Performance characteristics, continued

Intra-assay precision

Samples of known Hu Tau [pT181] concentration were assayed in replicates of 16 to determine precision within an assay.

Parameters	Sample 1	Sample 2	Sample 3
Mean (pg/mL)	35.6	126.5	492.2
SD	2.11	4.60	15.98
%CV	6	4	3

SD = Standard Deviation; CV = Coefficient of Variation

Inter-assay precision

Samples were assayed 48 times in multiple assays to determine precision between assays.

Parameters	Sample 1	Sample 2	Sample 3
Mean (pg/mL)	33.1	124.3	480.9
SD	3.21	4.58	15.29
%CV	10	4	3

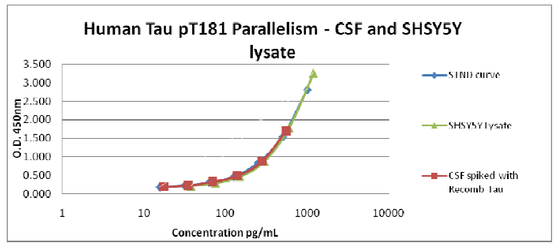
SD = Standard Deviation; CV = Coefficient of Variation

Sensitivity

The minimum detectable concentration of Hu Tau [pT181] is <10 pg/mL. This was determined by adding two standard deviations to the mean O.D. obtained when the zero standard was assayed 64 times.

Parallelism

Human CSF was spiked with Hu Tau [pT181] and both CSF samples and natural Hu Tau pT181 from SH-SY5Y neuroblastoma cell extract were serially diluted in Standard Diluent Buffer over the range of the assay. The optical density of each dilution was plotted against the standard curve. Parallelism between the natural and recombinant protein was demonstrated by the figure below and indicated that the standard accurately reflects natural Hu Tau [pT181] content in samples.

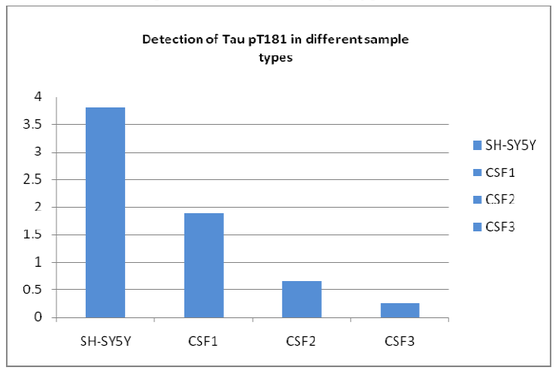


Specificity

The peptide blocking competition data presented show that only the phosphopeptide containing the phosphorylated threonine 181 could block the ELISA signal. The non-phosphorylated peptide sequence or other phosphopeptides from the Tau sequence did not block the signal.

	Non-Phospho Peptide	Phospho Peptide pT181	Non-specific Phospho Peptide pS396	Non-specific Phospho Peptide pT231	Non-specific Phospho Peptide pS199	Non-specific Phospho Peptide pS214
Standard 0	0.053	0.052	0.055	0.059	0.061	0.055
Standard 1	1.981	2.002	2.042	1.946	1.904	2.021
Peptide Blocking	1.91	0.333	2.04	1.894	1.929	2.06
	1.965	0.322	2.091	2.01	1.962	2.154

The Tau [pT181] ELISA kit is suitable for the measurement of Tau [pT181] in different sample matrixes. Human CSF and cell extract from neuroblastoma, were analyzed. Human CSF samples were spiked at various concentrations prior to performing assay. The data presented show that the kit detects various concentrations of Tau [pT181] in different sample types.



