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(57) Abstract: In certain embodiments, this invention provides methods and kits for detecting the level of at least one Tau protein in a biological sample.



TAU IMMUNOASSAY

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims priority of U.S. Provisional Application Serial No.
5 61/671397, filed on July 13, 2012, which is herein incorporated by reference.

BACKGROUND OF THE INVENTION

Protein accumulation, modifications and aggregation are pathological aspects
of numerous neurodegenerative diseases. The classical hallmarks of Alzheimer's
10 disease (AD) are extra-neuronal plaques consisting of precipitates or aggregates of
amyloid beta ($A\beta$), and intra-neuronal neurofibrillary tangles (NFTs) consisting of
Tau protein. During AD progression, levels of extracellular Tau increase as indicated
by elevated levels of Tau detected in the cerebrospinal fluid (CSF) (Blennow and
Hampel, 2003, *Lancet Neurol.*, 2(10):605-13; Shaw et al., 2009, *Ann Neurol.*,
15 65(4):403-13). Similar to AD, NFT pathology is observed in tauopathies including
frontotemporal dementia with Parkinson's disease with Tau mutations (FTDP-17),
progressive supranuclear palsy (PSP), corticobasal degeneration (CBD), and Picks
disease (Lee et al., 2001, *Annu Rev Neurosci.*, 24:1121-59).

Tau is a microtubule-associated protein expressed in the central nervous
20 system with a primary function to stabilize microtubules. There are six major
isoforms of Tau expressed mainly in the adult human brain, which are derived from a
single gene by alternative splicing. Under pathological conditions, the Tau protein
becomes hyperphosphorylated, resulting in a loss of tubulin binding and
destabilization of microtubules followed by the aggregation and deposition of Tau in
25 pathogenic neurofibrillary tangles (Mandelkow and Mandelkow, 2012, *Cold Spring
Harb Perspect Med.*, 2(7):a006247). Recently, there has been tremendous progress in
the characterization of CSF biochemical markers associated with AD. For example,
results from numerous studies have demonstrated that CSF $A\beta$ 42 levels decrease ~2
fold whereas Tau and pT181 Tau levels increase ~2 fold in AD compared to age-
30 matched controls. The changes in these biomarkers are evident prior to onset of
clinical symptoms and may be predictive of the conversion from predementia
(amnesic-MCI) to clinical AD (Mattsson et al., 2009, *JAMA.*, 302(4):385-93). In

addition to the clinical cognitive decline, brain changes in hippocampal volume as measured by magnetic resonance imaging (MRI), functional connectivity in the default-mode network as measured by functional MRI, and brain amyloid measurements using positron emission tomography also correlate with CSF Tau changes in AD subjects (Fagan et al., 2009, EMBO Mol Med., 1(8-9): 371–38; Apostolova et al., 2010, Neurobiol Aging., 31(8): 1284–1303; Weiner et al., 2010, Alzheimers Dement., 6(3):202-11.e7). Further, biochemical characterization of insoluble Tau from the AD brains and brains from other tauopathies demonstrate varying levels of phosphorylation, including the existence of 3-repeat and 4-repeat Tau isoforms and varying degrees of Tau cleavages. While Tau pathology is present in a diverse variety of human neurodegenerative disorders, robust elevations in CSF Tau are only evident in AD (Vanmechelen et al., 2011, Mechanisms of Ageing and Development, 122 (2001): 2005–2011; Sjögren et al., 2001, J Neurol Neurosurg Psychiatry, 70:624-630).

Although several Tau detection methods exist, they primarily use antibodies which bind to a mid-domain of Tau, and may not be able to detect changes in Tau fragments outside the mid-domain, for example, the N-terminal or C-terminal fragments. Thus, the present invention fulfills a need in the art by providing alternative methods useful for identifying and quantitating a Tau protein.

SUMMARY OF THE INVENTION

In certain embodiments, the present invention provides a method of detecting the level of at least one Tau protein in a biological sample, comprising: (a) contacting a biological sample from a subject with a first antibody which binds to an N-terminal portion of Tau 441 (SEQ ID NO: 1), and a second antibody which binds to a middle portion of Tau 441 (SEQ ID NO: 1), wherein the first antibody and the second antibody both bind to the Tau protein to form a complex; and (b) detecting the level of the Tau protein in the complex. For example, the first antibody binds to an N-terminal portion of amino acid residues 9-158 of Tau 441 (SEQ ID NO: 1).

Optionally, the first antibody binds to amino acid residues 9-18 of SEQ ID NO: 1. For example, the second antibody binds to a middle portion of amino acid residues 159-231 of Tau 441 (SEQ ID NO: 1). Optionally, the second antibody binds to amino

acid residues 194-198, residues 159-163, the phosphorylated threonine residue 181 or the phosphorylated threonine residue 231 of SEQ ID NO: 1. To illustrate, the first antibody is Tau12, and the second antibody is selected from BT2, HT7, Tau5, AT270, and PHF6. Optionally, the first antibody or the second antibody of the present
5 methods is immobilized on a solid substrate. Optionally, the first antibody or the second antibody comprises a label. For example, the biological sample is cerebrospinal fluid (CSF), blood, serum or plasma. Optionally, the subject has a Tau-related neurological disease, such as Alzheimer's disease.

In certain embodiments, the present invention provides a method of detecting
10 the level of at least one Tau protein in a biological sample, comprising: (a) contacting a biological sample from a subject with a first antibody which binds to amino acid residues 159-163 of Tau 441 (SEQ ID NO: 1), and a second antibody which binds to amino acid residues 194-198, residues 218-225, or the phosphorylated threonine residue 231 of Tau 441 (SEQ ID NO: 1), wherein the first antibody and the second
15 antibody both bind to the Tau protein to form a complex; and (b) detecting the level of the Tau protein in the complex. To illustrate, the first antibody is HT7, and the second antibody is selected from BT2, Tau5, and PHF6. Optionally, the first antibody or the second antibody of the present methods is immobilized on a solid substrate. Optionally, the first antibody or the second antibody comprises a label. For example,
20 the biological sample is cerebrospinal fluid (CSF), blood, serum or plasma. Optionally, the subject has a Tau-related neurological disease, such as Alzheimer's disease.

In certain specific aspects, the above-mentioned methods use a second antibody which binds to a phosphorylated threonine residue (e.g., at position 181 or
25 231). In such cases, the level of the phosphorylated Tau protein is detected by the present methods.

In certain embodiments, the present invention provides a method of identifying a subject having or at risk of having a Tau-related neurological disease, comprising: (a) isolating a biological sample from the subject; (b) detecting the level
30 of the Tau protein in the biological sample according to any of the above-mentioned methods; and (c) comparing the level of the Tau protein to a reference; and (d) if the

level of the Tau protein is increased compared to the reference, identifying the subject to have or at risk of having a Tau-related neurological disease. For example, the Tau-related neurological disease is Alzheimer's disease.

In certain embodiments, the present invention provides a method of
5 identifying a subject having or at risk of having a Tau-related neurological disease, comprising: (a) isolating a biological sample from the subject; (b) detecting a first level of the Tau protein in the biological sample using HT7 as the first antibody and BT2 as the second antibody; (c) detecting a second level of the Tau protein in the biological sample using HT7 as the first antibody and Tau5 as the second antibody;
10 (d) determining the ratio of the first level from (b) versus the second level from (c); (e) comparing the ratio to a reference; and (f) if the ratio is decreased compared to the reference, identifying the subject to have or at risk of having a Tau-related neurological disease. For example, the Tau-related neurological disease is Alzheimer's disease.

