Instructions for Use
Life Science Kits & Assays

phosphoTAU ELISA

analytikjena
An Endress+Hauser Company

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Manufacturer:
AJ Roboscreen GmbH
Hohmannstraße 7
04129 Leipzig
Made in Germany!

Phone +49 341 989734 0
Fax +49 341 989734 199

Distribution/Publisher:
Analytik Jena AG
Konrad-Zuse-Straße 1
07745 Jena · Germany

www.analytik-jena.com
info@analytik-jena.com

Contents

1 Introduction

1.1 Intended use

The phosphoTAU ELISA is an enzyme immunoassay intended for the quantitative determination of phosphorylated tau in human cerebrospinal fluid (CSF) for supporting diagnosis of Alzheimer's disease (AD). The development of Alzheimer's disease is characterized by three stages, as defined by the US National Institute on Aging workgroups:

- a preclinical stage of Alzheimer's Disease,
- the mild cognitive impairment (MCI) stage due to AD and
- dementia stage due to AD.

Phosphorylated tau in CSF shows at least comparable diagnostic specificity and sensitivity to other diagnostic available tests for Alzheimer's disease.

1.2 Warranty and technical support

The manufacturer guarantees the correct functioning of the kit for the applications described in the instruction for use (IFU). During the warranty period, phosphoTAU 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, Analytik Jena AG 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 phosphorylated tau in CSF.

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 instruction 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**
	**Expiration date**
	**Manufactured by**
	**For single use only**

The following abbreviations are used in the IFU:

AD	Alzheimer's disease
CSF	Cerebrospinal fluid
CV	Coefficient of variation
ELISA	Enzyme-linked immunosorbent assay
GLP	Good Laboratory Practice
HRP	Horseradish peroxidase
OD	Optical density
RT	Room temperature (18 - 25°C)
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 Latex 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 COMPONENTS OF THE KIT 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.

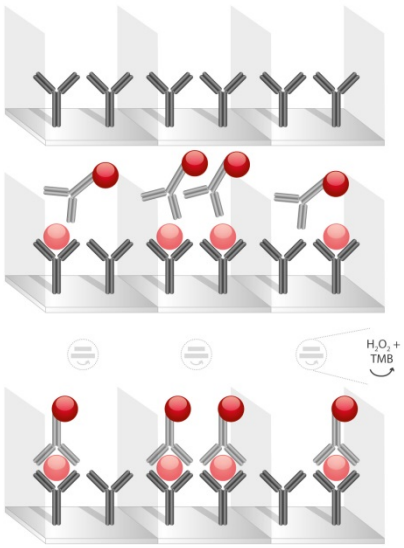
Avoid any contact with stopping solution. This may cause irritations to the skin and chemical burns.

FOR SINGLE USE ONLY!
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

This kit works by means of a monoclonal antibody that specifically recognizes phosphorylated tau protein within amino acids 1-200, immobilized on the surface area of the microtiter plate. Phosphorylated tau protein from samples, standards and controls is trapped by this antibody in presence of another peroxidase conjugated monoclonal anti-tau antibody that specifically binds to amino acids 155-165 of human tau protein. Amount of bound conjugated antibody is estimated using chromogenic substrate tetramethylbenzidine (TMB). The concentration of phosphorylated tau protein is proportional to the obtained optical density.



1. **Ready-to-use: Capture antibody coated on well plate**

2. **Binding of target antigen by capture antibody and incubation of HRP-conjugated antibody**

