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Review Article

High-specificity antibodies and detection methods for quantifying phosphorylated tau from clinical samples

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ABSTRACT

The ability to measure total and phosphorylated tau levels in clinical samples is transforming the detection of Alzheimer's disease (AD) and other neurodegenerative diseases. In particular, recent reports indicate that accurate detection of low levels of phosphorylated tau (p-tau) in plasma provides a reliable biomarker of AD long before sensing memory loss. Therefore, the diagnosis and monitoring of neurodegenerative diseases progression using blood samples is becoming a reality. These major advances were achieved by using antibodies specific to p-tau as well as sophisticated high-sensitivity immunoassay platforms. This review focuses on these enabling advances in high-specificity antibody development, engineering, and novel signal detection methods. We will draw insights from structural studies on p-tau antibodies, engineering efforts to improve their binding properties, and efforts to validate their specificity. A comprehensive survey of high-sensitivity p-tau immunoassay platforms along with sensitivity limits will be provided. We conclude that although robust approaches for detecting certain p-tau species have been established, systematic efforts to validate antibodies for assay development is still needed for the recognition of biomarkers for AD and other neurodegenerative diseases.

Statement of Significance: Levels of total and phosphorylated tau protein are believed to correlate with the onset of Alzheimer's disease. Detection of this protein is achieved through the use of antibodies, and it is important to understand how these antibodies distinguish phosphorylated tau from non-phosphorylated tau. Understanding antibody binding mechanisms and validating their specificity are crucial in the design of sensitive diagnostic assays. Such validation is essential to realize the full potential of recently developed high-sensitivity assay platforms.

KEYWORDS: phosphorylated tau; antibody specificity; antibody validation; Alzheimer's disease; neurodegeneration

INTRODUCTION

The observation of neurofibrillary tangles (NFTs) in neurons is a defining pathological feature of Alzheimer's disease (AD). These tangles consist of paired helical filaments (PHFs) of microtubule-associated protein tau [1,2]. Such tau inclusions are also observed in other neurodegenerative diseases including Pick's disease, progressive supranuclear palsy, chronic traumatic encephalopathy, and corticobasal degeneration. Dominantly inherited mutations in *MAPT* encoding tau have been discovered in genetically predisposed patients with frontotemporal dementia and parkinsonism linked to chromosome 17 (FTDP-17) [3], indicating that abnormal forms of tau are sufficient to cause neurodegeneration. At the molecular level, a common signature of NFTs is the hyperphosphorylation of tau [4,5].

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It is also hypothesized that tau posttranslational modifications such as hyperphosphorylation cause conformational variants that lead to tau inclusions [6].

Although a disease-modifying therapy for AD is not currently available, major advances in the diagnosis of AD have been made in the past few years. Many reports support that measuring the concentrations of amyloid β, tau and p-tau in the cerebrospinal fluid (CSF) can differentiate AD from normal aging and lead to the detection of AD many years prior to onset of cognitive impairment [7–9]. The most recent advances in this area are immunoassays capable of detecting p-tau at pg/mL (femtomolar) concentrations in plasma [10–12]. The levels of tau phosphorylated at threonine 181 (pThr181) in plasma correlated with CSF pThr181 tau and made it possible to differentiate AD from non-AD neurodegenerative diseases [10,13]. Plasma levels of pThr217 also emerged as a highly accurate biomarker for the diagnosis of AD [11], and for monitoring AD progression [14]. Considering the wide diversity of p-tau sites [15,16] and their high relevance to tau pathology [6,17,18], plasma p-tau biomarkers are expected to rapidly expand in the near future. Since obtaining plasma is far less invasive and cost-effective, these findings point to major advances in biomarker development for AD that will support early intervention strategies and drug efficacy assessment.

An essential component of high-sensitivity immunoassays is antibodies that selectively recognize the target in complex samples [19–24]. In identifying high-quality antibodies, much focus has been given to affinity due to the low concentrations of p-tau. However, antibody specificity—the ability to discriminate the target from other proteins—is as important as the tightness of binding. Achieving p-tau specificity is particularly challenging since the antibodies need to distinguish the presence of a single phosphorylated residue. This review will draw insights from structural studies on how specificity is achieved and engineering efforts to improve the affinity and specificity of p-tau antibodies. Findings from efforts to validate the specificity of p-tau antibodies, along with approaches used will be introduced. Finally, the new high-sensitivity detection methods that resulted in major improvements in p-tau detection sensitivity will be summarized.