In certain embodiments, the present invention provides a method of
15 identifying a subject having or at risk of having a Tau-related neurological disease, comprising: (a) isolating a biological sample from the subject; (b) detecting a first level of the Tau protein in the biological sample using HT7 as the first antibody and BT2 as the second antibody is BT2; (c) detecting a second level of the Tau protein in the biological sample using Tau12 as the first antibody is Tau12 and HT7 as the
20 second antibody; (d) determining the ratio of the first level from (b) versus the second level from (c); (e) comparing the ratio to a reference; and (f) if the ratio is decreased compared to the reference, identifying the subject to have or at risk of having a Tau-related neurological disease. For example, the Tau-related neurological disease is
25 Alzheimer's disease.

In certain embodiments, the present invention provides a kit comprising: (1) a first antibody which binds to an N-terminal portion of a Tau protein; (2) a second antibody which binds to a middle portion of a Tau protein; and (3) reagents necessary for facilitating an antibody-antigen complex formation. To illustrate, the first
30 antibody is Tau12, and the second antibody is selected from BT2, HT7, Tau5, AT270, and PHF6.

In certain embodiments, the present invention provides a kit comprising: (1) a first antibody which binds to amino acid residues 159-163 of Tau 441 (SEQ ID NO: 1); (2) a second antibody which binds to amino acid residues 194-198, residues 218-225, or the phosphorylated threonine residue 231 of Tau 441 (SEQ ID NO: 1); and (3) reagents necessary for facilitating an antibody-antigen complex formation. To illustrate, the first antibody is HT7, and the second antibody is selected from BT2, Tau5, and PHF6.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows detection of Tau fragments in human CSF. Human control and AD CSF subjected to RP-HPLC, fractions collected and run on SDS-PAGE gels followed by western-blotting with different Tau antibodies. A) HT7 (mid domain antibody). B) Tau 12 (N-terminal antibody). C) K9JA (C-terminal microtubule repeat domain antibody). D) IgG1 isotype control. On each blot, human recombinant Tau441 (Tau) is included in lane 1 and molecular weight markers (mw) in lane 2 followed by the HPLC fractions from 1 to 6 or HPLC fractions 7 to 11. Fractions 1 and 2 were pooled and run as a single sample, while fractions 3-10 were run as individual samples. Control CSF (C) and AD CSF (D) samples for each fraction were run side by side for comparison.

Figure 2 shows Tau and pTau ELISAs. Schematic of Tau 441 protein with the approximate location of various linear epitope antibodies Tau12, HT7, BT2, Tau5, AT120 and 77G7 and phospho-site specific antibodies AT270 (p181) and PHF6 (p231) indicated; additional antibody epitope and clone information included in Table 1. Antibody combinations used for the different Tau and pTau ELISAs are shown. For each assay, capture antibodies are highlighted in red, detection antibodies in black and the minimal Tau region required (aa numbering based on Tau 441) is indicated. Antibodies used in the INNOTEST/INNO-BIA AlzBio3 total Tau and p181 Tau assays are also shown for comparison.

Figure 3 shows characterization of Tau ELISAs. Representative Tau 441 standard curves (left panels) and CSF dilution linearity results (right panels) shown for: A) HT7-BT2, B) HT7-Tau5, C) Tau12-BT2 and D) Tau12-HT7 Tau ELISAs. On each standard curve graph, Tau 441 calibrators (Standards) and results for CSF

sample dilutions (Samples) are shown. The assay lower limit of quantitation (LLQ, vertical dashed line) is also indicated. On each dilution linearity graph, dilution-corrected Tau levels for a pooled control CSF sample and a pooled AD CSF sample relative to sample dilution are shown. The vertical dashed lines indicate the dilution determined to be optimal for CSF analysis.

Figure 4 shows characterization of HT7+77G7 Tau ELISA. Representative Tau 441 standard curve (left panel) and CSF dilution linearity results (right panel) shown. On the standard curve graph, Tau 441 calibrators (Standards) and results for CSF sample dilutions (Samples) are shown. The assay lower limit of quantitation (LLQ, vertical dashed line) is also indicated. On the dilution linearity graph, Tau levels for a pooled control and pooled AD CSF samples tested with or without a 100 pg/ml Tau 441 spike are shown.

Figure 5 shows characterization of pTau assays. Representative pTau standard curves (left panels) and CSF dilution linearity results (right panels) shown for: A) HT7-AT270, B) HT7-PHF6, and C) Tau12-AT270 pTau ELISAs. On each standard curve graph, pTau calibrators (Standards) and results for CSF sample dilutions (Samples) are shown. The assay lower limit of quantitation (LLQ, vertical dashed line) is also indicated. On each dilution linearity graph, dilution-corrected pTau levels for a pooled control and a pooled AD CSF samples relative to sample dilution are shown. The vertical dashed lines indicate the dilution determined to be optimal for CSF analysis.

Figure 6 shows Tau and pTau levels in 20 AD and 20 control CSF samples. A set of 20 AD and 20 age-matched normal control CSF samples were analyzed using the Tau ELISAs (HT7-BT2, HT7-Tau5, Tau12-BT2, Tau12-HT7 and HT7-77G7) and pTau ELISAs (HT7-AT270, HT7-PHF6 and Tau12-AT270). Dashed lines indicate the assay LLQ corrected for CSF dilution. Statistics based on 2-tailed Student's t test comparison of log-transformed data, * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Figure 7 shows Tau peptides used for epitope mapping. A set of 29 overlapping peptides spanning the length of human Tau 441 were generated, coupled to beads and used to map the epitope of Tau antibody 77G7 using a Luminex-based multiplex assay.

Figure 8 shows mapping the Tau epitope of antibody 77G7. Left panel) 77G7 exhibited binding to human Tau 441 and the C-terminal human Tau fragment aa 231-441 but not to the Tau fragments aa 1-125 or aa 126-230; 77G7 also bound to the other Tau isoforms (data not shown). Mid-domain Tau antibody HT7 exhibited binding to Tau 441 and fragment aa 126-230 as expected. Binding was not observed with the anti-IL6 control antibody. Right panel) A set of 29 overlapping peptides spanning the length of human Tau 441 were generated, coupled to beads and used in a Luminex-based multiplex assay to screen 77G7, HT7 and anti-IL6 control. 77G7 exhibited binding to peptide 22 (aa 316-335) and much lower level binding to peptide 25 but none of the other peptides (right panel and data not shown). HT7 exhibited binding to peptide 11 (aa 150-170) but none of the other peptides (right panel and data not shown) as expected while the control anti-IL6 antibody did not react with any of the peptides tested.

Figure 9 shows verification of signal specificity in Tau ELISAs by immunodepletion. Pooled CSF was immunodepleted with Tau antibody HT7 (IP) or treated with protein A/G beads alone (control). Samples were analyzed in Tau ELISAs A) HT7-BT2, B) HT7-Tau5, C) Tau12-BT2 and D) Tau12-HT7. Data represents mean $\pm$ SEM from 3-4 determinations. Dashed lines indicate the assay LLQ corrected for CSF dilution.

Figure 10 shows spike recovery in Tau ELISAs. Pooled CSF samples were treated with Tau 441 spikes ranging from 10 - 800 pg/ml. Spiked samples and a matching untreated control were analyzed in Tau ELISAs. A) HT7-BT2, B) HT7-Tau5, C) Tau12-BT2 and D) Tau12-HT7 and spike recovery determined (%). Data represents mean $\pm$ SEM from 3 determinations. Dashed lines indicate 100% spike recovery.

Figure 11 shows verification of signal specificity in pTau ELISAs by immunodepletion and peptide competition. Pooled CSF samples from healthy control subjects (black bars) or AD patients (red bars) were immunodepleted with Tau antibody HT7 (IP) or protein A/G beads alone (control and AD). CSF samples were also treated with pT181 or pT231 peptides for competition analysis. Samples were analyzed in pTau ELISAs A) HT7-AT270, B) HT7-PHF6, and C) Tau12-AT270.

Data represents mean $\pm$ SEM from 3 determinations. Dashed lines indicate the assay LLQ corrected for CSF dilution.