3. **Direct detection using a HRP-conjugated antibody**

4 Performance assessment

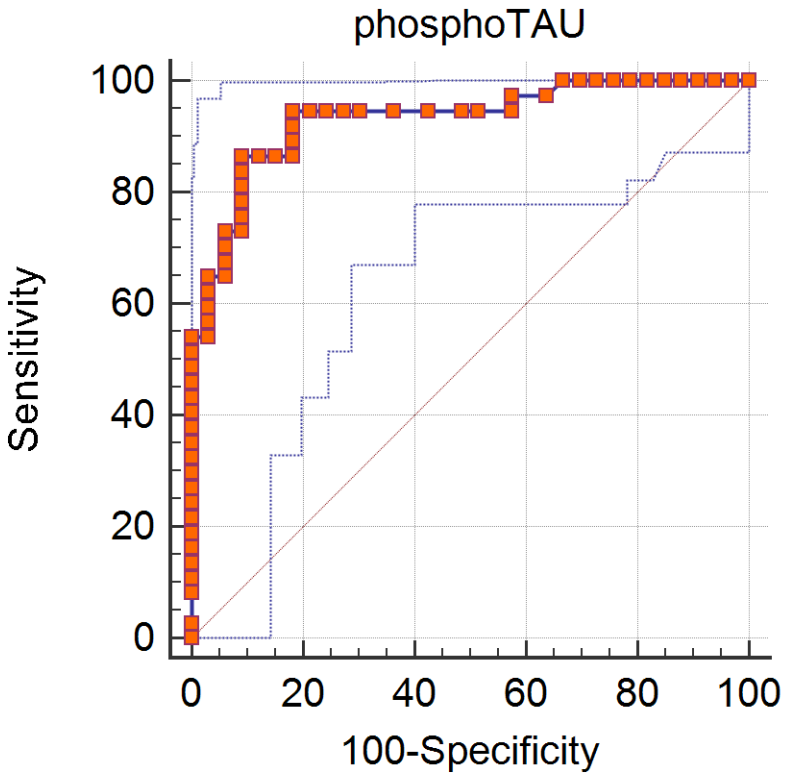
The table below shows typical data for calibration curves. Do not use for calculation!

Standard	Phosphorylated tau (pg/ml)	3 h Incubation 150 rpm (21,5 ± 3,5 °C)	
		OD_{mean}	OD/OD_{max} (%)
D3.1	1500	2.444	100
D3.2	260	0.638	26
D3.3	120	0.343	14
D3.4	70	0.203	8.3
D3.5	60	0.141	5.8
D3.6	30	0.082	3.4

The table summarizes sensitivity and specificity related to determined cut-offs.

Analytical Sensitivity (Limit of Detection)	30 pg/mL	Mean signal negative control (blank) + 3x SD
Cut-off	**62 pg/mL**	Each laboratory must establish its own cut-off values.
Clinical sensitivity	86.5 %	AD/MCI patients (n=37)
Clinical specificity	90.9 %	Controls (n=33)

Verification of significant differences ($p<0.001$) of phosphorylated tau concentration (phosphoTAU ELISA) in CSF samples of patients with Alzheimer's disease (AD, n=37) as well as control patients (CO, n=33).



5 Kit components

Component	Σ 96	Description
Immunoplate **D1**	12 x 8	Coated immuno strips containing anti-phosphorylated tau antibody, blocked and stabilized. Ready to use.
40X Wash buffer **D2**	1 x 50 ml	40X Wash buffer containing PBS, protein detergent and proclin 300.
Standards	6 x 3	Dryed phosphorylated tau standards for preparing a standard curve for quantification of phosphorylated tau in unknown cerebrospinal fluid samples. Containing PBS, protein and proclin 300.
D3.1 	3	1500 pg phosphorylated tau
D3.2 	3	260 pg phosphorylated tau
D3.3 	3	120 pg phosphorylated tau
D3.4 	3	70 pg phosphorylated tau
D3.5 	3	60 pg phosphorylated tau
D3.6 	3	30 pg phosphorylated tau
Negative control **D4** 	1 ml	Negative control (blank), containing TRIS, detergent and Sodium-Merthiolat. Ready to use.
2X HRP conjugate **D5** 	4 ml	Monoclonal anti-Tau antibody conjugated with horseradish peroxidase, 2X concentrate containingTRIS buffer, albumin, detergent and stabilizers (Ka-thon, Bronidox).
Assay buffer **D6**	50 ml	Dilution buffer containing carbonate, protein, detergent and proclin 300. Ready to use.

