Version 19-04/24

User's Manual

Human High-temperature requirement factor A3 ELISA kit

REF **DEIA-BJ1053**

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RUO

This product is for research use only and is not intended for diagnostic use.

For illustrative purposes only. To perform the assay the instructions for use provided with the kit have to be used.

Creative Diagnostics

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PRODUCT INFORMATION

Intended Use

The ELISA Kit from CD provides a highly sensitive and specific quantitative determination of HtrA3 in serum, tissue (Placenta) and cell culture supernatants. For research use only, not for use in diagnostic procedures.

General Description

Human HtrA3 (high temperature requirement A3) is a serine protease of the HtrA family. HtrA3 was initially identified in the developing placenta both in mouse and in human as a serine protease associated with pregnancy. HtrA3 is now known to inhibit trophoblast invasion during placental development, and regulate ovarian development, granulosa cell differentiation and luteinisation. Studies in mice have also suggested that HtrA3 inhibits TGF-β signalling during embryo development. It has two isoforms [long (HtrA3-L) and short (HtrA3-S)] and recently, HtrA3 was identified as a potential diagnostic marker for early detection of preeclampsia, a life-threatening pregnancy-specific disorder.

Principles of Testing

This quantitative assay is based on a two site sandwich format. A highly specific monoclonal antibody against HtrA3 is immobilised on the plate. HtrA3 will be bound to the wells, other components of the sample are removed by discarding/drying by tapping 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 tapping 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 yellow colour is read in a microtiter plate reader at 450 nm. The concentration of HtrA3 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.

Reagents And Materials Provided

1. Microtiter plate: The plate contains 6×16 strips coated with monoclonal anti-HtrA3.

2. Assay Buffer 2.5: Bottle contains 25 ml buffer with additives.

3. 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.

4. HtrA3 Standard (lyophilised): The vial contains 200 ng of lyophilized HtrA3. It has to be reconstituted with 1 ml of HtrA3 Standard Buffer prior to use to get a 200 ng/ml standard solution.

5. HtrA3 Standard Buffer: Solution to reconstitute HtrA3 lyophilized.

6. Serum Standard 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!

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

8. Biotinylated Antibody: Solution (150 µl) contains biotinylated monoclonal anti-HtrA3 antibody in buffer with additives. The antibody has to be diluted 100-fold with Detection Buffer before use.

9. Conjugate Solution: This solution (300 µl) contains a highly polymeric streptavidin-peroxidase conjugate with preservatives. It has to be diluted 100-fold with Detection Buffer prior to use.

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

11. Stop Solution: Bottle contains 12 ml 0.25 M H_2SO_4. Ready to use.

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

Materials Required But Not Supplied

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

Storage

1. All components of the kit can be stored in the refrigerator (2-8°C).
2. **Note! Wash buffer concentrate can precipitate in refrigerator.** We recommend stored at 18-35°C
3. **HtrA3 Standard Buffer** should be used immediately or stored at -20°C.
4. Once reconstituted, the **HtrA3 Standard** should be used immediately or stored at -80 °C.
5. Diluted **Biotinylated 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.

Specimen Collection And Preparation

1. Serum:

a. 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.

b. Dilute the serum samples minimum 1:10 with Serum Standard Diluent, depending on the possible concentration of the analyte.

2. Cell culture supernatant:

a. Centrifuge the samples to remove any particles. The supernatants can be stored at -80°C. Avoid freeze-thaw cycles.

b. Dilute the samples minimum 1:10 or more with Assay Buffer 2.5, depending on the possible concentration of the analyte. For measuring these samples, use the standards prepared with Assay Buffer 2.5.

Reagent Preparation

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!

1. Assay Buffer 2.5: After equilibration to room temperature, the buffer is ready to use.

2. 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.

3. HtrA3 Standard (lyophilised): Add 1 ml HtrA3 Standard Buffer to the standard tube (brown lid) and allow the contents to dissolve for 5-10 minutes. Gently mix, but avoid foaming of the reagent!

4. HtrA3 Standard Buffer: After equilibration to room temperature, the buffer is ready to use.

5. Serum Standard Diluent: After equilibration to room temperature, the reagent is ready to use.

6. Detection Buffer: After equilibration to room temperature, the reagent is ready to use.

7. Biotinylated Antibody: Dilute Biotinylated Antibody 100-fold with Detection Buffer. For a whole plate add 120 µl from the antibody solution tube (orange lid) to 12 ml Detection Buffer. When running half a plate, add 60 µl antibody solutions to 6 ml Detection Buffer.

8. Conjugate Solution: Dilute the provided Conjugate Solution 200-fold with Detection Buffer. For the whole plate, add 60 µl of the conjugate tube (blue lid) to 12 ml Detection Buffer. When running half a plate, add 30 µ l of the conjugate to 6 ml of Detection Buffer.

9. TMB Substrate and Stop Solution: After equilibration to room temperature, the reagents are ready to use.

10. Preparation of standards with Assay Buffer 2.5:

(For cell culture supernatants)

a. Label 7 tubes with 0.78, 1.56, 3.12, 6.25, 12.5, 25 and 50 ng/ml.

b. Pipette 375 µl of Assay Buffer 2.5 into the 50 ng/ml tube, in the remaining tubes pipette 250 µl of Assay Buffer 2.5.

c. Pipette 125 µl of the stock standard (200 ng/ml) into the 50 ng/ml tube and mix thoroughly.

d. Pipette 250 µl of the 50 ng/ml HtrA3 standard into the tube labelled with 25 ng/ml and mix thoroughly.

e. Repeat this dilution procedure with the other standard tubes.

f. The blank value (0 ng/ml) is obtained by using only Assay Buffer 2.5.

g. The stock solution is not part of the standard curve and can be stored at -20°C.

