substrate to each well.
18. Seal the plate with foil provided and incubate in the dark at room temperature for 10 minutes.
19. Stop the reaction by adding 100 µl of Stop Solution to each well.
20. Read the plate at 450 nm (540, 570 or 620 nm reference filter) within 30 minutes. Reading of the plate without reference may yield higher absorbencies and thus may be less accurate.

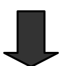
Assay Protocol Summary

Part 1: HtrA1/Protease Reaction

Prepare reagents of the Protease Module, the HtrA1-working standards

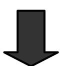


Carry out proteolytic reactions (15 min) of BA-ß-Casein-Beads with HtrA1-working standards and test samples. Stop reactions on ice with Detection Buffer



Part 2: ELISA

Prepare reagents of the ELISA Module and Fragment working standards



Pipette 100 µl of standards and stopped proteolytic reactions in duplicates into wells of the microtiter plate. Incubate 120 min at room temperature on a shaker

Aspirate and wash 4 times

Add 100 µl of diluted Antibody Solution HH1 to each well. Incubate 90 minutes at room temperature on a shaker.

Aspirate and wash 4 times.

Add 100 µl of diluted Antibody Solution (AM) to each well. Incubate for 30 minutes at room temperature on a shaker.

Aspirate and wash 6 times.

Add 100 µl of TMB Substrate to each well. Incubate for 10 minutes at room temperature **in the dark** without shaking!

Add 100 µl Stop Solution to each well. Read at 450 nm within 30 minutes (when possible with reference filter at 620 nm).

Data Processing

For the evaluation of test sample protease activity, absorbance values of proteolytic reactions catalyzed by test samples are compared with absorbance values of HtrA1 standard reactions. The specific activities of standard HtrA1 and sample protease are calculated from concentrations of product Biotinylated ß-Casein-Fragment formed during HtrA1/protease-catalyzed reactions. The calculations are illustrated using representative data.

Calculation of the concentration of active test sample protease

1) Calculate the average absorbance for each standard HtrA1 reaction. Absorbance data for reactions with standard HtrA1 should be similar to that shown in Table 1.

HtrA1 (nM) in standard reactions	OD (450 nm)
0	0.029
0.312	0.033
0.625	0.049
1.250	0.114
2.500	0.256
5.000	0.500
10.000	1.285
20.000	2.504

Table 1: Typical assay data for standard HtrA1 reactions

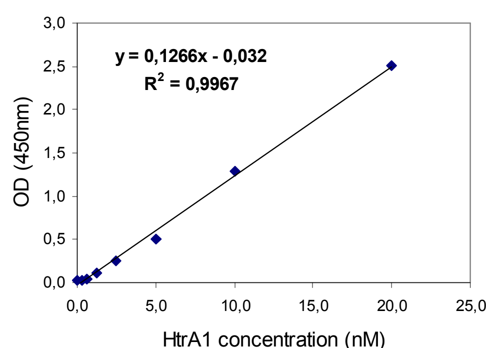


Fig. 2: Typical HtrA1 standard curve

2) Plot the mean absorbance against the concentration of HtrA1 (nM). The curve shape should be similar to Fig. 2. The concentration of active test sample protease equivalent to the activity of standard HtrA1 in proteolytic reactions can be read directly from the graph or calculated by regression analysis.
3) Multiply the concentration value of your sample by dilution factors to obtain the effective protease concentration in the original test sample.

Calculation of BA-ß-Casein-Fragment concentration formed in proteolytic reactions

1) Calculate the average absorbance for each set of BA-ß-Casein-Fragment standard wells. The data should be similar to that shown in Table 2.
2) Plot the mean absorbance against the concentration of BA-ß-Casein-Fragment standard. The curve shape should be similar to Fig. 3. The concentrations of BA-ß-Casein-Fragment corresponding to absorbencies of HtrA1/Protease reactions (Table 2, Fig. 3) can be read directly from the graph or can be calculated using appropriate computer software.
3) Multiply the concentration values for BA-ß-Casein-Fragment by the dilution factor 40 to obtain the concentration of BA-ß-Casein-Fragment formed in protease-catalyzed reactions.