Kit components

Component	Σ 96	Description
Control high **D7** 	3	Dryed phosphorylated tau high positive control. Containing PBS, protein and proclin 300.
Control low **D8** 	3	Dryed phosphorylated tau low positive control. Containing PBS, protein and proclin 300.
Staining solution **D9**	20 ml	TMB/peroxide solution. Ready to use.
Stop solution **D10**	25 ml	1 M sulphuric acid. Ready to use.
Sealing tape	1	
Instruction for use	1	

6 Preparation of components

6.1 1X Wash solution

Dilute 40X wash buffer **D2** using de-ionized or bi-distilled water before the first wash step of the immunoassay.

Volume of 1X Wash solution	Volume of 40X Wash buffer D2	Volume of de-ionized or bi-distillated water
400 ml	10 ml	390 ml
600 ml	15 ml	585 ml
800 ml	20 ml	780 ml
1000 ml	25 ml	975 ml

6.2 Standards D3.1-D3.6

Add 0.5 ml of assay buffer **D6** to each standard vial **D3.1 – D3.6** and mix quickly, e.g. within 2 s by vortex.

6.3 Controls D7 and D8

Add 0.5 ml of assay buffer **D6** to each control vial **D7** and **D8** and mix quickly, e.g. within 2 s by vortex.

6.4 1X HRP conjugate

Dilute 2X HRP conjugate **D5** at 1:2 (v/v) with assay buffer **D6**. Mix by means of shaking the tube.

Number of immuno strips	Volume of 2 x HRP D5	Volume of dilution buffer D6
1 - 4	1 ml	1 ml
5 - 8	2 ml	2 ml
9 - 12	3 ml	3 ml

7 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 life time 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 immuno strips after opening of bag, taking out of strips and closing of bag.	At 6 ± 4 °C up to 4 weeks.
D2	1X Ready-to-use wash solution.	At 6 ± 4 °C up to 4 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 1X HRP-conjugate.	At 6 ± 4 °C up to 4 h.

8 Components not included in the kit

- Calibrated micropipettes with CV < 3 %
  Volume: 10-100 µL; 100-1000 µL
- 8-channel micropipette with reagent reservoirs
- Plate shaker 100-1500 rpm.
- Vortex mixer
- Automated or semi-automated ELISA plate washing system
- Bi-distilled or de-ionized water
- Paper towels, pipette tips and timer
- ELISA plate reader for reading absorbance at 450 and 620 nm
- Polypropylene tubes for sample dilution

9 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. Allow staining solution **D9** to reach room temperature (21.5 ± 3.5 °C). Mix assay buffer **D6** and 2X HRP conjugate **D5** by vortex before use.

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

It is advised to test in duplicate and to use a pipetting scheme.

Solution of 1X HRP conjugate, staining solution **D9** and stop solution **D10** should transferred by 8-channel micropipette to all wells.

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

10 Specimen collection and storage

The Alzheimer's Biomarker Standardization Initiative provides the following recommendations for the pre-analytical and analytical aspects for AD biomarker testing in CSF (Vanderstichele et al., Standardization of preanalytical aspects of cerebrospinal fluid biomarker testing for Alzheimer's disease diagnosis: a consensus paper from the Alzheimer's Biomarkers Standardization Initiative. Alzheimers Dement. 2012 Jan; 8(1):65-73).

10.1 Specimen collection

Lumbar puncture may be performed at the vertebral body L3-L5 with the patient either sitting or lying down. Use a small diameter (0.7 mm and 22 G), preferably not traumatic needle. A small-gauge needle will make a

smaller hole in the Dura mater, aiding healing, and not traumatic needle will reduce the chance of blood contamination in the CSF.

Each laboratory should use one kind of polypropylene tubes only. Glass or polystyrene tubes should in no circumstances be used. Tubes of the smallest volume should be used, and these should be filled to at least 50 % of their volume. It is important to have carefully recorded and validated details concerning each stored sample so that any investigator when using these samples has a precise history of the sample.