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Product label explanation of symbols and warnings

REF	Catalog Number	LOT	Batch code		Temperature limitation		Use by		Manufacturer		Consult instructions for use		Caution, consult accompanying documents

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27 August 2014

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Human Tau [pT181] phosphoELISA™ Kit

Catalog. no. KHO0631 **Quantity:** 96 tests

Pub. Part No. PR328 **Pub. No**. MAN0004022 **Rev** 1.00

Description

The Human Tau [pT181] ELISA Kit is a solid-phase sandwich Enzyme Linked Immuno Sorbent Assay (ELISA), and is to be used for the quantitative determination of Hu Tau [pT181] in human cerebrospinal fluid (CSF), buffered solution, or cell culture medium. The assay will recognize both natural and recombinant Hu Tau [pT181].

Tau is a microtubule-associated protein of considerable importance to neuronal axons of vertebrate brain. Human tau exists as six different isoforms that result from alternative splicing of a single transcript derived from a gene located on chromosome 17. The molecular weights of the tau isoforms range from 48–68 kDa. Tau protein is highly soluble and normally attached to axonal microtubules. Tau stabilizes the microtubules and makes them rigid. Tau interacts with actin in the cytoskeleton and neuronal outgrowth, anchors enzymes such as protein kinases and phosphatases, and regulates intracellular vesicle transport.

Contents and storage

The components included in the ELISA kit are listed below. Upon receipt, store the kit at 2°C to 8°C.

Components	Quantity
Hu Tau [pT181] Standard, recombinant Hu Tau-441 expressed in *E. coli* and SMCC Conjugated to phospho-peptide T231. Contains 0.1% sodium azide. Refer to vial label for quantity and reconstitution volume.	2 vials
Standard Diluent Buffer. Contains 0.1% sodium azide; red dye*.	25 mL
Antibody Coated Wells. 12 × 8 Well Strips.	1 plate
Hu Tau [pT181] Detection Antibody. Contains 0.1% sodium azide; blue dye*.	6 mL
Anti-Rabbit IgG HRP (100X). Contains 3.3 mM thymol.	0.125 mL
HRP Diluent. Contains 3.3 mM thymol; yellow dye*.	25 mL
Wash Buffer Concentrate (25X)	100 mL
Stabilized Chromogen, Tetramethylbenzidine (TMB)	25 mL
Stop Solution	25 mL
Plate Covers, adhesive strips	3

*To help monitor the addition of reagents to the reaction wells and avoid any pipetting errors, we provide colored Standard Diluent Buffer, Detection Antibody, and HRP Diluent. The colored dye does not interfere with the test results.

CAUTION! This kit contains materials with small quantities of sodium azide. Sodium azide reacts with lead and copper plumbing to form explosive metal azides. Upon disposal, flush drains with a large volume of water to prevent azide accumulation. Avoid ingestion and contact with eyes, skin and mucous membranes. In case of contact, rinse affected area with plenty of water. Observe all federal, state, and local regulations for disposal.

Materials required but not provided

- Cell Extraction Buffer (Cat. no. FNN0011)
- Distilled or deionized water
- Microtiter plate reader (at or near 450 nm) with software
- Plate washer–automated or manual (squirt bottle, manifold dispenser, or equivalent)
- Calibrated adjustable precision pipettes and glass or plastic tubes for diluting solutions

Before starting

Review the **Procedural guidelines** and **Plate washing directions** in the *ELISA Technical Guide* available at **www.lifetechnologies.com/manuals** for details prior to starting the procedure.

Note: Reagents are lot-specific. Do not mix or interchange different reagent lots from various kit lots.

For Research Use Only. Not for use in diagnostic procedures.

Manufacturing Site • 7335 Executive Way • Frederick • MD 21704 • E-mail: **techsupport@lifetech.com**

Dilute wash buffer

1. Allow the Wash Buffer Concentrate (25X) to reach room temperature and mix to redissolve any precipitated salts.
2. Dilute 1 volume of the Wash Buffer Concentrate (25X) with 24 volumes of deionized water (e.g., 50 mL may be diluted up to 1.25 liters, 100 mL may be diluted up to 2.5 liters). Label as Working Wash Buffer.
3. Store the concentrate and the Working Wash Buffer in the refrigerator. Use the diluted buffer within 14 days.