Figure 12 shows spike recovery in pTau ELISAs. Pooled CSF samples were treated with pT181 or pT231 spikes ranging from 12.5 - 200 pg/ml. Spiked samples and a matching untreated controls were analyzed in pTau ELISAs. A) HT7-AT270, B) HT7-PHF6, and C) Tau12-AT270 and spike recovery determined (%). Data represents mean $\pm$ SEM from 3 determinations. Dashed lines indicate 100% spike recovery.

Figure 13 shows analysis of Tau and pTau levels in 20 AD and 20 control CSF samples. A set of 20 AD and 20 age-matched normal control CSF samples were analyzed using INNO-BIA AlzBio3. Statistics based on 2-tailed Student's t test comparison of log-transformed data (Tau and pTau) or untransformed data (A β 42). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

DETAILED DESCRIPTION OF THE INVENTION

In certain embodiments, the present invention relates to novel methods of identifying the level of at least a Tau protein in a biological sample (e.g., CSF) from a human subject. In other embodiments, the present invention provides novel methods of identifying a subject having or at risk of having a Tau-related neurological disease (e.g., AD).

Tau fragments have been detected in CSF primarily by using western blotting and ELISA assays. One study showed that there was no intact Tau and that the majority of the Tau fragments contained the amino (N)-terminus in human CSF samples (Johnson et al 1997). Another report showed evidence of multiple bands in the 20-40 Kd range by western blotting using HT7, a mid-domain Tau antibody (Hanisch et al 2010). Another report demonstrated that carboxy (C)-terminal Tau fragments are evident in CSF from traumatic brain injury patients but not in the normal controls (Zemlan et al 1999). A proteomic analysis of Tau in CSF from an AD patient using immunoprecipitation with mass-spectrometry demonstrated that the majority of Tau sequence detected is in the N-terminal half of the molecule (Portelius et al 2008). Alterations in cleaved N-terminal Tau fragments were evident in brain extracts from PSP distinct from those in CBD samples. In this study, Applicants used

reverse-phase high performance liquid chromatography to enrich and concentrate Tau prior to western-blot analysis. Multiple N-terminal and mid-domain fragments of Tau were detected in pooled CSF with apparent sizes ranging from <20 kDa to ~40 kDa. The pattern of Tau fragments in AD and control samples were similar. In contrast, 5 full-length Tau and C-terminal-containing fragments were not detected. Taken together, these results suggest that Tau in CSF may exist as shorter fragments, and that their abundance may be altered with disease and thus serve as biomarkers for such disease (e.g., AD).

Currently, the majority of the reported CSF Tau data uses the total Tau assays 10 in commercially available kits such as the Luminex-based AlzBio3 triplex (Olsson et al 2005) and Innostest enzyme linked immunosorbant assays (ELISAs) (Hulstaert et al 1999, Vanderstichele et al 2006). The antibodies used in these assays have epitopes localized to a mid-domain region of the Tau protein and, thus, are unable to detect the presence or alterations in the amino (N)- and carboxy (C)-terminal regions of the Tau 15 protein. Similar to the total Tau assays, the majority of the reported studies on CSF phospho-Tau utilize the pT181 assay in the Innostest ELISA kit and the Luminex-based AlzBio3 triplex kit. In addition to CSF pT181 Tau, levels of pT231 Tau show a robust increase in AD subjects compared to age matched controls (Kohnken et al 2000). CSF pT231 Tau is also significantly elevated in AD compared to other 20 neurodegenerative diseases (Buerger et al 2002). The CSF pT231 assay uses a combination of CP27 and Tau1, mid-domain Tau antibodies as capture antibodies, and CP9, a pT231 specific antibody for detection (Kohnken et al 2000).

Since the existing Tau assays utilize antibodies which bind to a mid-domain region of the Tau protein, these assays may not be able to detect certain Tau 25 fragments (e.g., N-terminal or C-terminal fragments). In this study, Applicants have developed novel assays to investigate CSF Tau and pTau as biomarkers for neurodegenerative diseases (e.g., AD). For example, five Tau ELISAs and three pTau ELISAs were developed to detect and quantitate different overlapping regions of the Tau protein. The discriminatory potential of each assay was determined using 20 AD 30 and 20 age-matched control CSF samples. Of the Tau ELISAs, the two assays specific for Tau containing N-terminal sequences, amino acids 9-198 (numbering based on Tau 441) and 9-163, exhibited the highest ROC AUCs measured.

Significant discrimination was also observed with pTau assays measuring amino acids 159-p181 and 159-p231. These results demonstrate that Tau in CSF occurs as a series of fragments and that discrimination of AD from control is dependent on the subset of Tau species measured.

5 In order that the present invention may be more readily understood, certain terms are first defined. Additional definitions are set forth throughout the detailed description.

As used herein, the term “A β ” refers to amyloid beta.

As used herein, the term “A β ₄₂” refers to Amyloid Beta 1-42.

10 As used herein, the term “Tau 441” refers to the native full-length Tau protein corresponding to the following amino acid sequence:

MAEPRQEFVMEHDHAGTYGLGDRKDQGGYTMHQDQEGDTDAGLKE
SPLQTPTEDGSEEPGSETSDAKSTPTAEDVTAPLVDEGAPGKQAAAQPHTEIPE
GTTAAEEAGIGDTPSLEDEAAAGHVTQARMVSKSKDGTGSDDKKAKGADGKTK
15 IATPRGAAPPGQKGQANATRIPAKTPPAPKTPPSSGEPPKSGDRSGYSSPGSPG
TPGSRSRTPSLPTPPTREPKKVAVVRTPPKSPSSAKSRLQTAPVPMPLKKNVKS
KIGSTENLKHQPGGGKVQIINKKLDLSNVQSKCGSKDNIKHVPGGGSVQIVYK
PVDLSKVTSKCGSLGNIHHKPGGGQVEVKSEKLDKDRVQSKIGSLDNITHVP
GGGNKKIETHKLTFRENAKAKTDHGAEIVYKSPVVSGDTSRHLNSVSSTGSI
20 DMVDSPQLATLADEVSSASLAKQGL (SEQ ID NO:1).

The terms “Tau protein” and “Tau polypeptide” are used interchangeably, and refer to any of the six non-cleaved isoforms of the Tau protein that have a molecular weight in the range of 48 to 68 kDa. In addition, the term “Tau protein” or “Tau polypeptide”, as used herein, includes a Tau fragment, a Tau variant, and a modified
25 form of human Tau protein (such as a phosphorylated Tau protein).

As used herein, the term “Tau fragment” refers to a Tau protein with a reduced molecular weight compared to the full-length Tau protein and can be comprised of any interior portion of the full-length Tau protein (e.g., the N-terminal portion, the C-terminal portion, and/or the middle portion). Exemplary Tau fragments include a Tau
30 fragment comprising at least residues 9-163 of SEQ ID NO: 1, a Tau fragment comprising at least residues 9-181 of SEQ ID NO: 1, a Tau fragment comprising at least residues 9-198 of SEQ ID NO: 1, a Tau fragment comprising at least residues

159-181 of SEQ ID NO: 1, a Tau fragment comprising at least residues 159-198 of
 SEQ ID NO: 1, a Tau fragment comprising at least residues 159-225 of SEQ ID NO:
 1, and a Tau fragment comprising at least residues 159-231 of SEQ ID NO: 1.

As used herein, the term “pT181” refers to a Tau protein, polypeptide or
 5 fragment that is phosphorylated at the threonine residue at amino acid position 181.

As used herein, the term “pT231” refers to a Tau protein, polypeptide or
 fragment that is phosphorylated at the threonine residue at amino acid position 231.

