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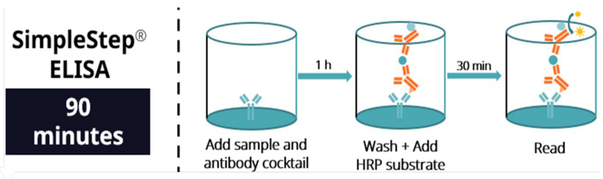
High sensitivity quantification of phosphorylated Tau in Alzheimer's Disease samples using chemically peptide-ligated protein standards

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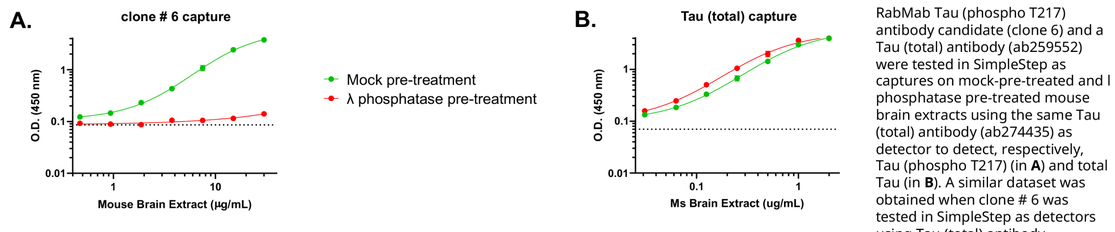
Introduction

Quantitative ELISA kits require standards to extrapolate the absolute concentrations of the analyte in the sample of interest. This is also the case for Abcam's SimpleStep® ELISA kits (SSEs), one-step one-wash sandwich ELISAs in which tagged capture antibody, the HRP-labelled detector antibody, and the sample are added simultaneously into the assay well coated with an anti-tag antibody.

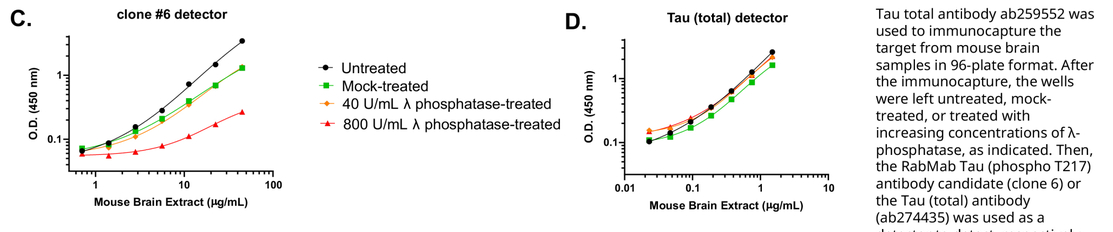


We have developed SimpleStep kits to quantify a variety of protein and peptide targets; these kits include recombinant- or native-purified proteins as kit standards. However, kits that assay protein post-translational modifications (PTMs), including phosphorylation, represent many challenges, including the preparation of appropriate standards. We previously developed a method to generate protein standards for Abcam's SimpleStep® sandwich ELISA kits, enabling quantification of specific phosphorylation. This approach uses a chemical ligation to attach a synthetic phosphopeptide of an amino acid sequence identical to the sequence surrounding the phosphorylated site of interest to a recombinant protein. Here, we expand the application of these chemically ligated proteins (CLPs) to screen and validate rabbit monoclonal antibody candidates for phospho- and sequence-specificity, facilitating their integration into SimpleStep® kits. We demonstrate this strategy in the development of ELISA kits targeting Alzheimer's Disease (A.D.) biomarker Microtubule-associated protein tau (Tau), specifically phosphorylated at T181, S199, T217, T231, and S396 sites. These phospho-Tau CLPs also enable assessment of cross-reactivity and interference with other Tau phospho proteins and the non-phosphorylated protein. We use the developed kits to measure relative PTM levels in A.D. brain extracts. Finally, we apply the high sensitivity SimpleStep Ignite™ chemiluminescence platform to the developed kits to measure relative PTM levels in individual A.D. patient biofluid samples, highlighting their utility in biomarker research and clinical studies.

