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Development of a Sensitive and Specific Phospho-Tau 217 [Tau (pT217)] Biomarker Assay for Alzheimer's Disease in Plasma and Serum

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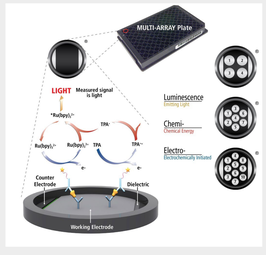
PURPOSE

As the population of older adults continues to increase in the United States and throughout the world, the incidence of Alzheimer's Disease (AD) is also on the rise. As a result, early detection of AD using simple tests such as blood-based biomarker assays is of increased interest. Although effective treatment strategies are currently limited, early detection will be important for implementing future AD therapies and essential for supporting the testing of new, experimental therapies. Many studies have shown that elevated levels of phosphorylated Tau (pTau) isoforms in serum, plasma, and CSF samples may be useful as early indicators of AD onset for researchers. More specifically, Tau phosphorylated at threonine 181 [Tau (pT181)] or threonine 217 [Tau (pT217)] are promising early-stage biomarkers of AD, with Tau (pT217) potentially having more predictive value than Tau (pT181), although this is still under investigation. Due to the relative ease of plasma and serum collection compared with CSF, there is a preference for the use of blood-based matrices for biomarker measurements. However, the detection of Tau (pT217) in plasma and serum is difficult due to the low circulating levels of this protein. Also, the adoption of Tau (pT217) as a biomarker for AD has been hampered by a lack of commercial assays with high sensitivity and specificity. Here we report the development of a highly specific and sensitive assay for the detection of Tau (pT217) in human serum and plasma.

METHODS

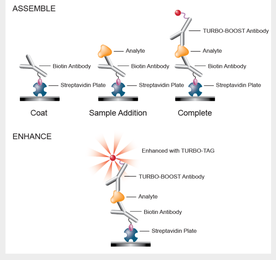
MSD's electrochemiluminescence (ECL) detection technology uses SULFO-TAG™ labels that emit light upon electrochemical stimulation initiated at the electrode surfaces of MULTI-ARRAY® and MULTI-SPOT® microplates.



Electrochemiluminescence Technology

- Minimal non-specific background and strong responses to analyte yield high signal-to-background ratios.
- The stimulation mechanism (electricity) is decoupled from the response (light signal), minimizing matrix interference.
- Only labels bound near the electrode surface are excited, enabling non-washed assays.
- Labels are stable, non-radioactive, and directly conjugated to biological molecules.
- Emission at ~620 nm eliminates problems with color quenching.
- Multiple rounds of label excitation and emission enhance light levels and improve sensitivity.
- Surface coatings can be customized.

Immunization and antibody engineering activities were conducted to produce many new clones against Tau (pT217). Selected antibodies were labeled individually with biotin (to serve as a capture antibody) and TURBO-BOOST® (to serve as a detection antibody for the ultrasensitive S-PLEX® assay format), and they were tested in various combinations and orientations for their ability to detect pT217. The S-PLEX platform uses an enhanced, electrochemiluminescence reporter technology for detection. Antibody pairs were chosen that included both a pan-specific total Tau antibody and an antibody specific for the phosphorylated T217 site. Furthermore, antibodies were tested for specificity against non-phosphorylated Tau protein and the phosphorylated T217 site using a mutated pTau protein, in which the threonine was replaced with an alanine at position 217. After a final antibody pair was chosen, the assay was optimized and used to measure pT217 in serum and plasma from healthy and diseased samples.



RESULTS

Clone Selection for pT217

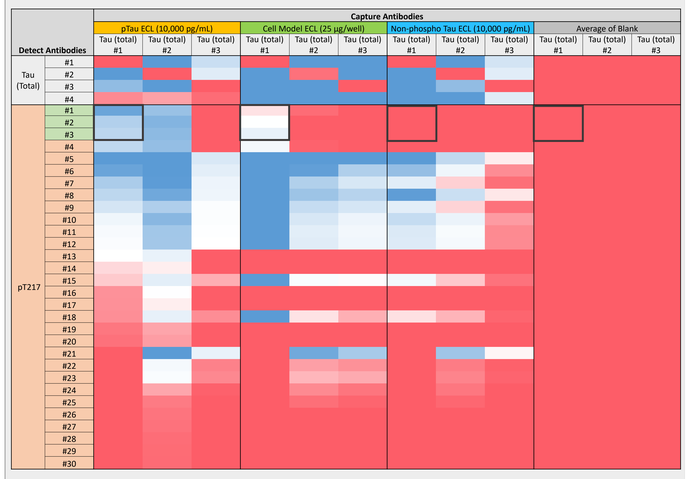


Table 1: Mice were immunized with KLH-conjugated peptide and resulting antibody clones were screened for reactivity against pT217 using 3 different Tau (total) capture antibodies. The 30 top-performing pT217 antibody clones are shown. Three antibody pairs were chosen for further screening (boxed) that had high ECL signal generation against phosphorylated Tau (orange) and a cell model that has natural expression of phosphorylated Tau (dark green), while having low reactivity against non-phosphorylated Tau (blue) and blanks (gray). ECL signal scale: blue>white>red.