As used herein, the term “antibody” is used in the broadest sense, and includes
 monoclonal antibodies, polyclonal antibodies, multispecific antibodies (*e.g.*,
 10 bispecific antibodies), full-length antibodies, antibody fragments (*e.g.*, “antigen-
 binding portion”), and single chains antibodies. An “antibody” refers to a
 glycoprotein comprising at least two heavy (H) chains and two light (L) chains inter-
 connected by disulfide bonds, or an antigen binding portion thereof. Each heavy
 chain is comprised of a heavy chain variable region (abbreviated herein as V_H) and a
 15 heavy chain constant region. The heavy chain constant region is comprised of three
 domains, C_{H1} , C_{H2} and C_{H3} . Each light chain is comprised of a light chain variable
 region (abbreviated herein as V_L) and a light chain constant region. The light chain
 constant region is comprised of one domain, C_L . The V_H and V_L regions can be
 further subdivided into regions of hypervariability, termed complementarity
 20 determining regions (CDR), interspersed with regions that are more conserved,
 termed framework regions (FR). Each V_H and V_L is composed of three CDRs and
 four FRs, arranged from amino-terminus to carboxy-terminus in the following order:
 FR1, CDR1, FR2, CDR2, FR3, CDR3, FR4. The variable regions of the heavy and
 light chains contain a binding domain that interacts with an antigen. The constant
 25 regions of the antibodies may mediate the binding of the immunoglobulin to host
 tissues or factors, including various cells of the immune system (*e.g.*, effector cells)
 and the first component (C1q) of the classical complement system.

The term “antigen-binding portion” of an antibody (or simply “antibody
 portion”), as used herein, refers to one or more fragments of an antibody that retain
 30 the ability to specifically bind to an antigen (*e.g.*, a Tau protein). It has been shown
 that the antigen-binding function of an antibody can be performed by fragments of a
 full-length antibody. Examples of binding fragments encompassed within the term

“antigen-binding portion” of an antibody include (i) a Fab fragment, a monovalent fragment consisting of the V_H , V_L , C_L and C_{H1} domains; (ii) a $F(ab')_2$ fragment, a bivalent fragment comprising two Fab fragments linked by a disulfide bridge at the hinge region; (iii) a Fd fragment consisting of the V_H and C_{H1} domains; (iv) a Fv fragment consisting of the V_L and V_H domains of a single arm of an antibody, (v) a dAb fragment (Ward et al., *Nature*, 341:544-546 (1989)), which consists of a V_H domain; and (vi) an isolated complementarity determining region (CDR).

Furthermore, although the two domains of the Fv fragment, V_L and V_H , are coded for by separate genes, they can be joined, using recombinant methods, by a synthetic linker that enables them to be made as a single protein chain in which the V_L and V_H regions pair to form monovalent molecules (known as single chain Fv (scFv); see e.g., Bird et al., *Science*, 242:423-426 (1988); and Huston et al., *Proc. Natl. Acad. Sci. USA*, 85:5879-5883 (1988)). Such single chain antibodies are also intended to be encompassed within the term “antigen-binding portion” of an antibody. These antibody fragments are obtained using conventional techniques known to those with skill in the art, and the fragments are screened for utility in the same manner as are intact antibodies.

The term “biological sample” refers to any source of biological material, for example, body fluids, brain extract, peripheral blood or any other sample comprising a Tau protein. The term “body fluid” refers to all fluids that are present in the human body including, but not limited to, whole blood, blood components (such as serum, plasma, blood cells, and platelets), urine, lymph, and cerebrospinal fluid (CSF).

The terms “Tau-related neurological disorder” and “tauopathy” are used interchangeably, and refer to any form of dementia that is associated with a Tau pathology. Alzheimer's disease and certain forms of Frontotemporal dementia (Pick's disease, sporadic Frontotemporal dementia and Frontotemporal dementia with Parkinsonism linked to chromosome 17) are the most common forms of tauopathy. Other tauopathies include, but are not limited to, Progressive supranuclear palsy (PSP), Corticobasal degeneration (CBD), and Subacute sclerosing panencephalitis.

30 Immunoassays

In certain aspects, the invention relates to methods for identifying the level of at least one Tau protein (e.g., a Tau fragment). An immunoassay often requires

biologically specific capture reagents, such as antibodies, to capture the analytes or a biomarker of interest (e.g., a Tau protein). Antibodies can be produced by methods well known in the art, e.g., by immunizing animals with the biomarker (e.g., a Tau protein) as an antigen.

5 The methods can employ an immunoassay, e.g., an enzyme immunoassay (EIA), enzyme-linked immunosorbant assay (ELISA), radioimmunoassay (RIA), indirect competitive immunoassay, direct competitive immunoassay, non-competitive immunoassay, sandwich immunoassay, agglutination assay or other immunoassay describe herein and known in the art (see, e.g., Zola, *Monoclonal Antibodies: A*
10 *Manual of Techniques*, pp. 147-158, CRC Press, Inc. (1987)). The immunoassays may be fluorescence-based or enzyme-based immunoassays. Immunoassays may be constructed in heterogeneous or homogeneous formats. Heterogeneous immunoassays are distinguished by incorporating a solid phase separation of bound analyte from free analyte or bound label from free label. Solid phases can take a variety of forms well
15 known in the art, including but not limited to tubes, plates, beads, and strips. One particular form is the microtiter plate. The solid phase material may be comprised of a variety of glasses, polymers, plastics, papers, or membranes. Particularly desirable are plastics such as polystyrene. Heterogeneous immunoassays may be competitive or non-competitive (*i.e.*, sandwich formats) (see, e.g., U.S. Patent No. 7,195,882).

20 For example, in the SELDI-based immunoassay, a biospecific capture reagent for the biomarker is attached to the surface of a mass spectrometry (MS) probe, such as a pre-activated ProteinChip array. The biomarker is then specifically captured on the biochip through this reagent, and the captured biomarker is detected by mass spectrometry.

25 To illustrate, the immunoassay may be a sandwich immunoassay, a single antibody immunoassay (often run in a competitive or “competition” mode for immunoreactive binding sites), or a double sandwich immunoassay or ELISA.

 In a specific embodiment, reverse-phase HPLC (RP-HPLC) coupled with western blot is used to detect multiple CSF fragments of Tau using mid-domain, C-
30 terminal and N-terminal region antibodies by Western blotting. RP-HPLC can

eliminate matrix interference and enrich analytes for western blot, ELISA, and other measurements.

In another embodiment, CSF Tau can be fractionated by RP-HPLC, appropriate fractions concentrated and then run by electron-spray ionization mass-
5 spectrometry (ESI-MS) or Tau peptide fragmentation and quantitative mass-identification.

In another embodiment, the methods of the present invention are used to screen for clinically significant biomarkers, for example novel Tau fragments, which are associated with a neurological disease. In a preferred embodiment, the disease is a
10 tauopathy. In a further embodiment, the tauopathy is associated with a disease selected from the group consisting of Alzheimer's disease, Parkinson's disease, including frontotemporal dementia with Parkinson's disease with Tau mutations (FTDP-17), progressive supranuclear palsy (PSP), corticobasal degeneration (CBD), and Picks disease. In a further embodiment, the methods of the present invention may
15 be used for clinical diagnosis of a tauopathy.

In certain specific embodiments, the present invention provides a method of detecting the level of at least one Tau protein in a biological sample, comprising: (a) contacting a biological sample from a subject with a first antibody which binds to an N-terminal portion of Tau 441 (SEQ ID NO: 1), and a second antibody which binds to
20 a middle portion of Tau 441 (SEQ ID NO: 1), wherein the first antibody and the second antibody both bind to the Tau protein to form a complex; and (b) detecting the level of the Tau protein in the complex. For example, the first antibody binds to an N-terminal portion of amino acid residues 9-158 of Tau 441 (SEQ ID NO: 1). Optionally, the first antibody binds to amino acid residues 9-18 of SEQ ID NO: 1.
25 For example, the second antibody binds to a middle portion of amino acid residues 159-231 of Tau 441 (SEQ ID NO: 1). Optionally, the second antibody binds to amino acid residues 194-198, residues 159-163, the phosphorylated threonine residue 181 or the phosphorylated threonine residue 231 of SEQ ID NO: 1. To illustrate, the first antibody is Tau12, and the second antibody is selected from BT2, HT7, Tau5, AT270,
30 and PHF6. When the methods use a second antibody which binds to a phosphorylated threonine residue (e.g., at position 181 or 231), the level of the phosphorylated Tau protein is detected by the present methods. Optionally, the first antibody or the

second antibody of the present methods is immobilized on a solid substrate.

