ROBOSCREEN DIAGNOSTICS

Instructions for Use

Human P231 TAU ELISA

Enzyme immunoassay for quantitative determination of amino acid 231 (T) phosphorylated human tau protein in biological samples.

For research use only

Rev. 15/2021

Order Number:

847-0104000112	96 reactions

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1 Introduction

1.1 Intended use

The Human P231 TAU ELISA is designed to detect and quantify the level of amino acid 231 (T) phosphorylated tau protein in cell free biological samples. All contents of the Human P231 TAU ELISA are produced under the guidelines of quality control accordingly to the DIN EN ISO 13485 requirements.

The Human P231 TAU ELISA is for research use only and is not intended as diagnostic test.

1.2 Warranty and technical support

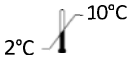
The manufacturer guarantees the correct functioning of the kit for the applications described in the instructions for use (IFU). During the warranty period, Human P231 TAU ELISA allows for precise and reproducible data collection in connection with superior sensitivity. Any warranty claims shall only be valid if the general principles of Good Laboratory Practice (GLP) and the manufacturer's recommendations are observed.

To improve the application and design, Roboscreen GmbH reserves the right of product replacement or modification. The manufacturer may be contacted at any time for questions and problems or technical support concerning the quantification of amino acid 231 (T) phosphorylated tau protein.

CONSULT INSTRUCTIONS FOR USE
This package insert must be read carefully prior to use. Package insert instructions must be followed accordingly. Reliability of results cannot be guaranteed if there are any deviations from the instructions in this package insert.

1.3 Notes on the use of this instructions for use

For easy reference and orientation, the IFU uses the following warning and information symbols as well as the shown methodology:

	REF Catalogue number
	Content Contains sufficient reagents for <N> tests
	Storage conditions
	Consult instructions for use
	Expiry date
	Manufactured by
	For single use only

The following abbreviations are used in the IFU:

AD	Alzheimer’s disease
CSF	Cerebrospinal fluid
CTRL	Control
ELISA	Enzyme-linked immunosorbent assay
GLP	Good Laboratory Practice
HRP	Horseradish peroxidase
IFU	Instruction for use
mAb	Monoclonal antibody
OD	Optical density
RT	Room temperature (18-25°C)
STD	Standard
TMB	Tetramethylbenzidine

2 Safety precautions

We recommend reading this chapter thoroughly before using this kit, to ensure the safety of the user and error-free utilization.
Any safety instructions and additional information of this IFU must be observed at all times.

Read and make sure you understand the operating instructions completely and thoroughly before carrying out the test. Use the currently valid version from the kit.

Notify the respective supplier in writing within one week from receiving the merchandise, should the test pack be substantially damaged. Damaged components must not be used to carry out the assay, however, they should be kept until the transport damages are finally settled.

Comply with Good Laboratory Practice and safety regulations. Wear laboratory coats, disposable gloves and safety goggles whenever the need arises.

Reagents of this kit which contain hazardous substances may cause irritations to eyes and skin. See indications under chapter *5 – Kit components, storage and expiry date* and on the labels. Safety data sheets of this product are available upon request.

Chemicals and prepared or used reagents shall be disposed of as hazardous waste in compliance with the respective national regulations.

The cleaning staff has to be instructed by experts with regard to any potential risks and the appropriate handling of such substances.

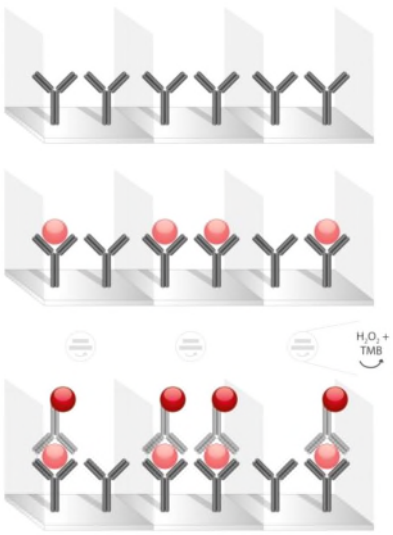
Avoid any contact with stop solution. This may cause irritations to the skin and chemical burns.

This kit is made for single use only!

ATTENTION!
Don’t eat or drink components of the kit!
The kit shall only be handled by educated personnel in a laboratory environment!

