Biodiagnostika
Oligonukleotide
Markierte Biomoleküle

BIOTEZ

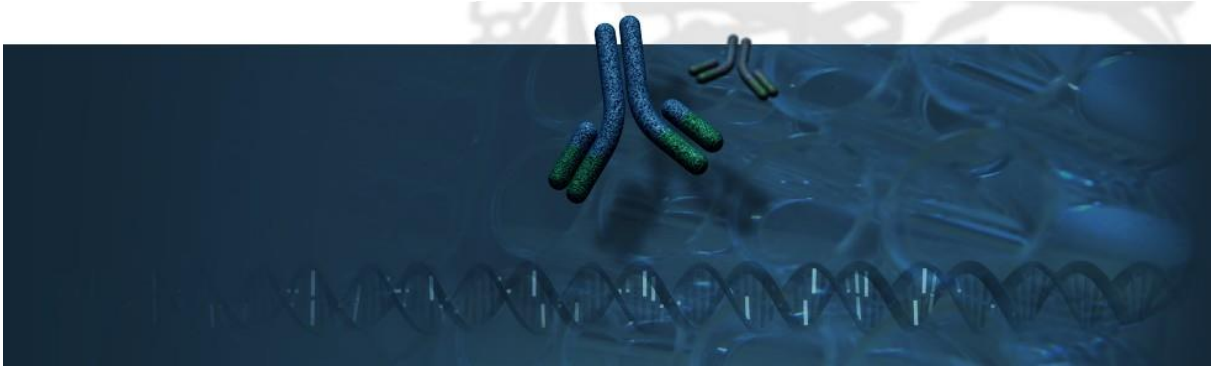
HtrA1 ELISA Assay Kit

REF Cat. No.: 30 516 101

For quantitative determination of human HtrA1

96 Determinations

2°C – 8 °C



For research use only
Not for diagnostic use

Contents

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This manual is valid from November, 2013

Short Review

Human HtrA1 protease (previously termed PRSS11) was initially identified in human fibroblasts (1) and belongs to the high temperature requirement factor A (HtrA) family of serine proteases that can be distinguished from other serine proteases by sequence homology, by the presence of a trypsin-type protease domain and one or two carboxyterminal PDZ domains and by their oligomeric architecture.

The loss of mammalian HtrA activity is correlated with severe diseases, including arthritis, cancer, familial ischemic cerebral small vessel disease and age related macular degeneration, as well as Parkinson's disease and Alzheimer's disease (2-7).

Epigenetic silencing occurs in various cancers, and the loss of HtrA1 correlates with decreased sensitivity to anticancer drugs and increased cell migration (3, 8, 9). In addition, overexpression of HtrA1 inhibited proliferation *in vitro* and tumour growth *in vivo* (10). These data suggest that HtrA1 might function as a tumour suppressor.

In the extracellular matrix, HtrA1 cleaves numerous secreted proteins, such as fibronectin, decorin, fibromodulin, aggrecan, type II collagen, biglycan, clusterin, a disintegrin and metalloproteinase domain-containing 9 (ADAM9), vitronectin, α-2-macroglobulin and the amyloid precursor protein fragment Aβ (7,11-14). The degradation of extracellular matrix components and the strong upregulation of HtrA1 in samples from patients implicate HtrA1 in arthritic diseases, in which it might affect the degradation of cartilage as well as inflammation (2).

It is unknown, how the cellular distribution of HtrA1 is regulated. In addition, little is known about the interaction partners of mammalian HtrAs. It will be important to identify proteins that function as determinants of the cellular localization, substrate specificity and regulation of HtrA proteases (15).

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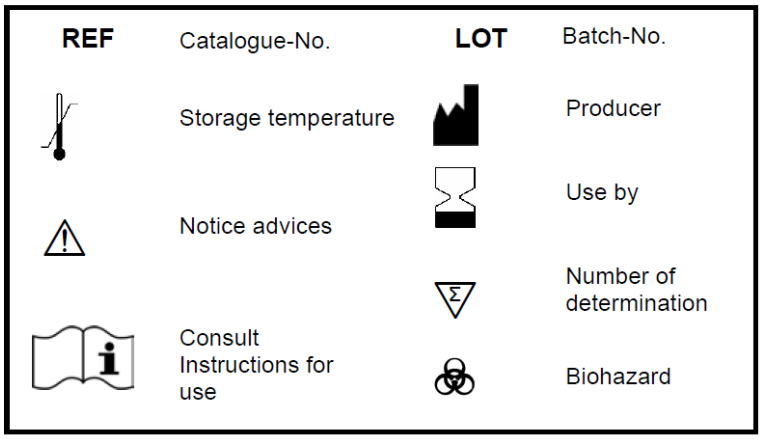