Prepare secondary antibody

Note: Prepare the secondary antibody within 15 minutes of usage.

The Anti-Rabbit IgG HRP (100X) is in 50% glycerol, which is viscous. To ensure accurate dilution:

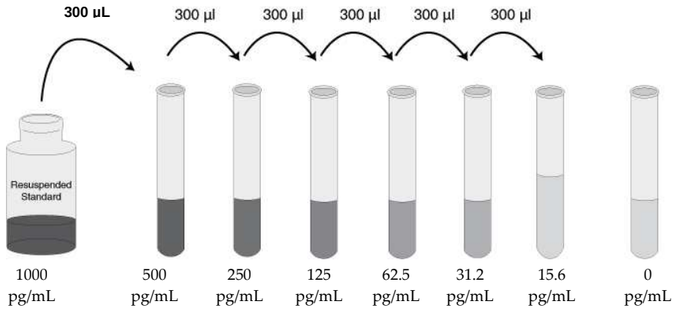
1. For each 8-well strip used in the assay, pipet 10 µL Anti-Rabbit IgG HRP (100X) solution, wipe the pipette tip with a clean absorbent paper to remove any excess solution, and dispense the solution to a tube containing 990 µL of HRP Diluent. Mix thoroughly.
2. Return the unused Anti-Rabbit IgG HRP (100X) to the refrigerator.

Dilute the standards

Note: The Hu Tau [pT181] Standard was calibrated using GSK-3β-phosphorylated, recombinant Hu Tau-441 protein expressed in *E. coli.*

1. Reconstitute Human Tau [pT181] Standard with Standard Diluent Buffer. Refer to the standard vial label for instructions. Swirl or mix gently and allow the contents to sit for 10 minutes to ensure complete reconstitution. Label as 1,000 pg/mL Hu Tau [pT181]. Use the standard within 1 hour of reconstitution.
2. Add 300 µL Standard Diluent Buffer to each of 6 tubes labeled as follows: 500, 250, 125, 62.5, 31.2, and 15.6 pg/mL of Hu Tau [pT181].
3. Make serial dilutions of the standard as described below in the dilution diagram. Mix thoroughly between steps.

Aliquot and store any remaining reconstituted standard at –80ºC for further use. Standard can be frozen and thawed **one time only** without any loss of activity.



Prepare cell lysate

The following extraction procedure is suitable for use with several cell lines using the Cell Extraction Buffer.

1. To the Cell Extraction Buffer (Cat. no. FNN0011), add the following protease inhibitors just prior to use:
   - 1 mM PMSF (stock is 0.3 M in DMSO).
   - Protease inhibitor cocktail (e.g. Sigma Cat. no. P-2714, reconstitute according to manufacturer's guideline). Add 250 µL per 5 mL Cell Extraction Buffer.

   Note: The stability of Cell Extraction Buffer with protease inhibitors is 24 hours at 4°C. PMSF is very unstable and must be added prior to use, even if added previously.
2. Collect cells in phosphate buffered saline by centrifugation (non-adherent) or scraping from culture flasks (adherent).
3. Wash cells twice with cold PBS. Discard the supernatant and collect the cell pellet. (At this point the cell pellet can be frozen at -80°C.)
4. Lyse the cell pellet in Cell Extraction Buffer for 30 minutes on ice with vortexing at 10 minute intervals. The volume of Cell Extraction Buffer depends on the cell number in cell pellet and expression and phosphorylation levels of the protein.

   For example, 10^7 SH-SY5Y cells can be extracted in 0.5 mL of Cell Extraction Buffer to recover 1 mg/mL of total protein. Under these conditions, cell extract dilutions from 1:10–1:100 with Standard Diluent Buffer are sufficient for Tau [pT181] detection.
5. Transfer lysates to microcentrifuge tubes and centrifuge at 13,000 rpm (13793 × g) for 10 minutes at 4°C.
6. Aliquot the clear lysates to clean microcentrifuge tubes for assay. Lysates can be stored at -80°C. Avoid multiple freeze-thaw cycles.