INSIGHTS FROM STRUCTURAL ANALYSIS AND ENGINEERING OF ANTIBODIES TARGETING PHOSPHORYLATED TAU

Since the first report on the structure of an antibody fragment bound to a phosphorylated epitope [25], several following studies expanded our understanding on how antibodies recognize phosphorylation sites [26–31]. The majority of these structures are that of p-tau antibodies, reflecting interest in the target. The main feature of these antibodies is their tight association with the phosphate group of the modified residues (Fig. 1). In antibodies that bind promiscuously to the non-phosphorylated target site, the phosphate group faces away from the antibody–antigen interface (Fig. 1a) [29] or a free phosphate molecule was bound near the phosphorylation site (Fig. 1b) [32]. The fact that these antibodies were raised using phosphorylated peptides as antigens illustrates the importance of performing negative selections to remove nonselective binders during the antibody screening process [33,34]. The structural analyses revealed that the antibodies engage the phosphate group using diverse complementarity determining region (CDR) residues including those in CDR H1, H2, H3, or L1 (Fig. 1c–f). A majority of antibodies use a single CDR loop for phosphate recognition, but for the antibody dmCBTAB-22.1 (targeting phospho-serine (pSer) 422 tau), residues in CDR H1 and H3 both form hydrogen bonds with the phosphate (Fig. 1f) [35]. A unique example is the antibody AT8, which binds to three phosphate groups from pSer202, pThr 205, and pSer208 of tau (Fig. 1g) [27]. Residues from CDR L1, L2, H1, and H3 interact with the three phosphates. Another key aspect of phosphate binding sites is the frequent presence of positively charged residues (i.e., lysine and arginine) (see surface charge in Fig. 1), glycine (indicated as "G" in Fig. 1), tyrosine, threonine, and histidine. Most of the antibodies possess one or more lysine or arginine residues that form a salt bridge with the phosphate group (Fig. 1c–e and g), although the antibody dmCBTAB-22.1 relies only on hydrogen bonds (Fig. 1f). Glycine within the CDR often forms hydrogen bonds with the phosphate group (Fig. 1c, e, and f) and is commonly observed in other phosphate binding proteins [36–38].

Remarkably, Koerber et al. [26] demonstrated that phospho-specific antibodies can be designed by incorporating a phosphate-binding motif into an antibody CDR. First, they identified an antibody structure with CDR H2 that possesses a conformation similar to that of an anion-binding motif. After finding antibodies containing such CDRs that interact with an aspartate or glutamate in the antigen, they diversified the sequence of the CDR site to tune the antibody binding toward pSer, pSer/pThr, or phospho-tyrosine. They then expanded the pSer and pSer/pThr binding antibodies by diversifying non-phospho interacting residues in CDR H2, H3, and L3. These pools of antibodies were screened against 10 distinct phosphosites leading to successful identification of phospho-specific binders for 7 of the 10. This study pioneered the idea of modular antibody paratopes—a region that binds the phosphate group (phospho-recognition) and another region that binds the surrounding amino acids (sequence recognition). Whether the modularity will prevail in targeting other posttranslational modifications remains to be tested.

These studies suggest that a balance in stability of phospho-recognition and sequence recognition is critical for specificity. A single CDR that captures the phosphate group imparts the ability to weakly interact with a wide range of phospho-peptides to antibodies [26]. In contrast, strong sequence recognition is likely to result in promiscuous binding to non-phosphorylated epitopes. This knowledge was highly valuable in engineering high-affinity p-tau antibodies [34]. Since the phosphate group is small relative to the surrounding amino acid residues in the phospho-epitope, a hypothesis emerged that antibody affinity maturation may over-stabilize the sequence recognition interactions. To test this, Li et al. [34] performed directed evolution of a high-specificity pThr231 tau