3 Test principle

The Human P231 TAU ELISA is based on a sensitive sandwich ELISA using a monoclonal antibody specific to all human tau protein isoforms for capturing tau protein. As detection antibody a HRP labelled monoclonal antibody specific to amino acid 231 (T) phosphorylated tau protein is used. Amount of bound conjugated antibody is estimated using chromogenic substrate tetramethylbenzidine (TMB). The concentration of human T231 phosphorylated tau protein is proportional to the obtained optical density. Controls are included for the proof of reproducibility and evaluation of the assay within labs.



1. Ready to use: Monoclonal capture antibody specific to human tau protein coated on well plate

2. Binding of human tau protein by capture antibody.

3. Detection of bound human T231 phosphorylated tau protein by HRP-conjugated antibody

4 Example of use

Phosphorylation of Tau protein at threonine 231 has been shown to be characteristic in post-mortem brain tissue of patients with AD and it can be sensitively detected in CSF. Therefore, it may serve as a biomarker to support the diagnosis of AD. In the study shown below CSF p231-Tau was significantly higher in patients with dementia due to AD (AD-D) than in those with dementia due to other causes (demented controls – DC) (Santos J, 2019).

Variables		Entire Cohort	DC	AD-D	p value
Patients		106	19	27	-
t-tau	pg/ml (SD)	428.2 (289.6)	332.8 (163.0)	707.78 (273.35)	<0.0001
Ptau181	pg/ml (SD)	56.42 (29.02)	45.79 (15.30)	83.96 (26.72)	<0.0001
Ptau231	pg/ml (SD)	123.3 (49.6)	105.42 (37.40)	172.72 (52.24)	<0.0001

Table 1: Comparison between AD-D and DC; p values were calculated with Man-Whitney test or Fisher exact test for nominal variables.

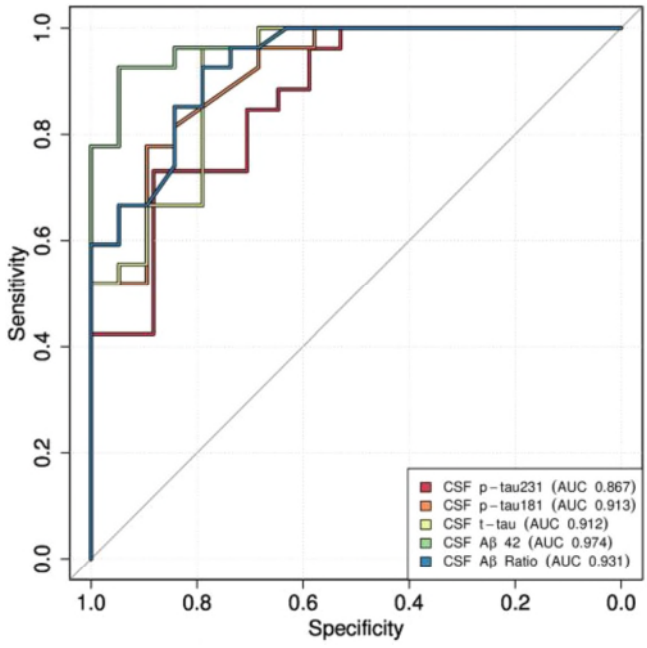


Figure 1: ROC Curve analysis of the sensitivity and specificity of the three tau markers as discriminators of AD-D and DC.

5 Kit components, storage and expiry date

5.1 Kit components

Component	Σ 96	Description
D1 Immunostrips	12 x 8	Coated immunostrips containing anti-human tau protein antibody, blocked and stabilized. Ready to use.
D2 40X Wash buffer	1 x 50 ml	40X Wash buffer containing PBS, detergent and proclin 300.
D3.1 – D3.6 Standards	6 x 3	Lyophilized T231 phosphorylated tau protein standards for preparing a standard curve for quantification of human T231 phosphorylated tau protein in unknown biological samples. Containing PBS, protein and proclin 300.
D3.1	3	300 pg T231 phosphorylated tau protein
D3.2	3	200 pg T231 phosphorylated tau protein
D3.3	3	100 pg T231 phosphorylated tau protein
D3.4	3	70 pg T231 phosphorylated tau protein
D3.5	3	40 pg T231 phosphorylated tau protein
D3.6	3	20 pg T231 phosphorylated tau protein
D4 Negative Control	1 ml	Negative control (blank), containing Na-Merthiolat. Ready to use.
D5 20X HRP conjugate	1 ml	HRP conjugated mAb anti-human T231 phosphorylated tau protein, 20X concentrate, containing TRIS buffer, detergent and stabilizers (Kathon, Bronidox).