Intended Use

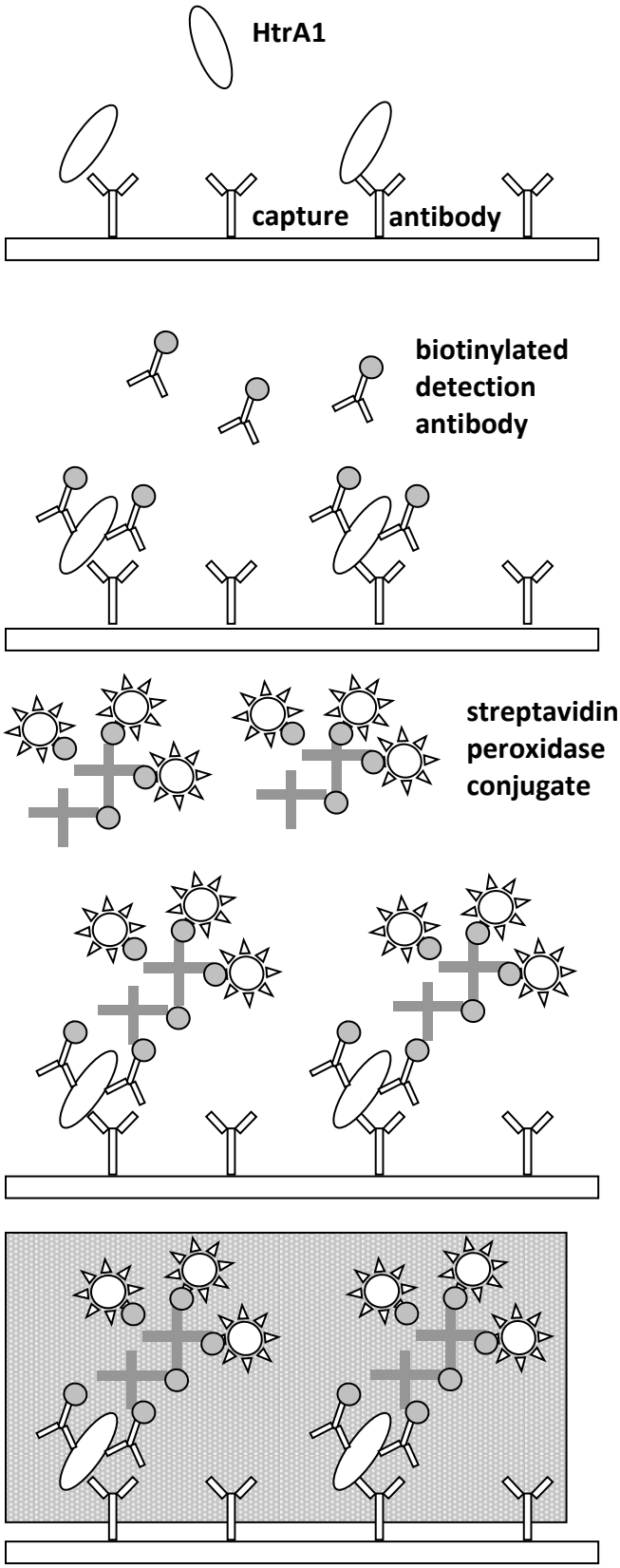
The HtrA1 ELISA from BioTeZ Berlin-Buch GmbH provides a highly sensitive and specific quantitative determination of HtrA1 in serum, tissue (Placenta) and cell culture supernatants.

The calibration curve covers the range from 25 ng/ml to 0.391 ng/ml.

This quantitative assay is based on a two site sandwich format. A highly specific monoclonal antibody against HtrA1 is immobilised on the plate. HtrA1 will be bound to the wells, other components of the sample are removed by discarding/drying by taping and washing of the plate. The analyte is detected in two steps using a secondary biotin-labeled monoclonal antibody and a highly polymerised streptavidin-peroxidase conjugate. Any excess is removed by discarding/drying by taping and washing after each detection step. The amount of peroxidase bound to each well is determined by the addition of TMB Substrate. The reaction is stopped by adding the Stop Solution and the resultant colour is read in a microtiter plate reader at 450 nm. The concentration of HtrA1 in a sample is determined by interpolation from the standard curve.