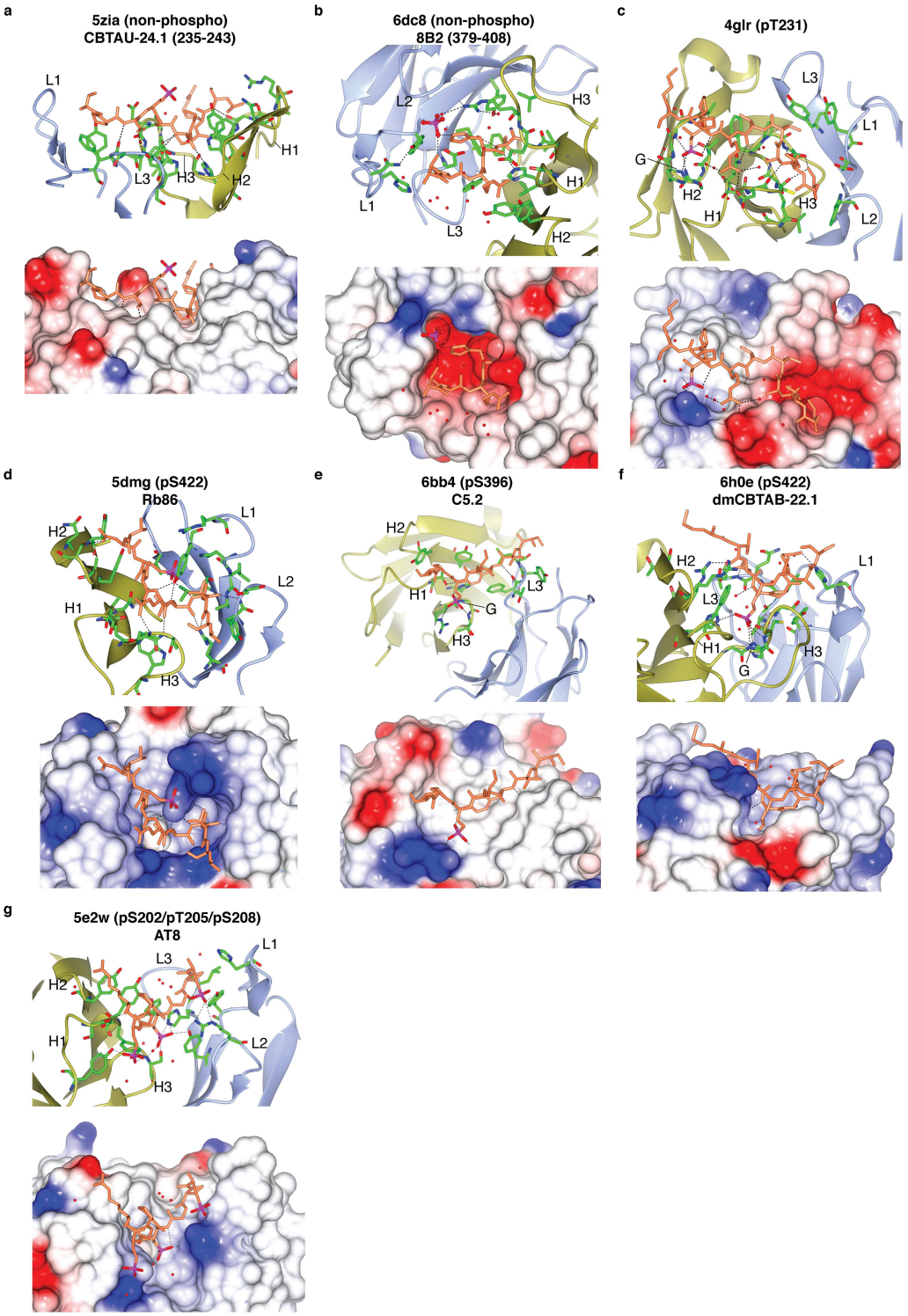

Figure 1. Structural analysis of p-tau antibodies. Each panel is labeled with the corresponding PDB ID, name of antibody clone, and phospho-site recognized by the antibody. The panels were generated using CCP4MG [91]. Antibody complementarity determining regions L1-3 and H1-3 that interact with the p-tau peptide are indicated. The lower half of each panel shows surface charge (blue—positive, red—negative). The phosphorus in the phosphate group is indicated in magenta. Antibodies in panels **(a-b)** are not specific to p-tau. Antibodies in panels **(c–f)** interact with a single phosphorylated residue. Antibodies in panel **(g)** interact with three phosphorylated residues.