Optionally, the first antibody or the second antibody comprises a label. For example, the biological sample is cerebrospinal fluid (CSF), blood, serum or plasma.

Optionally, the subject has a Tau-related neurological disease, such as Alzheimer's
5 disease.

In certain other embodiments, the present invention provides a method of detecting the level of at least one Tau protein in a biological sample, comprising: (a) contacting a biological sample from a subject with a first antibody which binds to amino acid residues 159-163 of Tau 441 (SEQ ID NO: 1), and a second antibody
10 which binds to amino acid residues 194-198, residues 218-225, or the phosphorylated threonine residue 231 of Tau 441 (SEQ ID NO: 1), wherein the first antibody and the second antibody both bind to the Tau protein to form a complex; and (b) detecting the level of the Tau protein in the complex. To illustrate, the first antibody is HT7, and the second antibody is selected from BT2, Tau5, and PHF6. When the methods use a
15 second antibody which binds to a phosphorylated threonine residue (e.g., at position 181 or 231), the level of the phosphorylated Tau protein is detected by the present methods. Optionally, the first antibody or the second antibody of the present methods is immobilized on a solid substrate. Optionally, the first antibody or the second antibody comprises a label. For example, the biological sample is cerebrospinal fluid
20 (CSF), blood, serum or plasma. Optionally, the subject has a Tau-related neurological disease, such as Alzheimer's disease.

Optionally, the present methods utilize enzyme amplification for detecting the presence or the level of the antibody:antigen complex. Enzyme amplification can occur when the second antibody is linked to an enzyme. When an appropriate
25 substrate is introduced to the enzyme, the enzyme converts the substrate to a detectable product. Examples of suitable substrates include fluorescent substrates, chemiluminescent substrates and chromogenic substrates. Examples of suitable enzymes include, but are not limited to, horseradish peroxidase, alkaline phosphatase, β -galactosidase, and acetylcholinesterase.

Optionally, the present methods utilize fluorescence readout for detecting the
30 presence or the level of the antibody:antigen complex. For example, readout by

fluorescence can occur when a non-enzyme light emitting label is bound to the second antibody. The non-enzyme light emitting label can be any fluorescent label known within the art. Illustrative examples of a non-enzyme light emitting label include fluorescein, and rhodamine. A fluorescently labeled second antibody can be detected
5 when the fluorescent label is exposed to a light of the proper wavelength and can be quantified using known methods in the art including a fluorometer. Readout by fluorescence can also include enzyme amplification of a fluorogenic substrate. The relative fluorescence units (emitted photons of light) that are detected are typically proportional to the amount of analyte being measured. Suitable enzymes for enzyme
10 amplification of a fluorogenic substrate include, but are not limited to, alkaline phosphatase, β -galactosidase or peroxidase. A fluorogenic substrate may be chosen for its quantitative emission of light following excitation. Examples of suitable substrates include, but are not limited to, 4-methylumbelliferyl phosphate, 4-methylumbelliferyl galactoside, hydroxyphenylacetic acid, 3-p-
15 hydroxyphenylpropionic acid.

Optionally, the present methods utilize luminescence readout for detecting the presence or the level of the antibody:antigen complex. Readout by luminescence may include the presence of a luminescent-tagged second antibody that can be detected by the presence of luminescence that occurs during the course of a chemical reaction.
20 Examples of luminescent labeling compounds include, but are not limited to, luminol, isoluminol, acridinium ester, imidazole, acridinium salt and oxalate ester. Luminescence detection can also include enzyme amplification wherein an enzyme converts a substrate to a reaction product that emits photons of light instead of developing a visible color. Luminescence can be bioluminescence,
25 chemiluminescence, photoluminescence each which differ in the way the excited state is reached. For each type of luminescence, a suitable enzyme includes alkaline phosphatase, β -galactosidase or peroxidase. Examples of suitable substrates include luminol, polyphenols and acridine esters, luciferin, pyrogallol, purpurogallin, gallic acid, umbelliferone, 3-(2'-spiroadamantane)-4-methyl-4-(3'-phosphoryloxyphenyl)-1,
30 2-dioxetane, disodium salt and 3-(2'-spiroadamantane)-4-methoxy-4-(3'- β -D-galactopyranosyloxyphenyl)-1,2-dioxetane).

Optionally, the present methods utilize colorimetric readout for detecting the presence or the level of the antibody:antigen complex. Colorimetric detection results in a colored reaction product that absorbs light in the visible range. For colorimetric detection the rate of color development is proportional, over a certain range, to the amount of enzyme conjugate present. Examples of suitable enzymes for colorimetric detection include, but are not limited to, alkaline phosphatase, B-galactosidase or peroxidase. Examples of suitable enzymes include, but are not limited to, 5-bromo-4-chloro-3-indolyl-phosphate/nitroblue tetrazolium, p nitrophenylphosphate, 3,3',5,5' tetramethylbenzidine, 3,3',4,4' diaminobenzidine, 4-chloro-1-naphthol, TMB (dual function substrate), 2,2'-azino-di [3-ethylbenzthiazoline and o-phenylenediamine.

Antibodies

In certain aspects, the methods of the instant invention were developed through a systematic evaluation of Tau-specific antibodies to detect Tau fragments containing the N- or C-terminal regions of Tau in disease samples compared to control samples. The instant invention discloses multiple Tau fragment ELISA assays which have been developed using antibody pairs that are specific to the mid-domain and the N-terminal region (N-terminus to the beginning of microtubule repeat domain of Tau), respectively. In addition, the instant invention discloses a novel pT231 Tau assay (e.g., using the HT7 and the PHF6 antibodies) and an N-terminal version of the pT181 Tau assay (e.g., using the Tau12 and AT270 antibodies) to complement the well-established mid-domain pT181 Tau assay (Vanderstichele et al 2006). Using these assays, the existence of N-terminal and mid-domain Tau fragments in human CSF (but not the C-Terminal fragment) is demonstrated. In addition, a significant increase in the Tau fragment and pTau fragments in AD compared to age-matched controls is shown.

Antibody supports and test membranes are also known to the art. In one embodiment, the antibody support is a cuvette or a nitrocellulose membrane. Alternatively, membranes to which the antibodies are removably, or fixedly attached may be employed. The assays provided by this invention may utilize polyclonal or monoclonal antibodies to the selected antigens. They may use the same or different polyclonal or monoclonal antibodies for capture and/or detection. The antibodies may be labeled using labels known in the art. Preferably, the label is readily detectable.

Antibodies that bind either phosphorylated or unphosphorylated Tau are contemplated. Antibodies that bind both phosphorylated and unphosphorylated Tau are also contemplated. Examples of antibodies that bind Tau are listed in Table 1.

Kits

5 In certain embodiments, the present invention provides kits that can be used in the assays described above, which comprise at least two antibodies (monoclonal or polyclonal) against a Tau protein, as well as reagents necessary for facilitating an antibody-antigen complex formation and/or detection. For example, a kit of the present invention is a packaged combination including the basic elements of: (a)
10 capture reagents comprising at least one anti-Tau antibody (herein referred to as a “capture antibody”); and (b) at least one detectable (labeled or unlabeled) anti-Tau antibody that binds to a different epitope on Tau. Optionally, the kit may further comprise reagents necessary for facilitating an antibody-antigen complex formation. Optionally, the kit may further comprise instructions on how to perform the assay
15 using these reagents.