Component	Σ 96	Description
D6 Assay buffer	50 ml	Assay buffer containing PBS, protein, detergent and proclin 300. Ready to use.
D7 Control high 	3	Human T231 phosphorylated tau protein high positive control (CTRL), containing PBS, protein and proclin 300.
D8 Control low 	3	Human T231 phosphorylated tau protein low positive control (CTRL), containing PBS, protein and proclin 300.
D9 Staining solution	20 ml	TMB/peroxide solution. Ready to use.
D10 Stop solution	25 ml	1 M sulphuric acid. Ready to use.
Sealing tape	2	
Instruction for use	1	

5.2 Storage and expiry date

The kit is delivered at ambient temperature and should be stored at 6 ± 4 °C. Protect from heat and direct sunlight. Under these conditions, the kit has a shelf life as indicated on the kit box while retaining its endurance and stability.

Prepared kit components have the following expiry dates:

Component	Preparation step	Expiry date
D1	Coated immunostrips after opening of bag, taking out strips and closing of bag.	At 6 ± 4 °C up to 4 weeks.
D2	Ready to use 1X wash solution.	At 6 ± 4 °C up to 2 weeks.
D3.1-D3.6	Standards D3.1-D3.6 dissolved in D6.	At 6 ± 4°C up to 4 h.
D7, D8	Controls D7 and D8 dissolved in D6.	At 6 ± 4°C up to 4 h.
D5	Ready-to-use HRP-conjugate 1:20 diluted.	At 6 ± 4°C up to 4 h.

6 Components not included in the kit:

- Calibrated micropipettes with CV < 3 %
  Volume: 0.5-10 µl; 10-100 µL; 100-1000 µL
- Calibrated 8-channel micro-pipette with reagent reservoirs
- Plate shaker* 100 – 1000 rpm; e.g. Rotamax 120
- Vortex mixer
- Automated or semi-automated ELISA plate washing system
- Bidistilled or deionized water
- Paper towels, pipette tips and timer
- ELISA plate reader for reading absorbance at 450 and 620 nm
- Polypropylene tubes for sample dilution

* Shakers have different forces due to their specifications (deflection in mm) at a frequency of 150 rpm (see note page 16)

7 Preparation of components

Immunostrips **D1**, negative control **D4**, assay buffer **D6**, staining solution **D9** and stop solution **D10** are **ready to use** components.

7.1 1X Wash solution

Mix 40X wash buffer **D2** by 2-3 x inversing and dilute **D2** with bidistilled or deionized water 1:40 as described below before the first wash step of the immunoassay.

Number of immunostrips	Volume of 1X Wash solution	Volume of 40X Wash buffer D2	Volume of bidistilled or de-ionized water
1-4	400 ml	10 ml	390 ml
5-8	600 ml	15 ml	585 ml
9-12	800 ml	20 ml	780 ml

7.2 Standards D3.1 – D3.6

Add **1.0 ml** of assay buffer **D6** to each standard vial **D3.1 – D3.6** and mix quickly, e.g. within 2 s by vortex.

7.3 Controls D7 and D8

Add **1.0 ml** of assay buffer **D6** to each control vial **D7** and **D8** and mix quickly, e.g. within 2 s by vortex.

7.4 1X HRP conjugate

Dilute 20X HRP conjugate D5 at 1:20 ratio with assay buffer D6. Mix by shaking the tubes.

Number of immunostrips	Volume of 20X HRP D5	Volume of assay buffer D6
1 – 4	0.3 ml	5.7 ml
5 – 8	0.5 ml	9.5 ml
9 - 12	0.6 ml	11.4 ml

8 Procedure notes

Any improper handling of samples or modification of the test procedure may influence the results. The indicated volumes, incubation times, temperatures and pretreatment steps must be followed strictly regarding this instruction.

Be sure that required reagents, materials and devices are prepared ready at the appropriate time.

1X HRP conjugate D5, staining solution D9 and stop solution D10 should be transferred by 8-channel micropipette or a pipette with reservoir (multistep pipette) to all wells of the immunostrips.

Washing should be done by 8-channel micropipette or ELISA plate washer. Avoid drying and over stressing of wells and control exact washing of all wells.

All measurements can be done in **single determinations**; however a dual determination increases the safety of the results and allows additional evaluations for the precision of the measurements. For duplicate determinations, R^2 of the standard curve should be ≥ 0.99.

It is recommended to use a pipetting scheme to apply all STD, CTRL and samples.

8.1 Ready to use components

- Allow negative control **D4**, assay buffer **D6**, staining solution **D9** and stop solution **D10** to reach RT and mix by vortex before use.