antibody for improved affinity and assessed the binding specificity of the identified variants. Although the wild-type antibody did not bind to the non-phospho-peptide, over half of the high-affinity variants showed detectable binding to a non-phospho-peptide with the target sequence. None of the high-affinity variants showed binding to a

phospho-peptide containing scrambled sequence, indicating that over-stabilization of phosphate recognition did not occur. Based on the fact that not all variants with improved affinity showed binding to the non-phospho-peptide, a second stage of screening was conducted for the absence of non-phospho-peptide binding. This led to the identification of a high-specificity pThr231 tau antibody with a picomolar dissociation constant. These results again highlight the importance of specificity validation in developing phospho-specific antibodies.

APPROACHES FOR ANTIBODY SPECIFICITY VALIDATION

Although the need for antibody specificity validation is widely recognized, published data on antibody validation have been scarce. Across different areas of research, antibody validation has been identified as a major bottleneck in improving reproducibility of research outcomes [39–41]. However, the very aspect that makes antibodies so useful—a wide range of applications—presents a formidable challenge in standardizing their validation. For example, Kalina et al. [42] and Pillai-Kastoori et al. [43] express the importance of validating antibodies using their intended experiment. Furthermore, comprehensive antibody validation has been demonstrated in the case of histone modification targeting antibodies [44–48]. Due to the importance of combinatorial recognition of multiple nearby modification sites [46,49], peptide microarrays were used to systematically validate site-specificity [44,46,50]. The collective dataset is a rare example of a quantitative assessment of antibody specificity [44]. Other studies validated histone antibody binding in the context of cellular background proteins using Western blotting and chromatin immunoprecipitation [47]. These studies also reported data on antibodies that lack specificity or failed to bind, providing a valuable guide for selecting antibodies. Acknowledging these aspects, we herein focus our attention to antibodies that target p-tau.

Though few validation studies have been published for p-tau antibodies, they provide insight into the types of nonspecific binding observed. Methods used for specificity validation include immunoblotting approaches and a whole cell immunocytochemistry assay [51–53]. Immunoblotting has been performed using synthetic peptide sequences, cell lysate, and extracted PHF tau [52,53]. This method allows characterization of nonspecific binding to non-tau proteins (using cell lysate), non-phosphorylated tau (using phosphatase treated PHF tau), and binding to other known phospho-sites (using synthetic peptides). Ercan et al. [52] observed that, in addition to some antibodies not binding to their specific sites, other antibodies may be specific but their binding could be inhibited by other modifications in the vicinity of the binding site, which could lead to false negative signal. Another important finding was the frequent nonspecific binding to unmodified peptides. As an example, all tested antibodies that claimed to target pSer262 also bound to unmodified peptide, leading to no antibody validated for the pSer262 site [52]. Cell lysates and tissue from tau knockout (TKO) mice [54] provide an effective means to assess nonspecific binding of p-tau antibodies to other nontarget proteins. Petry et al. [53] showed that p-tau antibodies show nonspecific binding to TKO mouse brain lysate proteins via Western blotting. Non-specific binding observed in certain p-tau antibodies could be greatly reduced by using heat stable fractions of the lysates [53]. Heating complex samples such as cell lysates can deplete other proteins that cause nonspecific binding since tau is highly heat stable [55–57]. These results highlight the importance of validating the specificity of p-tau antibodies and demonstrate how assay conditions impact apparent nonspecific binding.

Li et al. [51] developed a whole cell immunocytochemistry assay which utilizes human embryonic kidney (HEK) 293FT cells and flow cytometry to provide a quantitative measurement of specificity (Φ). The approach measures the fraction of specific and nonspecific cell staining intensity within a single sample using flow cytometry. This is achieved by quantifying both the binding signal to cells expressing the wild-type tau and cells expressing tau with an alanine point mutation at the target phospho-site. Since the point mutation itself may disrupt antibody binding, the authors also measured binding to cells expressing wild-type tau treated with a phosphatase. This method measures nonspecific binding to different p-tau sites across the entire tau protein, binding to non-phospho tau, and binding to other cellular proteins. Li et al. [51] found that some p-tau antibodies showed nonspecific binding to irrelevant cellular proteins in HEK293FT cells and mouse primary hippocampal neurons. By performing confocal microscopy and Western blot experiments, Li et al. confirmed antibodies AT270 (targeting pThr181) and 1H6L6 (targeting pThr231) bound to cells not expressing tau.