11. Preparation of standards with Serum Standard Diluent

(For the highly sensitive measurement of serum samples)

a. Label 7 tubes with 0.78, 1.56, 3.12, 6.25, 12.5, 25 and 50 ng/ml.

b. Pipette 375 µl of Serum Standard Diluent into the 50 ng/ml tube, in the remaining tubes pipette 250 µl of Serum Standard Diluent.

c. Pipette 125 µl of the stock HtrA3 standard (200 ng/ml) into the 50 ng/ml tube and mix thoroughly.

d. Pipette 250 µl of the 50 ng/ml standard into the tube labelled with 25 ng/ml and mix thoroughly.

e. Repeat this dilution procedure with the other standard tubes.

f. The blank value (0 ng/ml) is obtained by using only Serum Standard Diluent.

g. The stock solution is not part of the standard curve and can be stored at -20°C.

Assay Procedure

Assay Notes:

1. 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!
2. For the highly sensitive determination of HtrA3 in serum, use the Serum Standard Diluent to prepare your standard curve. For all other samples, use Assay Buffer 2.5.
3. 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.
4. Use only reagents from the same lot for each assay. This is especially important when running more than one plate per sample group.
5. A separate standard curve must be run on each plate.
6. Mix all reagents thoroughly prior to use, but avoid foaming!
7. Keep the wells sealed with the foil except when adding reagents and during reading.
8. Any variation in the protocol can cause variation in binding!
9. The kit should not be used beyond the expiration date on the kit label.
10. 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.
11. 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.
12. EDTA (0.5 mM - 4 mM) does not inhibit the assay!

Procedure:

1. Prepare reagents and standards as described in the sections above. For assessing serum samples, prepare the standard using the Serum Standard 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 Assay Buffer 2.5.
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 (50 ng/ml) in duplicate in the wells using a clean pipette tip for each standard. Assay Buffer 2.5 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 tapping 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 tapping 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 tapping 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 10 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 absorbance and thus may be less accurate.

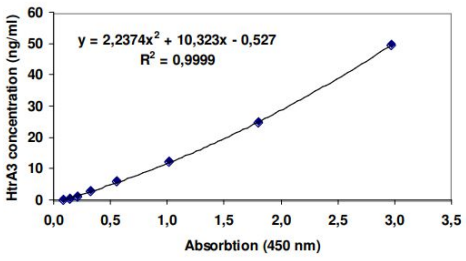
Calculation

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

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).
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.

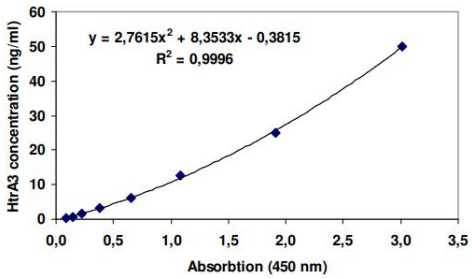
Typical Standard Curve

Standard (ng/ml)	Standard curve in Assay Buffer 2.5	
	Absorbance (450 nm)	Average
50.00	2.960 / 3.064	3.012
25.00	1.881 / 1.935	1.908
12.50	1.038 / 1.118	1.078
6.25	0.6311 / 0.669	0.650
3.12	0.369 / 0.393	0.381
1.56	0.224/ 0.223	0.223
0.78	0.150 / 0.151	0.150
0	0.086 / 0.086	0.086



Typical assay data and Typical standard curve (prepared with Assay Buffer 2.5).

Standard (ng/ml)	Standard curve in Serum Standard Diluent	
	Absorbance (450 nm)	Average
50.00	2.952 / 2.992	2.972
25.00	1.774 / 1.829	1.802
12.50	1.003 / 1.029	1.017
6.25	0.557 / 0.564	0.561
3.12	0.323 / 0.331	0.327
1.56	0.207 / 0.209	0.208
0.78	0.138 / 0.141	0.140
0	0.084 / 0.084	0.084



Typical assay data and Typical standard curve (prepared with Serum Standard Diluent).

Precision

1. Within assay precision

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

Standard (ng/ml)	Standard deviation	%CV	N
50	3.68	7.6	10
25	1.24	5.3	10
12.5	0.64	5.8	10
6.12	0.25	4.5	10

2. Between assay precision

The between assay variation was measured by assaying.

Standard (ng/ml)	Standard deviation	%CV	N
50	4.94	9.7	10
25	1.89	7.1	10
12.5	0.84	6.9	10
6.12	0.49	8.4	10

Sensitivity

The sensitivity was determined up to 700 pg/ml.

Specificity

The HtrA3 ELISA has a high sensitivity and high specificity for quantitative determination of HtrA3.

The assay recognizes HtrA3 full length (50 kDa). It does not cross-react with human HtrA1 or HtrA4.

Linearity

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

Sample Type	Added concentration (ng/ml)	Measured (ng/ml)	Recovery (%)
Assay Buffer	50	48.4	97
	25	23.5	94
	12.5	11.0	88
	6.25	5.4	86
Serum	50	52.6	105
	25	29.7	119
	12.5	15.2	122
	6.25	7.2	115

Precautions

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

The Serum Standard Diluent contains human serum that has been negatively tested for contaminations of 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.

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