BA-ß-Casein-Fragment (nM)	OD (450 nm)
0	0.021
0.039	0.041
0.078	0.072
0.156	0.152
0.312	0.350
0.625	0.765
1.25	1.730
2.5	3.310

Table 2: Typical data for BA-ß-Casein-Fragment standard

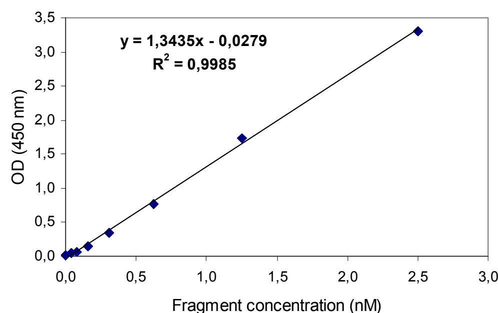


Fig. 3: Typical standard curve for BA-ß-Casein Fragment

Calculation of specific activity of HtrA1/ protease

The concentration of BA-ß-Casein-Fragment formed in HtrA1/protease-catalyzed reactions is plotted against HtrA1 concentration (nM). For standard HtrA1 the relation should be liniar in the initial part of the reaction and similar to that shouwn in Fig. 4. The slope of the linear dependence gives the specific activity of HtrA1. The slope of the linear dependence gives the specific activity of HtrA1. From Fig. 4 a value of 0.251 nM BA-ß-Casein Fragment/min x nM HtrA1 is calculated. When related to mg enzyme, the value is 5.018 mU BA-ß-Casein Fragment /mg HtrA1.

HtrA1 (nM) in standard reactions	BA-ß-Casein frag. conc nM /15 min	BA-ß-Casein frag. conc x DF* nM /15min
0	0.042	**1.690**
0.312	0.045	**1.803**
0.625	0.057	**2.281**
1.250	0.106	**4.222**
2.500	0.211	**8.450**
5.000	0.393	**1.717**
10.000	0.977	**39.082**
20.000	1.885	**75.390**

Table 3: Typical data for calibration curve.
*DF- Dilution Factor of 40x

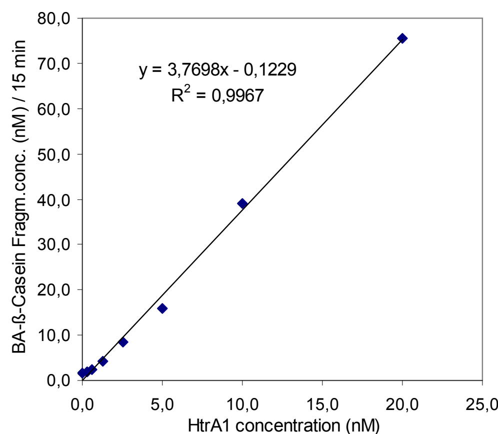


Fig. 4: Typical curve to determine the activity of recombinant human HtrA1

Troubleshooting

Problem	Recommendation
Low absorbance	• 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	• 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	• 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

Additional Information

Specificity - Universal Protease Activity ELISA is based on a universal substrate for proteases, beta casein. The HtrA1 standard provided in the assay is recombinant human HtrA1. The kit was tested for MMPs, ADAMTSs, HtrA1, 3, 4. All these proteases give in ELISA absorbance values above the control levels.

Sensitivity – The sensitivity is dependent on substrate specificity similar to the relationship lock-key, pH, temperature and ionic strength. This kit is based and evaluated on HtrA1- activity. The reaction time and rate can differ with variable conditions.
The sensitivity of the detection of recombinant HtrA1 was determined as 0.312 nM HtrA1. The sensitivity of the detection of BA-ß-Casein fragment was determined as 0.039 nM.

Related Products

For enrichment of proteases you can use:

Cat.No.: 30 501 101 EXTRACT HtrA1 mini
Cat.No.: 30 501 102 EXTRACT HtrA1 maxi
Cat.No.: 30 501 501 EXTRACT HtrA3 mini
Cat.No.: 30 501 502 EXTRACT HtrA3 maxi
Cat.No.: 30 501 401 EXTRACT HtrA4 mini
Cat.No.: 30 501 402 EXTRACT HtrA4 maxi

For detection of proteases you can use our antibodies, like:

Cat.No.: 31 601 004 anti-HtrA1
Cat.No.: 31 605 004 anti-HtrA3
Cat.No.: 31 604 004 anti-HtrA4

More antibodies information you can find on our catalogue:
(http://www.biotez.de/images/biotez/downloads/Catalogue_BioTeZ_2016.pdf)

For determination of concentration, you can use our ELISA kits:

Cat.No.: 30 516 101 HtrA1 ELISA
Cat.No.: 30 516 501 HtrA3 ELISA
Cat.No.: 30 516 401 HtrA4 ELISA
Cat.No.: 30 518 101 MMP13 ELISA

For determination of protease activity, you can use our activity ELISAs:

Cat.No.: 30 510 111 Activity Aggrecanase Assay
Cat.No.: 30 510 211 Activity Sensitive Aggrecanase Assay

For more products, please use our catalogue.

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