8.2 Reconstitution of reagents

- 1X wash solution should be prepared before the first wash step.
- Standards D3.1 – D3.6 and controls D7 and D8 should be reconstituted before starting the test.
- 1X HRP conjugate should be prepared before starting the second incubation.

8.3 Specimen preparation and dilution

- Allow samples to reach RT before use.
- Mix samples before use by vortexing for 6-10 s.
- For dilution of specimen use of known and pre-tested polypropylene tubes only is recommended.
  Alternatively, specimen can be diluted directly onto immunostrips by transfer of D6 followed by transfer of specimen for well.
- A sample dilution between **1:2** and **1:4** is recommended.
- To inhibit reactions from specimen e.g. serum to mouse immunoglobulin Roboscreen offers Blocking solution on request.
- Samples showing an OD higher than OD of highest standard D3.1 should be diluted more using assay buffer D6.
- Samples showing an OD lower than OD of lowest standard D3.6 should be diluted less.

Immunoassay procedure

1. Transfer 100 µl of each pre- diluted **sample** from polypropylene tube onto Immunostrips. For dilution of samples directly in the plate pipet assay buffer **D6** followed by samples and mix 3-5 x using pipet. Pipetting of duplicates of each sample is recommended.

2. Transfer 100 µl of each reconstituted standards **D3.1-D3.6**, controls **D7** and **D8** and blank **D4** onto immunostrips. Pipetting of duplicates is recommended.

NOTE
Avoid contamination of reagents, pipettes and wells/tubes by using different disposables between different samples and components. Do not interchange caps. Do not re-use any well, tube or reagent.

3. Cover the strips with sealing tape and incubate for 20 ± 2 h at RT and 150 ± 15 rpm.

NOTE
Shakers have different forces due to their deflection at a frequency of 150 rpm. The calculation of the acceleration (a) according to the formula $a = 4 \pi^2 rn^2$ should give a value of 2.5 m/s^2.

Radius (mm)	Number of revolutions (rpm)
10	150
5	212
3	274
1,5	387
0.5	671

π^2 = 9.87, r in m (10 mm = 0,01 m) and n in r/s (150 rpm/60 = 2,5 r/s)

4. Remove cover and wash **3** times with 300 µl Wash buffer manually or by use of a plate washer.

NOTE
Pull off the sealing tape carefully to avoid cross-contamination.

5. Transfer 100 µl of 1:20 diluted HRP conjugate **D5** into each well.

6. Cover the strips with sealing tape or lid and incubate at RT for 90 min.

7. Remove cover and wash **5** times with 300 µl Wash buffer manually or by use of a plate washer.

NOTE
Staining should be performed immediately after washing step 7 within 5 min.

8. Pipette 100 µl of **staining solution D9** into each well.

9. Incubate plate at RT in the dark for 30 min.

10. Stop the substrate reaction by adding 150 µl of **stop solution D10** into each well.

11. Measurement of absorbance: Mix plate with shaker of the reader for 3-5 s and let it settle down for 5 s. Measure the OD at 450 nm using 620 nm as reference wave length within 10 minutes after termination of the reaction.

Note
In high concentrated controls or samples staining components may be precipitated some time after termination. In this case additional mixing before reading is recommended.

9 Data analysis

The OD of the measured values is determined as the difference of the measured OD at 450 nm minus the OD at reference wavelength 620 nm ($OD_{450/620\ nm}$).

9.1 Quality criteria of the assay

- $OD_{450/620\ nm}$ value of negative control **D4** (blank) should be **< 0.2**.
- $OD_{450/620\ nm}$ values of positive controls **D7** and **D8** should be inside range corresponding to batch specific certificate.
- R^2 of the calibration curve should be ≥ 0.99.

9.2 Calculation of unknown T231 phosphorylated tau protein concentration

For the determination of the human T231 phosphorylated tau protein concentration in controls and samples the automatic data analysis by means of reader software, usually the logistic regression with 4 or 5 parameters or logit-log method is recommended. The standard curve typically shows a linear progression between the plateau of the highest standard **D3.1** (300 pg/ml) and the lowest standard **D3.6** (20 pg/ml).

Note
Dilution factor has to be included for estimation of real concentration of T231 phosphorylated tau protein within samples.

Note
Samples with a measured OD smaller than the OD of the lowest standard D3.6 can be reported in terms of T231 phosphorylated tau protein concentration <20 pg/ml.

10 References

Santos J, et. al. 2019. Validation of a prototype tau Thr231 phosphorylation CSF ELISA as a potential biomarker for Alzheimer's disease. *J. Neural Transmission.* 2019, S. 126: 339–348.