One kit contains reagents for 96 determinations, thus allowing the measurement of one standard curve and 40 samples in duplicate.

Principle of the assay



Step 1: Incubation of standard or sample on the microtiter plate. Specific binding of HtrA1
(duration: 120 minutes)

Step 2: Detection of bound HtrA1 with biotinylated antibody
(duration: 90 minutes)

Step 3: Addition of streptavidin-peroxidase conjugate
(duration: 30 minutes)

Step 4: Color development after addition of TMB Substrate
(duration: 5-15 minutes)

Fig. 1: Scheme of the assay procedure

Safety warning and precautions

Warning: Stop Solution contains 0.25 M sulphuric acid. Wear eye, hand, face and clothing protection when using this material.

The Serum Diluent contains human serum that has been tested negatively for HAV, HBV, HCV and HIV. Wear suitable protective clothing, such as laboratory overalls and gloves and observe caution when working with this material.

All chemicals should be considered as being potentially hazardous. We therefore recommend that this product is handled only by those persons who have been trained in laboratory techniques and that it is used in accordance with the principles of good laboratory practice. Wear suitable protective clothing, such as laboratory overalls, safety glasses and gloves. Avoid contact with skin and eyes. In case of skin and eyes contact, wash immediately with water.

Storage

All components of the kit can be stored in the refrigerator (2-8 °C).

Note! Wash buffer concentrate can precipitate in refrigerator. We recommend stored at 18-35°C

Once reconstituted, the **HtrA1 standard** should be used immediately or stored at -20 °C.

Diluted **biotinylated detection antibody** and diluted **conjugate solution** should be prepared freshly directly before use. When running a partial plate, only suitable aliquots of these solutions should be made.

Components of the assay system

Microtiter plate: The plate contains 6 x 16 strips coated with monoclonal anti-HtrA1.

Diluent Buffer: Bottle contains 25 ml buffer with additives.

Wash Buffer Concentrate: Two bottles containing each 25 ml of a buffer concentrate. It has to be diluted 20-fold with distilled or deionised water before use.

Standard: The vial contains 100 ng of lyophilized HtrA1. It has to be reconstituted with 1 ml of HtrA1 Standard Buffer prior to use to get a 100 ng/ml stock solution.

HtrA1 Standard Buffer: Solution to reconstitute HtrA1 lyophilized.

Serum Diluent: This solution (20 ml) contains certified human serum in a buffer with additives. Ready to use. **Warning :** Component contains human serum! Wear eye, hand, face and clothing protection when using this material!

Detection Buffer: Solution (25 ml) consists of phosphate buffer containing additives.

Biotinylated Antibody: Solution (150 µl) contains biotinylated monoclonal anti-HtrA1 antibody in buffer with additives. The antibody has to be diluted 100-fold with Detection Buffer before use.

Conjugate Solution: This solution (300 µl) contains a highly polymeric streptavidin-peroxidase conjugate with preservatives. It has to be diluted 40-fold with Detection Buffer prior to use.

TMB Substrate: Bottle contains 12 ml of a TMB solution ready to use.

Stop Solution: Bottle contains 12 ml 0.25 M H_2SO_4. Ready to use. **Warning**: Stop Solution contains 0.25 M sulphuric acid. Wear eye, hand, face and clothing protection when using this material!

EQUIPMENTS REQUIRED BUT NOT PROVIDED

- Pipettes with disposable tips (100 µl, 500 µl and 1 ml), a multi-channel pipette (100 - 200 µl) would be appropriate
- Distilled or deionised water
- Horizontal orbital microplate shaker
- Microplate reader capable of measuring at 450 nm
- 500 ml graduated cylinder.

Sample preparation and storage

Serum:

- Serum samples may be stored at -80°C. When stored at -80°C, it is absolutely necessary to mix the samples thoroughly prior to measuring. Avoid freeze-thaw cycles.
- Dilute the serum samples minimum **1:10** with Serum Diluent, depending on the possible concentration of the analyte.

Cell culture supernatant

- Centrifuge the samples to remove any particles. The supernatants can be stored at -80°C. Avoid freeze-thaw cycles.
- Dilute the samples minimum 1:10 **or more with Diluent Buffer**, depending on the possible concentration of the analyte. For measuring these samples, use the **standards prepared with Diluent Buffer**.