These studies provide us with a collection of antibodies tested for their specificity. Taken together, they reveal that several commonly used p-tau antibodies either were not able to detect their site-specific modification or they bound to non-phosphorylated tau. Notably, the majority of commercially available p-tau antibodies are generated by rabbit immunization, and many of them remain polyclonal. Since the identity of polyclonal antibodies varies between batches, repeated validation is required. The list of validated monoclonal p-tau antibodies remains limited (Table 1). Given the fact that tau has 85 total serine, threonine, and tyrosine sites available for phosphorylation, 45 of which have been detected in neurons [58,59], systematic development and validation of p-tau antibodies is necessary. It is notable that antibody validation efforts have not yielded high-specificity antibodies for critical tau phosphorylation sites such as pThr181 and pSer262 (Table 1) [51,52].

APPROACHES FOR HIGH-SENSITIVITY DETECTION OF PHOSPHORYLATED TAU

Since the concentration of tau and p-tau in clinical samples is estimated to be in the femtomolar (pg/mL) range, many efforts have been made to improve assay sensitivity. In the past few years, several major improvements in immunoassays have enabled the detection of total and p-tau in human

Table 1. Validated monoclonal p-tau antibodies

Tau phospho-site	Antibodies	Validation method	References
pSer198	pSer198 (ab79540)	Peptide Array, Immunoblotting	Ercan et al. [52]
pSer199	2H23L4	Peptide Array, Immunoblotting	Ercan et al. [52]
pSer202/pThr205	AT8	Phi	Li et al. [51]
pThr231	AT180, PHF6, TG-3	Phi	Li et al. [51]
pSer396/pSer404	PHF1	Phi	Li et al. [51]
pSer404	pSer404 (ab92676)	Phi	Li et al. [51]
pSer422	pSer422 (ab79415)	Peptide Array, Immunoblotting	Ercan et al. [52]

CSF as well as in plasma. Table 2 lists the performance of assays for detecting total tau and p-tau. Many different studies have used an enzyme-linked immunosorbent assay (ELISA) to measure tau and p-tau in human CSF targeting a variety of phospho-sites [60–66]. However, the reported sensitivity differs between studies and largely depends on the antibodies used (Table 2). Although ELISA was the first platform systematically developed for p-tau detection, the need for more sensitive detection methods has pushed researchers to develop alternative assays with ultra-sensitivity.

Several different methods use the principles of sandwich ELISA coupled with a highly sensitive detection method, including enhanced immunoassay using multi-arrayed fiberoptics (EIMAF), single-molecule array (SiMoA), the ELECSYS platform, and the Meso Scale Discovery (MSD) platform.

Previously known as surround optical fiber immunoassay (SOFIA), the enhanced immunoassay using multi-arrayed fiberoptics (EIMAF) uses the principle of a sandwich ELISA coupled with a highly sensitive detection method [67]. The EIMAF instrumentation collects the emission of a fluorescent protein using an assembly of optical fibers positioned to cover the entire optical radiation pattern of the sample. Scattered light is eliminated and the light from the sample is focused to a single optical fiber and detected using an amplifier [67]. Rubenstein et al. [68] used EIMAF coupled with rolling circle amplification (a-EIMAF). In rolling circle amplification (RCA), a circular DNA hybridizes to an oligonucleotide primer, which results in synthesis of a long repetitive linear DNA upon addition of DNA polymerases [69]. Subsequently, multiple fluorescent DNA probes can be hybridized *in situ* to the long synthesized DNA, resulting in signal amplification. In immunoassays, RCA can amplify the antibody-binding signal by conjugating an oligonucleotide primer to the detection antibody [70]. In a-EIMAF, the detection antibody is biotinylated and streptavidin is added, which allows a biotinylated DNA primer to initiate RCA. This enabled the detection of pThr181 tau levels in plasma, CSF, and serum with tremendously low reported limits of detection and quantification (Table 2) [68,71]. SiMoA uses paramagnetic beads conjugated with antibodies mixed with the protein of interest. Following a Poisson distribution, the number of the protein molecules is small enough, typically at least 10 times less than the number of paramagnetic beads, so that a singular bead is either bound to an individual molecule or not bound [72], allowing for "digital" detection of target molecules. The formed immunocomplexes are loaded into micro-fabricated wells specifically sized to hold individual beads and sealed with a substrate of reporter enzyme. The assay produces a "digitized" signal, with the wells being either "on" (fluorescent) or "off" (not fluorescent) [72,73]. Zetterberg et al. [74] were the first to use SiMoA to detect both normal and p-tau protein in CSF and plasma samples. Since then, the assay technology has been commercialized by the company Quanterix. Additionally, Tatebe et al. [13] employed the SiMoA technology using different antibodies to recognize tau phosphorylated at Thr181 (Table 2) with an impressive limit of detection of 0.0090 pg/mL.