The use of phosphatase-treated sample validates clone #6 for detecting of native phospho signal as both capture and detector

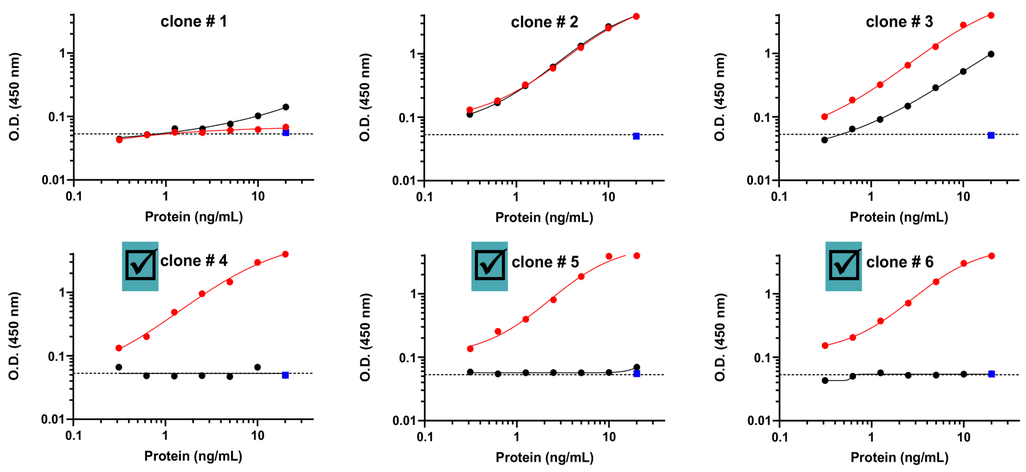


RabMab Tau (phospho T217) antibody candidate (clone 6) and a Tau (total) antibody (ab259552) were tested in SimpleStep as captures on mock-pre-treated and l phosphatase pre-treated mouse brain extracts using the same Tau (total) antibody (ab274435) as detector to detect, respectively, Tau (phospho T217) (in **A**) and total Tau (in **B**). A similar dataset was obtained when clone # 6 was tested in SimpleStep as detectors using Tau (total) antibody (ab259552) (data not shown).



Tau total antibody ab259552 was used to immunocapture the target from mouse brain samples in 96-plate format. After the immunocapture, the wells were left untreated, mock-treated, or treated with increasing concentrations of λ-phosphatase, as indicated. Then, the RabMab Tau (phospho T217) antibody candidate (clone 6) or the Tau (total) antibody (ab274435) was used as a detector to detect, respectively, Tau (phospho T217) (in **C**) and total Tau (in **D**).

Pair selection #1: Screening antibodies using Tau phosphopeptide CLP reveals phospho-specific clones #4, 5, and 6

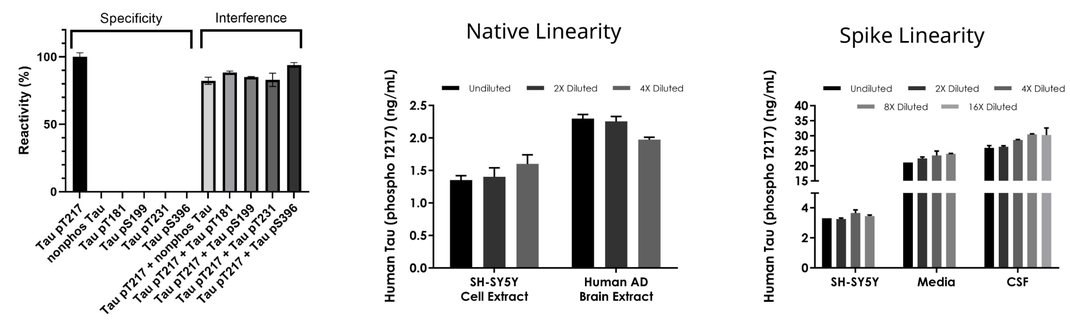


RabMab Tau (phospho T217) antibody candidates (clone # 1 – 6) were tested as captures on Tau (phospho T217) CLP (red circles), and on controls, Tau (non-phospho) CLP (black circles), and recombinant Tau protein (blue squares) using the same Tau (total) antibody (ab274435) as detector in SimpleStep. A similar dataset was obtained when the clones # 1 - 6 were tested in SimpleStep as detectors using the same Tau (total) antibody as capture (data not shown).