Effect of Dephosphorylation of pT217

Capture	Tau (total) #2	pT217 #1	pT217 #2	pT217 #3
Detect	Tau (total) #1	Tau (total) #1	Tau (total) #1	Tau (total) #1
Calibrator	ECL Signal	ECL Signal	ECL Signal	ECL Signal
pT217	13,992	14,006	22,680	4,753
pT217, dephosphorylated	32,027	1,393	2,718	753
pT217, no phosphatase ("mock")	23,989	14,403	28,141	9,143

Table 2: The 3 top-performing antibody pairs for pT217 from Table 1 were selected for further characterization and compared with a Tau (total) pair in the U-PLEX® format for screening using a calibrator concentration of 20,000 pg/mL. Phosphatase was used to remove phosphate groups from the pT217 calibrator and compared with one given "mock" treatment and the untreated calibrator. The total Tau assay did not show a reduction in signal, whereas all three pT217 antibody pairs showed a marked decrease in signal compared with the untreated or "mock" treated conditions (denoted in green text).

Antibody Orientation Comparison

	pT217 Antibody as **Capture**			pT217 Antibody as **Detect**		
	pT217 #1	pT217 #2	pT217 #3	pT217 #1	pT217 #2	pT217 #3
pT217 ECL Signal	749,608	1,014,595	963,225	176,117	90,613	62,625
Blank ECL Signal	774	865	2,132	321	9,604	12,898
Signal/Bkgd Ratio	968	1,173	452	549	9	5
eLLOD (pg/mL)	0.78	0.44	1.30	0.58	18.9	60.2

Table 3: The 3 top-performing antibody pairs for pT217 were tested in the ultrasensitive S-PLEX format for screening as capture or detect using a calibrator concentration of 2,110 pg/mL. Using pT217-specific capture antibodies provides the best signal/background ratios and sensitivity overall. Both pT217 clones #1 and #2 maintained low background signals in the assay (denoted in green text). Several pairs show elevated background signals and reduced sensitivity (denoted in red text). eLLOD = estimated lower limit of detection.

Specificity Testing

	pT217 Antibody as **Capture**		
	pT217 #1	pT217 #2	pT217 #3
pT217 ECL Signal	748,834	1,013,730	961,093
Non-Phosphorylated Tau ECL Signal	720	3,189	4,882
Non-Phosphorylated Tau Non-Specific %	0.10%	0.31%	0.51%
A217 Mutant ECL Signal	941	9,653	3,540
A217 Mutant Non-Specific Signal %	0.13%	0.95%	0.37%

Table 4: The 3 top-performing antibody pairs were tested in the S-PLEX format against non-phosphorylated Tau and phosphorylated Tau with threonine at the 217 position mutated to alanine (A217), using a calibrator concentration of 2,110 pg/mL. Both pT217 clones #2 and #3 have reduced specificity (denoted in red text). pT217 #1 was chosen as the final capture antibody and paired with the Tau (total) #1 antibody as the detect antibody (denoted in green text).

Concentration of pT217 in Plasma and Serum from Healthy and Diseased Samples

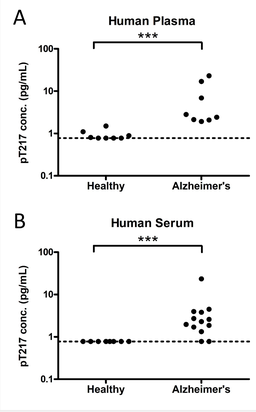


Figure 1.
Both healthy and diseased (A) plasma or (B) serum samples were collected from two independent cohorts. pT217 was quantified using the optimal antibody pair in the S-PLEX format. Individual data points are shown. Dotted line indicates estimated lower limit of detection. *** indicates $p \leq 0.001$.

RESULTS

Correlation of pT217 Concentration with Alzheimer's Severity

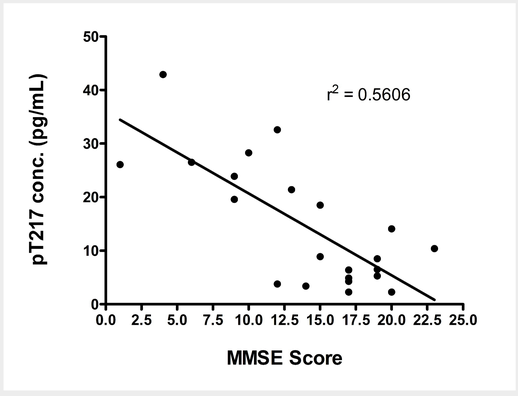


Figure 2: Serum was collected from a well-characterized cohort of diseased samples, and pT217 was quantified using the optimal antibody pair in the S-PLEX format. The concentrations of serum pT217 and the mini-mental state examination (MMSE) score were compared to determine the correlation of serum pT217 levels with disease severity using a linear regression. $p \leq 0.001$.

CONCLUSIONS

- An ultrasensitive assay for Tau (pT217) was developed.
- The assay is highly specific to Tau that is phosphorylated at the T217 site, while not recognizing total Tau.
- The assay is able to measure elevated levels of pT217 in diseased plasma and serum samples.

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