Examples of immunoassays that are based on electrochemiluminescence to produce a sensitive signal include ELECSYS and MSD. The ELECSYS immunoassay platform uses an antibody labeled with a ruthenium complex that can be magnetically captured onto the surface of an electrode of a measuring cell. A voltage is then applied to induce chemiluminescent emission, which is measured and correlated with the concentration of target molecules [75]. Lifke et al. [75] used the ELECSYS platform to detect both total tau and pThr181 tau in CSF and found that its automated nature makes it a more reliable alternative to other ELISA assays (Table 2). MSD also uses electrochemiluminescence but employs plates that are precoated with certain biomarkers or other proteins. Since they can be spot coated with working electrodes, each well can have a different number of spots. Having more spots increases the assay sensitivity, and the different plate designs allow for more customizability of the assay. Mielke et al. [76] used a small spot streptavidin plate from MSD to detect pThr181 tau in plasma samples (Table 2). A biotinylated capture antibody was added to the streptavidin MSD plate and a SULFO-TAG conjugated detection antibody allowed for the production of electrochemiluminescent signal.

Other developed methods that differ from the ELISA include Luminex xMAP technology, the superconducting quantum interference device (SQUID) immunomagnetic reduction assay (IMR), and the AlphaLISA. The Luminex

Table 2. Performances of assays for detecting total tau (t-tau) and phosphorylated tau (p-tau)

Assay	Capture Antibody	Binding Sites	Detection Antibody	Binding Sites	Reported Sensitivity	Sample	Reference
ELISA Tyramide Signal Amplification t-tau	HT7	aa 159-163	tau antiserum 92e	N/A	14 pg/ml	CSF	Yamamori et al. [85]
			R134d	N/A			
ELISA t-tau (INNOGENETICS)	AT120	N/A	HT7	aa 159-163	LoD[1]: 34 pg/ml LoQ[2]: 57 pg/ml (INNOTEST hTau User Manual)	CSF	Blennow et al. [62]
			BT2	aa 194-198			
ELISA p-tau (INNOGENETICS)	AT180	pThr231	HT7	aa 159-163	N/A		
	AT270	pThr181	AT120	N/A			
ELISA p-tau (INNOGENETICS) User Manual	HT7	aa 159-163	AT270	pThr181	LoD[1]: 13 pg/ml LoQ[2]: 20 pg/ml	CSF	INNOTEST User Manual [63]
Sandwich EIA (Ishiguro)	anti-tau mAb	N/A	anti-PT231PS235	pThr231/pSer235	N/A	CSF	Ishiguro et al. [60]
			anti-PS199	pSer199			
Sandwich ELISA (Kohnken)	PC1C6/Tau-1	aa 196-205	CP9	pThr231	N/A	CSF	Kohnken et al. [61]
	CP27	aa 130-150					
Sandwich ELISA (Vandermeeran) t-tau	AT120	N/A	rabbit anti-normal tau	N/A	< 5 pg/ml	CSF	Vandermeeren et al. [65]
Sandwich ELISA (Vandermeeran) p-tau	AT8	pSer202/pThr205	rabbit anti-normal tau	N/A	< 20 pg/ml		
Bienzyme-Substrate-Recycle ELISA t-tau	tau antiserum 92e	N/A	PC1C6/Tau-1	aa 196-205	0.75-200 pg (7.5 pg/ml)	CSF	Hu et al. [64]
Bienzyme-Substrate-Recycle ELISA p-tau	tau antiserum 92e	N/A	PHF-1	pSer396/404	0.5-50 pg (5 pg/ml)		
Overlapping ELISA p-tau	Tau12	aa 9-18	AT270	pThr181	LoQ[2]: 6 pg/ml	CSF	Meredith Jr. et al. [66]
	HT7	aa 159-163	AT270	pThr181	LoQ[2]: 2 pg/ml		
	HT7	aa 159-163	PHF-6	pThr231	LoQ[2]: 7.8 pg/ml		