We recommend to prepare many dilutions of the sample (e.g. 1:50, 1:100, 1:400) in order to get an absorption in a range of 0.7-1.7 , whereas the absorption values of the calibration curve are in a range of 0.1-2.6 . This is very important for the right calculation of the concentration of the samples.

Critical Parameters

- Allow samples and all reagents to equilibrate to room temperature (18-35°C) prior to performing the assay. This is especially a prerequisite for Wash Buffer and TMB Substrate!
- For the highly sensitive determination of HtrA1 in serum, use the **Serum Diluent** to prepare your standard curve. For all other samples, use Diluent 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 taping the inverted plate on dry absorbent surface (see technical advice on the following link: http://www.youtube.com/watch?v=FZirnCas17Y)
- Use only reagents from the same lot for each assay. This is especially important when running more than one plate per sample group.
- A separate standard curve must be run on each plate.
- Mix all reagents thoroughly prior to use, but **avoid foaming**!
- Keep the wells sealed with the foil except when adding reagents and during reading.
- Any variation in the protocol can cause variation in binding!
- The kit should not be used beyond the expiration date on the kit label.
- The values obtained by the samples should be within the standard range. If this is not the case, dilute the sample and repeat the assay.
- We take great care to ensure that this product is suitable for all validated sample types, as designated in this manual However, it is possible that in some cases, high levels of interfering substances may cause unusual results.
- EDTA (0.5 mM - 4 mM) **does not** inhibit the assay! However, the results obtained for determination of HtrA1-concentration in EDTA-plasma were much lower if compare with serum.

Please note: To prevent margin effects it is absolutely necessary to equilibrate all reagents **to room temperature (1 h incubation on the bench)** prior to use. For the dilution of the wash buffer concentrate use either distilled or deionised water. Always seal the plates with the provided foil during incubation!

Diluent Buffer: After equilibration to room temperature, the buffer is ready to use.
Wash Buffer: Dilute the contents of a 25 ml bottle to 500 ml with distilled or deionised water. Make sure that the whole content of the bottle is used by repeated washing.
Standard: Add 1 ml HtrA1 Standard Buffer to the standard tube (**violet lid**) and allow the contents to dissolve for 5-10 minutes. Gently mix, but avoid foaming of the reagent!
HtrA1 Standard Buffer: After equilibration to room temperature, the buffer is ready to use.
Serum Diluent: After equilibration to room temperature, the reagent is ready to use.
Detection Buffer: After equilibration to room temperature, the reagent is ready to use.
Biotinylated Antibody: Dilute Biotinylated Antibody 100-fold with Detection Buffer. For a whole plate add 120 µl from the antibody solution tube (red lid) to 12 ml Detection Buffer. When running half a plate, add 60 µl antibody solutions to 6 ml Detection Buffer.
Conjugate Solution: Dilute the provided Conjugate Solution 40-fold with Detection Buffer.
For the whole plate, add 300 µl of the conjugate tube (blue lid) to 12 ml Detection Buffer. When running half a plate, add 150 µl of the conjugate to 6 ml of Detection Buffer.
Substrate and Stop Solution: After equilibration to room temperature, the reagents are ready to use.

Preparation of standards with Diluent Buffer

(For cell culture supernatants)

1. Label 7 tubes with 0.39, 0.78, 1.56, 3.13, 6.25, 12.5 and 25 ng/ml.
2. Pipette 375 µl of Diluent Buffer into the 25 ng/ml tube, in the remaining tubes pipette 250 µl of Diluent Buffer.
3. Pipette 125 µl of the stock standard (100 ng/ml) into the 25 ng/ml tube and mix thoroughly.
4. Pipette 250 µl of the 25 ng/ml standard into the tube labelled with 12.5 ng/ml and mix thoroughly.
5. Repeat this dilution procedure with the other standard tubes.
6. The blank value (0 ng/ml) is obtained by using only Diluent Buffer.
7. The stock solution is not part of the standard curve and can be stored at -20°C

Preparation of standards with Serum Diluent

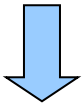
(For the highly sensitive measurement of serum samples)