 Optionally, the kit further comprises a solid support for the capture antibodies, which may be provided as a separate element or on which the capture antibodies are already immobilized. Hence, the capture antibodies in the kit may be immobilized on a solid support, or they may be immobilized on such support that is included with the
20 kit or provided separately from the kit. For example, the capture antibodies are coated on a microtiter plate. The detectable antibodies may be labeled antibodies detected directly or unlabeled antibodies that are detected by labeled antibodies directed against the unlabeled antibodies raised in a different species. Where the label is an enzyme, the kit will ordinarily include substrates and cofactors required by the
25 enzyme, where the label is a fluorophore, a dye precursor that provides the detectable chromophore, and where the label is biotin, an avidin such as avidin, streptavidin, or streptavidin conjugated to HRP or β -galactosidase with MUG.

 In a specific embodiment, the present invention provides a kit comprising: (1) a first antibody which binds to an N-terminal portion of a Tau protein; (2) a second
30 antibody which binds to a middle portion of a Tau protein; and (3) reagents necessary for facilitating an antibody-antigen complex formation. To illustrate, the first

antibody is Tau12, and the second antibody is selected from BT2, HT7, Tau5, AT270, and PHF6.

In another specific embodiment, the present invention provides a kit comprising: (1) a first antibody which binds to amino acid residues 159-163 of Tau 441 (SEQ ID NO: 1); (2) a second antibody which binds to amino acid residues 194-198, residues 218-225, or the phosphorylated threonine residue 231 of Tau 441 (SEQ ID NO: 1); and (3) reagents necessary for facilitating an antibody-antigen complex formation. To illustrate, the first antibody is HT7, and the second antibody is selected from BT2, Tau5, and PHF6.

Optionally, the kits may further comprise, as a positive control, a Tau protein (full-length or a fragment; phosphorylated or unphosphorylated). The kits may further comprise, as a negative control, a Tau fragment which does not bind to a capture antibody or a detection antibody. The kit may further comprise other additives such as stabilizers, washing and incubation buffers, and the like. The components of the kit will be provided in predetermined ratios, with the relative amounts of the various reagents suitably varied to provide for concentrations in solution of the reagents that substantially maximize the sensitivity of the assay. Particularly, the reagents may be provided as dry powders, usually lyophilized, including excipients, which on dissolution will provide for a reagent solution having the appropriate concentration for combining with the sample to be tested.

Diagnosing Tau-related neurological diseases

In certain embodiments, the present invention provides methods of identifying a subject having or at risk of having a Tau-related neurological disease (e.g., tauopathy). Such diagnostic methods utilize the above-mentioned immunoassays for detecting the level of at least one Tau protein (e.g., a Tau fragment) in a biological sample from a subject. The level of the Tau protein is then compared to a reference (e.g., a level of the Tau protein in a sample from a healthy control subject). If the level of the Tau protein is increased relative to the reference, then identifying the subject to have or at risk of having a Tau-related neurological disease. Optionally, such diagnostic methods utilize the above-mentioned immunoassays for detecting the level of at least two Tau proteins (e.g., two Tau fragments) in a biological sample from a subject. The ratio of two Tau protein levels is then compared to a reference

(e.g., the ratio of the two Tau protein levels in a sample from a healthy control subject). If the ratio of the two Tau protein levels is decreased relative to the reference, then identifying the subject to have or at risk of having a Tau-related neurological disease.

5 The terms “Tau-related neurological disorder” and “tauopathy” are used interchangeably herein, including, for example, Alzheimer's disease, Pick's disease, sporadic Frontotemporal dementia and Frontotemporal dementia with Parkinsonism linked to chromosome 17.

 In certain specific embodiments, the present invention provides a method of
10 identifying a subject having or at risk of having a Tau-related neurological disease, comprising: (a) isolating a biological sample from the subject; (b) detecting the level of the Tau protein in the biological sample according to any of the above-mentioned methods; and (c) comparing the level of the Tau protein to a reference; and (d) if the level of the Tau protein is increased compared to the reference, then identifying the
15 subject to have or at risk of having a Tau-related neurological disease. For example, the Tau-related neurological disease is Alzheimer's disease.

 In certain specific embodiments, the present invention provides a method of identifying a subject having or at risk of having a Tau-related neurological disease, comprising: (a) isolating a biological sample from the subject; (b) detecting a first
20 level of the Tau protein in the biological sample using HT7 as the first antibody and BT2 as the second antibody; (c) detecting a second level of the Tau protein in the biological sample using HT7 as the first antibody and Tau5 as the second antibody; (d) determining the ratio of the first level from (b) versus the second level from (c); (e) comparing the ratio to a reference ratio; and (f) if the ratio is decreased compared
25 to the reference ratio, then identifying the subject to have or at risk of having a Tau-related neurological disease. For example, the Tau-related neurological disease is Alzheimer's disease.

 In certain specific embodiments, the present invention provides a method of identifying a subject having or at risk of having a Tau-related neurological disease,
30 comprising: (a) isolating a biological sample from the subject; (b) detecting a first level of the Tau protein in the biological sample using HT7 as the first antibody and

BT2 as the second antibody is BT2; (c) detecting a second level of the Tau protein in the biological sample using Tau12 as the first antibody is Tau12 and HT7 as the second antibody; (d) determining the ratio of the first level from (b) versus the second level from (c); (e) comparing the ratio to a reference ratio; and (f) if the ratio is
5 decreased compared to the reference ratio, then identifying the subject to have or at risk of having a Tau-related neurological disease. For example, the Tau-related neurological disease is Alzheimer's disease.

Diagnosis of a Tau-related neurological disease can include any diagnostic tool that can be used to separate data points of healthy control individuals from data
10 points of diseased patients. Such detection tools can be based on optimizing either specificity or sensitivity or both. For example, detection of a Tau-related neurological disease includes use of Receiver Operating Characteristic (ROC) curve analysis. ROC curve analysis is a graphical plot of sensitivity vs. (1-specificity). The cut-off value for the assay can be derived from the ROC curve analysis at a point where the
15 sum of specificity and sensitivity is maximized. Optionally, the ratio of two Tau protein levels (measured by two assays) can be plotted in a scattergram to demonstrate the relationship between the ratio and the disease (e.g., AD).

The following examples are provided to illustrate certain embodiments of the invention. They are not intended to limit the invention in any way.

20

EXAMPLES

Methods

1. CSF samples

Pooled control and pooled AD CSF samples were generated by board-certified
25 laboratory technicians at the Clinical Neurochemistry Laboratory, the Sahlgrenska University Hospital in Mölndal, Sweden. Samples were categorized as control or AD on the basis of CSF Tau, pTau and A β 42 cut-points that are 90% sensitive and specific for AD (Tau > 350 ng/L, pTau > 80 ng/L and A β 42 < 530 ng/L; biomarker concentrations derived using INNOTEST ELISAs (Innogenetics, Ghent, Belgium))
30 [4].

Individual AD and age-matched CSF samples were purchased from Precision Med (Solana Beach, CA). Written and verbal consents were obtained from participants at screening and enrollment. For all patients, participants were > 55 years of age, in good general health having no other neurological, psychiatric or major medical diagnosis that could contribute significantly to cognitive impairment or dementia. For AD, patients were selected based upon a probable diagnosis of AD using NINCDS-ADRA criteria, a Hachinski score < (and equal to) 4 and with an MMSE between 14-26. Control subjects were classified as healthy, but no cognitive testing was performed.