Centrifugation is only required for visually hemorrhagic samples. Centrifuge immediately with recommended 2000 x g at RT for 10 min.

10.2 Specimen storage

It is recommended to freeze samples and store at -80 °C for long time storage. It is recommended to limit the number of freeze /thaw cycles to a maximum of 1-2. Samples should be stored no longer than 2 years.

Note
For dilution of CSF use polypropylene tubes or dilute directly onto immuno plate **D1**.

10.3 Specimen dilution

Samples showing an OD higher than OD of highest standard **D3.1** should be diluted before test procedure 1:2 or higher using assay buffer **D6.**

11 Test procedure

1. Transfer 50 µL of 1X **HRP conjugate** in each well followed by pipetting of 50 µL of each **standard, controls and patient samples** into the respective wells of plate. The sequence can be reversed.
2. Mix using pipette tip 2-3 times.
3. Cover plate with lid or foil.
4. Incubate immune plate for **3 h ± 10 min at 21.5 ± 3.5 °C** and **150 ± 50 rpm**.
5. Wash plate 5 x with 300 µL/well of **1X wash solution** using an automatic ELISA plate washer.

Note
Alternatively, when performed manually, discard incubation solution. Remove excess solution after washing by tapping immuno strip on paper towel.

6. Pipette 100 µL of **staining solution D9** into each well.
7. Incubate plate at RT in the dark for 30 min.
8. Stop the substrate reaction by adding 150 µL of **stop solution D10** into each well. Briefly mix contents by gently shaking the plate.
9. Measure optical density with a photometer at 450 nm using 620 nm as reference wave length within 15 min after stopping.

Note
In samples with a high concentration of phosphorylated tau formed dye may be precipitated due to intensive staining. Therefore, 15 min time lapse until the measurement takes place are recommended.

12 Data analysis

12.1 Quality criteria of the assay

- $OD_{450/620\ nm}$ of the negative control **D4** (**BLANK**) should be **<** 0.1
- Concentration of positive control **D7** and **D8** should be according to the certificate of analysis
- $OD_{450/620\ nm}$ of the standard **D3.1** should be >1.2
- R^2 of the calibration curve that should be ≥ 0.99

12.2 Calculation of unknown phosphorylated tau concentration

Use logarithmic values (LN) of standard ODs and standard concentrations for plotting them onto x-axis (OD) and y-axis (concentration) of a linear diagram or for using them in linear regression analysis to estimate concentration of controls and each sample. Logarithmic values of measured sample OD's have to be used for this regression analysis followed by exponentiation to calculate concentration in pg/ml or ng/l.

An automated method performed by common reader software could be also used for quantification; 4 or 5 parameter logistics or logit-log methods are recommended. The calibration curve typically shows a linear part between a plateau for highest standard **D3.1** (1500 pg/ml) and plateau for lowest standard **D3.6** (30 pg/ml).

Note
Measured samples with OD below OD of lowest standard **D3.6** without option to measure it more concentrated could be calculated for unknown Phosphorylated tau concentration as ½ of this lowest standard concentration = 15 pg/ml.

13 Expected values

Note
Expected values are calculated from data of first clinical validation stage. All data should to be handled as preliminary.

Variable	phosphoTAU
Classification variable	AD_CO
Sample size	70
AD group	37 (52,86%)
Control group	33 (47,14%)
Area under the ROC curve (AUC)	0,932
Standard Error [a]	0,0297
95% Confidence interval [b]	0,845 to 0,978
z statistic	14,522
Significance level P (Area=0.5)	<0,0001
Youden index J	0,7740
95% Confidence interval [a]	0,5915 to 0,8853
Associated criterion	>62
95% Confidence interval [a]	>59 to >90,7
Sensitivity	86,49
Specificity	90,91

engl. 10/18 – Analytik Jena AG, Jena

Headquarters

Analytik Jena AG
Konrad-Zuse-Str. 1
07745 Jena · Germany

Phone +49 3641 77 70
Fax +49 3641 77 9279
info@analytik-jena.com
www.analytik-jena.com

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