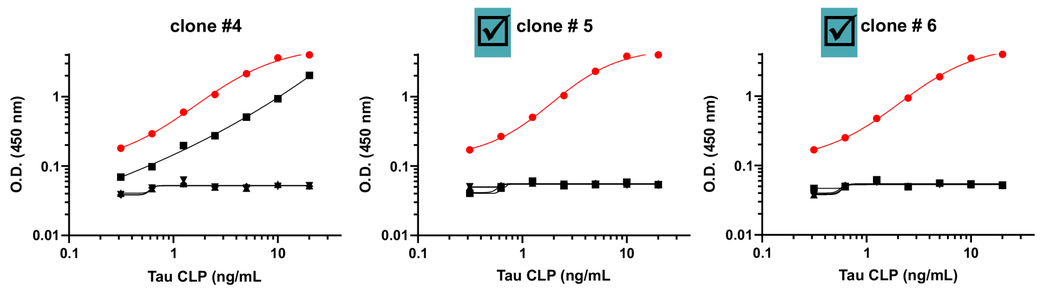
Example kit validation data for Human Tau (phospho T217)

SimpleStep validation requirements

- **Each validated sample type:**
  - ✓ Native (spike) linearity of dilution: 80 – 120%
  - ✓ Recovery: 80 – 120%
  - ✓ Unrelated capture interpolated values: < 20%
- **Precision**
  - ✓ intra and inter assay CVs: < 15 %
- **Specificity and Interference**
- **[Species Reactivity]**



Pair selection #2: Screening antibodies using variety of relevant phosphopeptide CLPs reveals phospho T217 sequence-specific clones #5 and 6



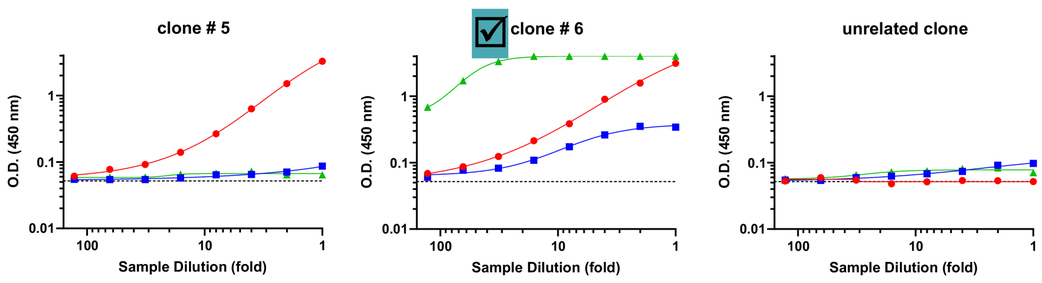
RabMab Tau (phospho T217) antibody candidates (clone # 4, 5, and 6) were tested as captures on Tau (phospho T217) CLP, Tau (phospho T181) CLP, Tau (phospho S199), Tau (phospho T231) CLP, and Tau (phospho S396) CLP using the same Tau (total) antibody (ab274435) as detector in SimpleStep. A similar dataset was obtained when the clones # 4, 5, and 6 were tested in SimpleStep as detectors using the same Tau (total) antibody as capture (data not shown).