10 2. HPLC fractionation of CSF for western-blotting

Human CSF was denatured in guanidine-HCl (VWR, West Chester, PA) to a final concentration of 6 M guanidine-HCl. 24 ml injections of the denatured CSF (6 ml CSF + 18 ml guanidine-HCl) were fractionated with an Agilent 1100 series HPLC running at 1.5 ml/min over a Poros R1/10 protein column (4.6 mm X 100 mm, Applied Biosystems, Foster City, CA) heated to 65° C. 30 x 2 ml fractions were collected for each sample using a water/acetonitrile gradient (0-60% acetonitrile over 35 min) in the presence of 0.1% (volume/volume) trifluoroacetic acid. Fractions were dried to completion in a SpeedVac Explorer (Thermo Savant, Pittsburgh, PA) overnight. Dried fractions were stored at -20° C until analysis. A similar strategy was used previously to extract and enrich A β peptides from human plasma and CSF and to eliminate matrix interference [50,51].

3. Western-blotting of fractions

Dried HPLC fractions were resuspended in tricine sample buffer, and separated on Novex 10-20% tricine gels according to manufacturer's directions. Gels were transferred onto 0.45 μ m polyvinylidene difluoride (PVDF) membrane (Invitrogen, Carlsbad, CA) in CAPS transfer buffer (pH 11.0) at room temperature for 90 min. Membranes were blocked in TBST (Tris-buffered saline with 0.1% (v/v) Tween-20) with 1% BSA (Thermo, Rockford, IL) for 1 hr. Membranes were probed with HT7 (Pierce), Tau12 (Covance, Dedham, MA), KJ9A (Dako, Carpinteria, CA), and mouse IgG1 monoclonal isotype control (Abcam, Cambridge, MA) conjugated to HRP (LYNX Rapid HRP Antibody Conjugation kit, AbD Serotec, Oxford, UK) in TBST with 1% BSA for 16 hrs at room temperature. Probed membranes were

developed using SuperSignal West Femto Maximum Sensitivity Substrate (Pierce, Rockford, IL).

4. INNO BIO AlzBio 3

INNO BIO AlzBio 3 was used to measure CSF $A\beta_{(1-42)}$ ($A\beta_{42}$), Tau and p-Tau (181) according to the manufacturer's instructions (Innogenetics, Ghent, Belgium). Briefly, suspension array bead sets were incubated with reference standards, QC samples, human CSF along with biotinylated reporter overnight. Excess unbound material was removed via vacuum filtration, followed by incubation with streptavidin-phycoerythrin (PE) for 60 min. The antibody/peptide complexes were detected via PE fluorescence as measured by a Bio-Rad BioPlex instrument running Luminex xPonent software. Analyte levels were quantified using an unweighted 4-parameter logistic (4PL) curve fit generated from the reference standards using BioPlex manager 5.0 software.

5. CSF Tau ELISAs

Tau ELISAs were developed using the following mouse monoclonal antibodies for capture: Tau12 (aa 9-18, SIG-39416, Covance, Princeton, NJ), HT7 (aa 159-163, MN1000, Thermo Scientific, Rockford, IL) or BT2 (aa 194-198, MN1010, Thermo Scientific, Rockford, IL). The antibody information is listed in Table 1 and "Supplemental Methods". The respective analytes were detected using the following alkaline phosphatase (AP) conjugated mouse monoclonal antibodies: BT2, HT7, Tau 5 (aa 218-225, SIG-39413, Covance, Princeton, NJ) or 77G7 (aa 316-335, SIG-34905, Covance, Princeton, NJ). Human Tau441 (Tau441) recombinant protein (rPeptide, Bogart, GA) was used to generate standard curves for each of the assays. Standards were run in two-fold serial dilutions in assay buffer containing 1% BSA (w/v) and 0.05% tween-20 (v/v) in Tris buffered saline (TBS), pH 8. The Tau441 standard curve range for each of the ELISAs was 400-2 pg/ml (Tau12-BT2 and HT7-Tau5), 1000-16 pg/ml (Tau12-HT7), 1000-4 pg/ml (HT7-BT2), 1000-8 pg/ml (HT7-77G7). Human CSF dilution linearity curves were run for each of the Tau ELISAs with CSF at 2-fold serial dilutions from 2- to 64-fold to determine the optimal sample dilution for each of the assays. Based on the results from the CSF linearity experiment, individual CSF samples were assayed at the following dilutions in assay buffer for

each of the total Tau assays: 2-fold (HT7-77G7), 20-fold (Tau12-HT7), 10-fold (HT7-Tau5), 25-fold (Tau12-BT2), and 30-fold (HT7-BT2).

Tau ELISAs were run as follows. High binding black 96 well plates (Costar 3925, Corning, NY) were coated by the addition of 2.5 µg/ml (BT2, HT7) or 5 µg/ml (Tau12) capture antibodies which were diluted in Tris buffered saline (TBS), pH 8. Plate sealers were attached then the plates were incubated at 37° C for 1 hr. Plates were washed with TBST (TBS containing 0.05% Tween-20) before blocking nonspecific binding sites with 3% bovine serum albumin (BSA; protease free, fraction V; Roche Biochemicals, Indianapolis, IN) (w/v) in TBS. Plate sealers were attached and the plates were incubated at room temperature for 2-4 hrs while shaking. Plates were washed with TBST before the addition of 50 µl per well diluted human CSF and human Tau441 standard curves which were each prepared in a final assay buffer concentration of 1% BSA (w/v) and 0.05% Tween-20 (v/v) in Tris buffered saline (TBS), pH 8. Plate sealers were attached; then assay plates containing human CSF and standard curves were incubated overnight at 4° C while shaking. Alkaline phosphatase (AP) conjugated BT2, HT7, Tau5 or 77G7 antibodies were diluted into assay buffer before being added to the assay plate (50 µl per well) to co-incubate with human CSF and hTau441 standard curves for 1 hr at room temperature while shaking. Plates were washed with TBST before being developed using alkaline phosphatase substrate (T2214; Applied Biosystems, Foster City, CA). Luminescence counts were measured using a Packard TopCount (PerkinElmer, MA). Log-transformed luminescence counts from individual samples were interpolated to concentration using a second-order polynomial fit to the respective standards (GraphPad Prism 5.00, GraphPad Software, San Diego, CA). CSF Tau levels were plotted after correction for dilution factor in the respective assays. Assay lower limit of quantitation (LLQ) was set based on the lowest calibrator demonstrating acceptable total error (bias + precision of < 30%).

6. Human Tau441 Spike Recovery in HT7-77G7 ELISA

A pooled CSF sample from AD patients and an age-matched pooled control CSF sample were 2-fold serially diluted from 2- to 256-fold in a final assay buffer concentration of 1% BSA (w/v) and 0.05% tween-20 (v/v) in Tris buffered saline (TBS), pH 8 before an aliquot of each was spiked with recombinant human Tau441

protein (rPeptide, Bogart, GA) at a final concentration of 100 pg/ml. Diluted CSF samples with and without Tau441 spike were assayed in the HT7-77G7 ELISA as described above.

7. CSF pTau ELISAs

5 Human CSF was analyzed in three different pTau assays: HT7-AT270 (p181) and HT7-PHF6 (p231) and Tau12-AT270 (p181). The HT7-AT270 and HT7-PHF6 ELISAs utilized HT7 (amino acids 159-163), while the Tau12-AT270 assay used Tau12 (amino acids 9-18), as the capture antibody, and the respective analytes were detected using alkaline phosphatase (AP) conjugated AT270 pT181 Tau antibody
10 (MN1050, Thermo, Rockford, IL) or PHF6 pT231 specific monoclonal antibody (SIG-39430, Covance, Dedham, MA); antibody information listed in Table 1. Standards for the 3 different assays were custom synthesized (Abgent Inc., San Diego, CA) and contained sequences with the respective capture and detection epitopes as follows: the HT7-AT270 and HT7-PHF6 assay standards used native human Tau
15 sequence of aa 155–207 and aa 155–236, respectively, with the Thr181 and Thr 231 residues being phosphorylated; the Tau12-AT270 assay standard consisted of aa 5-28 linked with a polyethylene glycol (PEG12) linker to aa 174-187, with a phosphorylated Thr181 residue. Standard purity was verified at the Keck Biotechnology Resource Laboratory at Yale University. Standards were run in 2-fold
20 serial dilution in assay buffer containing 0.3% BSA in PBS with 0.05% Tween, with a range of 125 to 1 pg/ml for HT7-AT270, and 500 to 4 pg/ml for HT7-PHF6 and Tau12-AT270 Tau assays. CSF samples were run neat for the HT7+pT231 Tau assay, while the Tau12+pT181 and HT7+pT181 Tau assays were run at 2- and 4-fold dilution in assay buffer.