Continued

Table 2. Continued

Assay	Capture Antibody	Binding Sites	Detection Antibody	Binding Sites	Reported Sensitivity	Sample	Reference
Overlapping ELISA t-tau	Tau12	aa 9-18	HT7	aa 159-163	LoQ[2]: 3.9 pg/ml	CSF	
	Tau12	aa 9-19	BT2	aa 194-198	LoQ[2]: 1.6 pg/ml		
	HT7	aa 159-163	BT2	aa 194-198	LoQ[2]: 7.8 pg/ml		
	HT7	aa 159-164	Tau5	aa 218-225	LoQ[2]: 7.8 pg/ml		
	HT7	aa 159-165	77G7	aa 316-335	LoQ[2]: 16 pg/ml		
ELISA p-tau Kawayabarashi	AntihTau441-E22A3 Rat IgG mAb	N/A	anti-hTau p181-Rk27A6 Rat monoclonal IgG Fab	pThr181	3.06 pg/ml	CSF	Kawarabayashi et al. [86]
a-EIMAF p-tau	RZ3	pThr231	DA9	aa 102-140	LoQ[2]: 0.002 fg/ml LoD[1]: 0.00001 fg/ml	Plasma, CSF, Serum	Rubenstein et al. [68]
SiMoA	Tau5	aa 218-225	HT7 BT2	aa 159-164 aa 194-198	0.02 pg/ml	Plasma	Zetterberg et al. [74]
SiMoA p-tau	Tau5	aa 218-225	AT270	pThr181	LoD[1]: 0.0090 pg/ml	Plasma	Tatebe et al. [13]
SiMoA p-tau User Manual pThr181	N/A	N/A	N/A	N/A	LoQ[2]: 1.204, 2.64 pg/ml LoD[1]: 0.756, 0.724 pg/ml	CSF	SiMoA User Manuals [87]
SiMoA p-tau User Manual pThr231	N/A	N/A	N/A	N/A	LoQ[2]: 1.23, 1.83 pg/ml LoD[1]: 0.284, 0.621 pg/ml	CSF	SiMoA User Manuals [88]

Continued

Table 2. Continued

Assay	Capture Antibody	Binding Sites	Detection Antibody	Binding Sites	Reported Sensitivity	Sample	Reference
ELECSYS t-tau	5.28.464	aa 150-230	PC1C6/Tau-1	aa 196-205	LoD[1]: 18.6 pg/ml LoQ[2]: 62.6 pg/ml	CSF	Lifke et al. [75]
ELECSYS p-tau	4.35.411 11H5V1	aa 170-205 pThr181	PC1C6/Tau-1	aa 196-205	LoD[1]: 1.96 pg/ml LoQ[2]: 3.9 pg/ml		
MSD p-tau	AT270	pThr181	SULFO-TAG-LRL	N/A	N/A	Plasma	Mielke et al. [76]
xMAP Technology t-tau	HT7	aa 159-163	AT120	N/A	45-1500 pg/ml (Bjornstal et al.)	CSF	Olsson et al. [79]
xMAP Technology p-tau	HT7	aa 159-163	AT270	pThr181	10-225 pg/ml (Bjornstal et al.)		
SQUID IMR t-tau	HT7	aa 159-164			< 1 pg/ml	Plasma	Chiu et al. [89]
SQUID IMR p-tau	AT270	pThr181			0.0196 - 10000 pg/ml	Plasma	Yang et al. [81]
AlphaLISA t-tau	(Donor): HT7	aa 159-163	(Acceptor): BT2	aa 194-198	N/A		Dujardin et al. [18]
AlphaLISA p-tau	(Donor): AT8	pSer202/pThr205	(Acceptor): HT7	aa 159-163	N/A		
AlphaLISA p-tau	(Donor): HT7	aa 159-163	(Acceptor): PHF-6	pThr231	N/A		