1. Label 7 tubes with 0.39, 0.78, 1.56, 3.13, 6.25, 12.5 and 25 ng/ml.
2. Pipette 375 µl of Serum Diluent into the 25 ng/ml tube, in the remaining tubes pipette 250 µl of Serum Diluent.
3. Pipette 125 µl of the stock standard (100 ng/ml) into the 25 ng/ml tube and mix thoroughly.
4. Pipette 250 µl of the 25 ng/ml standard into the tube labelled with 12.5 ng/ml and mix thoroughly.
5. Repeat this dilution procedure with the other standard tubes.
6. The blank value (0 ng/ml) is obtained by using only Serum Diluent.
7. The stock solution is not part of the standard curve and can be stored at -20°C

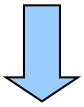
1. Prepare reagents and standards as described in the sections above. For assessing serum samples, prepare the standard using the Serum Diluent. **Remind that it is necessary to equilibrate the reagents to room temperature before use**.
2. Prepare the unknown samples as described above by appropriate dilution with Diluent Buffer.
3. Prepare the Microtiter plate by inserting the required amount of wells into the frame. **Note that you need 16 wells for the standard curve**.
4. Pipette 100 µl of the reconstituted standards (25 ng/ml) in duplicate in the wells using a clean pipette tip for each standard. Diluent Buffer serves as zero blank.
5. Pipette 100 µl of the prepared unknown samples in duplicate into the wells.
6. Seal the plate with the provided foil and incubate on a shaker at room temperature for exactly 120 minutes.
7. Wash by filling each well with Wash Buffer (200 µl), then remove by discarding/drying by taping inverted plate against clean paper towels. Take care that all wells are completely filled and emptied at each wash. Wash the wells 4 times with Wash Buffer.
8. Add 100 µl of diluted Biotinylated Antibody into each well.
9. Seal the plate and incubate on a shaker at room temperature for exactly 90 minutes.
10. Wash by filling each well with Wash Buffer (200 µl), then remove by discarding/drying by taping inverted plate against clean paper towels. Take care that all wells are completely filled and emptied at each wash. Wash the wells 4 times with Wash Buffer.
11. Add 100 µl of diluted Conjugate Solution into each well.
12. Seal the plate with the provided foil and incubate on a shaker at room temperature for exactly 30 minutes.
13. Wash by filling each well with Wash Buffer (200 µl), then remove by discarding/drying by taping inverted plate against clean paper towels. Take care that all wells are completely filled and emptied at each wash. Wash the wells 6 times with Wash Buffer.
14. Add 100 µl of TMB Solution to each well.
15. Seal the plate with foil provided and incubate in the dark at room temperature without shaking for 5-15 minutes.
16. Stop the reaction by adding 100 µl of Stop Solution to each well.
17. Read the plate at 450 nm (620 nm reference filter) within 30 minutes. Reading of the plate without reference may yield higher absorbances and thus may be less accurate.

Protocol summary

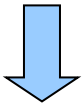
Prepare reagents, standards and samples as described, equilibrate reagents to **room temperature.**
For assessing human serum samples, prepare a
Serum Standard!



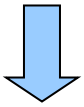
Pipette 100 µl standard or sample in duplicate into the wells. Incubate 120 minutes at room temperature on a shaker



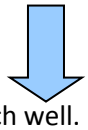
Discard/Dry by taping/Wash 4 times



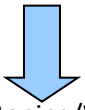
Add 100 µl of diluted Biotinylated Antibody to each well. Incubate 90 minutes at room temperature on a shaker.



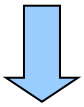
Discard/Dry by taping/Wash 4 times.



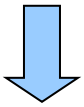
Add 100 µl of diluted Conjugate Solution to each well. Incubate for 30 minutes at room temperature on a shaker.



Discard/Dry by taping/Wash 6 times.



Add 100 µl of Substrate Solution to each well. Incubate for 5 - 15 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

Calculation of results

The calculation is illustrated using representative data: the assay data should be similar to that shown in table 1.

1. Calculate the average absorbance for each set of standard wells.
2. A standard curve is generated by plotting the mean absorbance (x axis) against ng/ml standard (y-axis, fig. 2).
3. The ng/ml values of the samples can be read directly from the graph or calculated by the regression coefficients.
4. Multiply the calculated ng/ml values by the dilution factor of the samples.