CLP Legend
- phospho T217
- phospho T181
- phospho S199
- phospho T231
- phospho S396

Developed human Tau (phospho) SimpleStep colorimetric kits for cell and tissue extracts

PTM Site	SimpleStep Kit abID	Range (MDD) pg/mL	Phospho Tau / Total Tau Ratio	
			Normal Hippocampus	A.D. Hippocampus
T181	ab325079	39.1 – 2,500 (10.5)	0.13	0.48
S199	ab323735	68.8 – 4,400 (16.3)	0.24	0.46
T217	ab318935	470 – 30,000 (90)	0.00	0.48
T231	ab322366	93.8 – 6,000 (21.0)	0.33	0.86
S396	ab324329	62.5 – 4,000 (7.8)	0.07	1.02

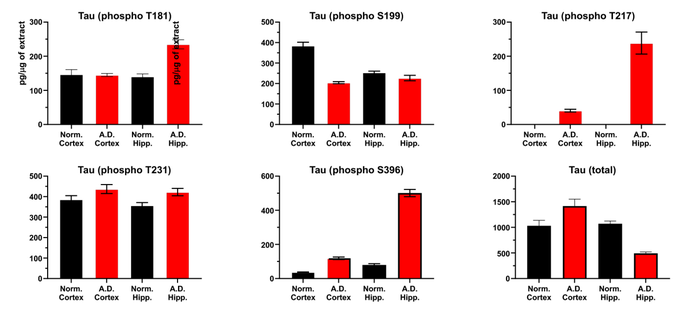
Pair selection #3: Screening antibodies against biological samples identifies clone # 6 as best candidate detecting native target



RabMab Tau (phospho T217) antibody candidates (clone # 5 and 6) and a Tau-unrelated antibody were tested in SimpleStep as captures on Tau (phospho T217) CLP, PC-12 cell extract, and mouse brain tissue extract using the same Tau (total) antibody (ab274435) as detector. A similar dataset was obtained when clones # 5 and 6 were tested in SimpleStep as detectors using the same Tau (total) antibody as capture (data not shown).

Sample Legend
- Tau (phospho T217) CLP 20 ng/mL top dose
- PC-12 Cell Extract 1 mg/mL top dose
- Mouse Brain Extract 1 mg/mL top dose

Utility of developed SimpleStep ELISA kits: Phospho Tau and total Tau concentrations in normal and A.D. brain cortex and hippocampus tissue extracts



Tau (phospho T181), Tau (phospho S199), Tau (phospho T217), Tau (phospho T231), Tau (phospho S396), and total Tau concentrations were measured, respectively, using ab325079, ab323735, ab318935, ab322366, ab324329 and ab273617 in serial dilutions of the same normal pooled (n=4) and A.D. individual (n=1) brain cortex and hippocampus tissue extracts in duplicates. The interpolated target concentrations, normalized to mg of extract, are shown (mean ± SD, n ≥ 4).

Human Tau (phospho) SimpleStep Ignite™ chemiluminescent ELISA kits for serum, plasma, supernatant and CSF show 6.9 – 16.4 increase in sensitivity

PTM Site	SimpleStep Ignite Kit abID	Sensitivity Increase (fold)	Range (MDD) pg/mL	Individual Post-mortem CSF Values (n=4)		
				Mean (pg/mL)⁺	Detectable (%)	Range (pg/mL)
T181*	ab325895	11.4	4.12 – 9,000 (1.06)	119.2	100	25.3 – 342.7
S199	ab325893	16.4	4.57 – 10,000 (3.92)	680.1	100	110.8 – 1,812.8
T231**	ab325892	12.8	6.86 – 15,000 (5.59)	2,259.0	100	590.8 – 5,651.9
S396*	ab325894	12.2	4.12 – 9,000 (2.27)	814.9	100	147.7 – 2,112.6

* Detectable in 1 out of 10 healthy individual serum. Not detectable (ND) in A.D. serum (n=5), A.D plasma (n=4), normal and A.D. (n=6) CSF.
** Detectable in 1 out of 10 healthy individual serum, and in one out of six A.D. CSF. Not detectable (ND) in A.D. serum (n=5), A.D plasma (n=4), normal CSF.
⁺ Mean of detectable

Summary of the three-step pair selection process showing CLP advantages for initial screening for phospho-specific antibody. Using this scheme, a single RabMab Tau (phospho T217) antibody was selected out of six candidates

1. Phospho specificity (CLP) → 2. Sequence specificity (CLP) → 3. Native signal (biologicals)

CLP advantage: Negative Controls * Sequence Controls * No Biologicals

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