Dilute sample

Dilute the cell extract samples 1:20 or greater in Standard Diluent Buffer (for example, dilute 5 µL sample into 95 µL buffer). This minimum 20–fold dilution step is necessary to avoid SDS interference with the assay. Optimize the dilution for each experimental system.

ELISA procedure

Allow all reagents to reach room temperature before use. Mix all liquid reagents prior to use. **Total assay time is 15-19 hours.**

IMPORTANT! Perform a standard curve with each assay.

Determine the number of 8-well strips required for the assay. Insert the strips in the frames for use. Re-bag any unused strips and frames, and store at 2 to 8°C for future use.

Bind antigen/add detector antibody

1. Add 50 µL of standards, diluted samples (see page 2) or controls to the appropriate microtiter wells.
2. Add 50 µL of Hu [pT181] Detection Antibody solution into each well.
3. Cover the plate with plate cover and incubate overnight (14-18 hours) at 4°C.
4. Thoroughly aspirate the solution from the wells and wash wells 4 times with diluted Wash Buffer.

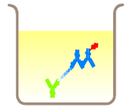
Add secondary antibody

5. Add 100 µL of diluted Anti-Rabbit IgG HRP (see page 2) to each well.
6. Cover the plate with plate cover and incubate for 30 minutes at room temperature.
7. Thoroughly aspirate solution from wells and wash wells 4 times with diluted Wash Buffer.

Add chromogen

8. Add 100 µL of Stabilized Chromogen to each well. The substrate solution begins to turn blue.
9. Cover the plate with plate cover and incubate for 30 minutes at room temperature in the dark.

Note: TMB should not touch aluminum foil or other metals.



Add stop solution

10. Add 100 µL Stop Solution. Tap side of the plate gently to mix. The solution in the wells changes from blue to yellow.

Read the plate and generate the standard curve

1. Read the absorbance at 450 nm. Read the plate within 2 hours after adding the Stop Solution.
2. Use curve-fitting software to generate the standard curve. A four parameter algorithm provides the best standard curve fit. Optimally, the background absorbance may be subtracted from all data points, including standards, unknowns and controls, prior to plotting.
3. Read the concentrations for unknown samples and controls from the standard curve. Multiply value(s) obtained for sample(s) by the appropriate factor to correct for the sample dilution.

   Note: If samples produce signals greater than that of the highest standard, then dilute samples in Standard Diluent Buffer and reanalyze. Multiply the concentration by the appropriate dilution factor.

Performance characteristics

Standard curve (example)

The following data were obtained for the various standards over the range of 0–1000 pg/mL Hu Tau [pT181].

Standard Hu Tau [pT181] (pg/mL)	Optical density (450 nm)
1000	3.343
500	1.261
250	0.646
125	0.344
62.5	0.219
31.2	0.166
15.6	0.131
0	0.098

Linearity of dilution

Human CSF samples spiked with Hu Tau [pT181] and and natural Hu Tau pT181 from SH-SY5Y neuroblastoma cell extracts were serially diluted in Standard Diluent Buffer over the range of the assay. Linear regression analysis of samples versus the expected concentration yielded a correlation coefficient of 0.99 in both.

CSF				SH-SY5Y Cell Lysate			
Dilution	Measured (pg/mL)	Expected (pg/mL)	% Expected	Dilution	Measured (pg/mL)	Expected (pg/mL)	% Expected
Neat	556.99	556.99	100	1/64	1196.90	1196.90	100
1/2	261.60	278.50	93.93	1/128	588.14	598.45	98
1/4	128.66	139.25	92.39	1/256	266.44	299.22	89
1/8	70.82	69.62	101.72	1/512	120.43	149.61	80
1/16	37.10	34.81	106.57	1/1024	59.89	74.81	80
1/32	20.27	17.41	116.44	1/2048	30.55	37.40	82