Standard (ng/ml)	Standard curve in Diluent Buffer	
	Absorbance (450 nm)	Average
25.00	2.171/2.033	2.102
12.50	1.008/1.033	1.021
6.25	0.476/0.508	0.492
3.125	0.259/0.255	0.257
1.56	0.167/0.166	0.167
0.78	0.123/0.127	0.125
0.39	0.109/0.109	0.109
0	0.095/0.098	0.097

Table 1: Typical assay data

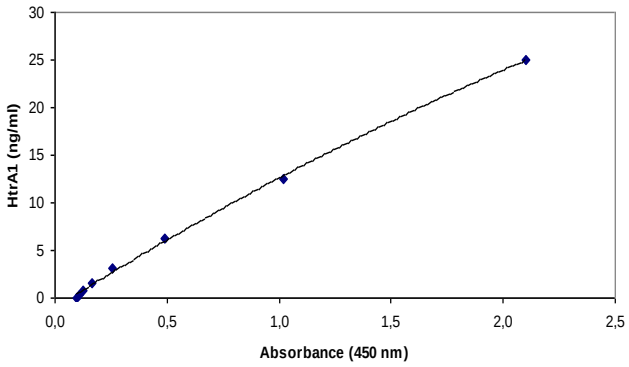


Fig. 2: Typical standard curve (prepared with Diluent Buffer).

Specificity

The HtrA1 ELISA has a high sensitivity and high specificity for quantitative determination of HtrA1.

The assay recognizes HtrA1 full length (50kDa) and the truncated form of HtrA1 (141-480 aa, 37 kDa).

It does not cross-react with human HtrA3 or HtrA4.

Sensitivity

The sensitivity was determined by as 391 pg/ml.

Precision profile

The precision profile was calculated as CV% from the mean and standard deviation of absorbance for each standard. The results for the determination in Diluent Buffer and Serum Diluent.

Standard (ng/ml)	%CV Diluent Buffer	N
25	0,7	13
12,5	2,4	13
6,25	4,1	13
3,12	4,2	13
1,56	6,6	13
0,78	11	13
0,39	14	13

Standard (ng/ml)	%CV Serum Diluent	N
25	0,3	6
12,5	1,9	6
6,25	3,3	6
3,12	6	6
1,56	2,4	6
0,78	3,2	6
0,39	7,7	6

Linearity

The following samples below were measured after dilution with Diluent Buffer and Serum Diluent to assess linearity of the assay.

Sample Type	Added concentration (ng/ml)	Measured (ng/ml)	Recovery (%)
Diluent Buffer	25 12.5 6.25 3.12	24.8 13.15 5.82 2.32	99 105 93 74
Serum	25 12.5 6.25 3.12	24.9 12.8 6.1 2.9	100 102 98 93

Reproducibility

Within assay precision

The within assay precision was measured by assaying four control samples in 10 duplicates in Diluent Buffer on one plate

Standard (ng/ml)	Standard deviation	%CV	N
25	1.6	7.2	10
12.5	0.9	7.3	10
6.25	0.6	10	10
3.12	0.2	8.2	10

Between assay precision

The between assay variation was measured by assaying

Standard (ng/ml)	Standard deviation	%CV	N
20	1.9	9.5	10
10	0.8	8.0	10
5	0.5	10	10
2,5	0.3	10	10

Recovery

The recovery of HtrA1 standard spiked to levels throughout the range of the assay in serum.

Matrix	Recovery range (%)	Average (%)
Serum (n=6)	84-104%	94%

Troubleshooting

Problem	Potential cause	Recommendation
Low absorbance	• Wrong wavelength • Enzyme conjugate out of date/reagents improperly stored • Improper incubation time and temperature • Reagents not equilibrated to RT • Reagents not correctly prepared	• Check reader wavelength • Control the expiration date/storage conditions • Control the incubation time and temperature • Check equilibration of reagents to RT • Check preparation of reagents
High absorbance/ high zero standard value (>0.15 OD)	• Incomplete washing • Improper removing of residual fluid • Improper incubation time and temperature • Reagents not equilibrated to RT • Reagents not correctly prepared	• Ensure that every well is completely filled/emptied during each washing step • Check that plates are blotted on tissue paper after each washing step • Control the incubation time and temperature • Check equilibration of reagents to RT • Check preparation of reagents
Flat curve/poor reproducibility	• Wrong wavelength • Enzyme conjugate out of date/reagents improperly stored • Improper preparation of working standards • Pipette errors • Contamination of components by use of unclean reservoirs/used pipette tips • Margin effects by using of cold substrate solution • Washing incomplete	• Check wavelength • Control the expiration date/storage conditions • Check preparation of standards • Check pipette calibration • Use separate reservoirs and always new pipette tips • Equilibrate substrate to room temperature • Ensure sufficient washing procedure

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