[1]LoD: Limit of Detection—lowest concentration that can be distinguished from blank sample [90]
[2]LoQ: Limit of Quantification—lowest concentration that can be reliably detected, and which meets a predefined goal for bias [90]

xMAP technology is a microsphere-based flow cytometric method that features beads covalently coupled with different antibodies to capture target proteins [77,78]. This allows the assay to measure several different target proteins in one test since each microsphere has spectrally specific fluorescence. Olsson et al. [79] used this technology to design a multiplex assay that measures pThr181 tau in CSF with a sensitivity of 10 pg/mL. The IMR utilizes the magnetic properties of magnetic nanoparticles with a sensor known as the SQUID to measure concentration-dependent signal [80]. This assay takes advantage of a magnetic property known as multiple-frequency alternating current (AC) magnetic susceptibility, X_{AC}, which changes when antibody-coated magnetic nanoparticles interact with the target antigen. The change in X_{AC} is measured by the SQUID and then correlated with concentration [80]. Yang et al. [81] have used IMR to detect pThr181 tau in plasma successfully with a limit of detection as low as 0.0196 pg/mL (Table 2). AlphaLISA, which depends on luminescent oxygen channeling chemistry and was initially described for its use in the luminescent oxygen channeling immunoassay (LOCI) [82,83], has also been adopted for the detection of total and p-tau. This method uses a "donor" bead and an "acceptor" bead. The donor bead donates a singlet oxygen to the acceptor bead after excitation at a wavelength of 680 nm [82]. The singlet oxygen then reacts with the acceptor bead to emit a signal with a wavelength of 615 nm. For this reaction to occur, the two beads need to be in close proximity to one another. To ensure this, both beads are coated with antibodies that bind specifically to the antigen of interest. For its use to detect total tau and p-tau, Dujardin et al. [18] used three separate antibody variations with AlphaLISA to detect total tau, pSer202/pThr205, and pThr231.

These advanced assay technologies enable the detection of total tau and p-tau in human CSF and plasma samples without tenuous enrichment steps. Since antibodies are essential in these assays, antibody validation should accompany their development. Accurate measurement of total and p-tau has great potential to track biomarkers and monitor disease progression in AD and other neurodegeneration. Moreover, they may enable early detection of AD long before symptom onset [84], which will open a new window for therapeutic intervention.

CONCLUSIONS

Plasma biomarkers are transforming our ability to detect AD and other forms of neurodegeneration early and to monitor the disease progression. This new capability will enable clinical trials during early stages of neurodegeneration and the assessment of drug efficacy in delaying or preventing its progression. P-tau biomarkers are especially valuable, given their diversity and relevance in pathology of neurodegeneration. Now that platforms with sensitivities able to detect p-tau in plasma have been developed (Table 2), efforts to validate assay specificity and expand the panel of p-tau biomarkers are in critical need. Antibodies are an essential part of highly sensitive immunoassays for the recognition of AD biomarkers, including p-tau. In addition to affinity, the specificity of the antibodies used is just as important in discriminating p-tau species. Through the analysis of p-tau antibody structures and molecular engineering efforts, it is becoming clear that optimizing affinity and specificity should go hand in hand. A major bottleneck is the lack of antibody specificity validation, and a number of studies have been carried out that show some widely used p-tau antibodies do not specifically bind to the intended phospho-site. While some important phospho-site specificity has been validated in monoclonal antibodies (pS198, pS199, pS202/pT205, pT231, pS396, pS404, pS422) (Table 1), many phospho-sites remain without validated antibodies. These results point to the importance of validating antibody specificity when choosing which antibodies to use in diagnostic immunoassays. Although the methods differ, antibody validation should accompany all assay developments to ensure robust detection of the target p-tau species.

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CONFLICT OF INTEREST STATEMENT

None declared.

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