25 pTau ELISAs were run as follows. Black high-binding plates (Costar, Corning, NY) were coated with 2.5 µg/ml of capture antibody HT7 or Tau12 in carbonate-bicarbonate buffer at pH 9.4 (Thermo, Rockford, IL). After overnight incubation at 4° C, plates were washed with PBS and nonspecific binding sites were blocked using 3% BSA in PBS buffer for at least 4 hrs at 4° C. Standards and CSF
30 samples (50 µL) were added to plates, followed by 50 µL of AP conjugated AT270 (pThr181) and PHF6 (pThr231) Tau antibodies for the respective assays. After overnight incubation at 4° C, plates were washed with PBS (containing 0.05% tween)

and developed using alkaline phosphatase substrate (T2214; Applied Biosystems, Foster City, CA). Luminescence counts were measured using Envision (Perkin Elmer, MA). Log-transformed luminescence counts from individual samples were interpolated to concentration using a 3rd order polynomial fit to the respective standards (GraphPad Prism 5.00, GraphPad Software, San Diego CA). CSF pTau levels were plotted after correction for dilution factor in the respective assays. Assay lower limit of quantitation (LLQ) was set based on the lowest calibrator demonstrating acceptable total error (bias + precision of < 30%).

8. Statistics

Statistical calculations were performed using GraphPad Prism 5.00 (GraphPad Software, San Diego, CA). Differences in biomarker levels between AD and control samples were examined using both parametric (unpaired two-tailed Student's t-test) and nonparametric (Receiver operating characteristic (ROC) area under the curve (AUC)) analysis. Data was log-transformed prior to t-test comparisons to correct for non-Gaussian distributions as determined by the D'Agostino & Pearson normality test. Statistical comparison of ROC AUC values was based on the method of Hanley and McNeil [52]. Results were considered significant for p-values < 0.01.

Supplemental Methods

1. Epitope mapping

A multiplexed immunoassay was developed on the Luminex platform for mapping Tau antibody epitopes. The assay consisted of 41 different antigens, each covalently coupled to a unique Luminex bead set using standard amine coupling protocols. These antigens included all six human Tau isoforms (rPeptide, Bogart, GA), a set of 29 synthetic overlapping Tau sequence peptides (GenScript, Piscataway NJ) spanning the length of human Tau 441 (Fig. 7), and 3 Tau fragments (aa 1-125, 126-230, 231-441) generated using standard in vitro transcription/ translation system. Beads were conjugated using a two-step carbodiimide procedure. Briefly, 1 mL of beads (1.25×10^7 /mL) was pelleted by centrifugation for 1 min at 10000 x g at 4° C in an Eppendorf 5415D centrifuge (Westbury, NY). Beads were washed and resuspended in 500 µL of 0.1M sodium phosphate buffer pH 4.8 (activation buffer) followed by 15 seconds of vortexing and 15 seconds of sonication. Beads were

washed 2x times with activation buffer, resuspended in 200 μ L of freshly prepared 5 mg/mL of EDC (1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride, inactivation buffer), and incubated in a rotator for 20 min at RT protected from light. Beads were then washed and resuspended in 500 μ L of antigen in PBS (100 μ g for the

5 Tau isoforms and 20 μ g for the peptides) and incubated for 2 hrs at RT in a rotator protected from light. Beads were then washed and incubated with 0.5 mL of blocking buffer (PBS, 1% (w/v) BSA, 0.02% (w/v) Tween-20) in a rotator for 1-hour at RT protected from light. Finally, the beads were counted with a hemacytometer and resuspended in blocking buffer at 2×10^6 beads/mL. Beads were stored protected

10 from light at 4° C. Prior to testing, the bead sets were mixed together to form a suspension array. Antibodies for epitope mapping were incubated with the bead mix, washed, and subsequently incubated with PE-labeled anti-mouse IgG (H+L) reporter antibodies. The beads were then analyzed on a Bioplex Luminex 100 instrument (Bio-Rad Laboratories, Hercules, CA)

15 2. Immunodepletion and spike recovery

Human control CSF was diluted 2-fold into assay buffer (final concentration of 1% BSA in TBS with 0.05% Tween-20) before an aliquot was removed and HT7 was added to a final concentration of 1 μ g/mL. CSF and HT7 were co-incubated for 1 hour at 4° C before being added to protein A/G agarose (Thermo Fisher Scientific,

20 Rockford, IL) at a 9:1 ratio. The protein A/G beads were blocked with 10 volumes of 2% BSA (w/v) in TBS for 1 hour before use. The remaining diluted CSF sample that was not immunodepleted with HT7 was incubated with protein A/G beads to serve as a control. CSF and protein A/G beads were incubated overnight at 4° C. Beads were spun out before the supernatant was collected, diluted and assayed in CSF Tau

25 ELISAs. CSF was assayed at a 3-fold (HT7+Tau5), 5-fold (Tau12+BT2 and Tau12+HT7) or 10-fold dilution (BT2+HT7).

For pTau ELISAs, pooled CSF samples from healthy controls and AD subjects were immunodepleted with Tau antibody HT7 conjugated to CNBr activated Sepharose 4B beads or unconjugated inactive sepharose 4B beads (GE Healthcare,

30 Pittsburgh, PA) following overnight incubation at 4° C in a rotating holder. Next day, CSF samples were spun at 1000 rpm for 5 min and supernatants (depleted CSF) were run in respective pTau assays. For phospho-peptide depletion studies, CSF samples

- were spiked with 100 ng/ml of pT181 (PPAPK-T(PO4)-PP) or pT231 (KVAVVR-T(PO4)-PPK) peptides and run in respective pTau assays (HT7-AT270, HT7-PHF6, and Tau12-AT270). For spike-recovery studies, control CSF samples diluted in 0.3% BSA/PBS were spiked with the respective pTau standards over a range of
- 5 concentrations and run in ELISA's along with non-spiked samples. Spike recovery was estimated as a percent recovery of signal above basal signal levels in non-spiked CSF samples.

Results

10 1. Tau fragments detected in human CSF by western-blotting

- To determine the nature of Tau in human CSF, a reverse-phase high performance liquid chromatography column (RP-HPLC) was used to enrich the relatively low abundance Tau protein prior to western-blot analysis. Equal volumes of pooled control and AD CSF samples were fractionated and then western-blot
- 15 analyzed with antibodies specific for different regions of the protein; a summary of the antibodies used is shown in Table 1.

Table 1. Tau and pTau antibodies

Clone	Vendor	Cat #	Epitope ¹	Species	Reference
BT2	Thermo Scientific	MN1010	194-198	Mouse	[38,49];
HT7	Thermo Scientific	MN1000	159-163	Mouse	[38]
Tau5	Covance	SIG-39413	218-225	Mouse	[53 ,54]
Tau12	Covance	SIG-39416	9-18	Mouse	[55]
KJ9A	Dako	A 0024	243-441	Rabbit	
77G7	Covance	SIG-39405	316-335	Mouse	this paper
PHF6	Covance	SIG-39430	pT231	Mouse	[56]
AT270	Thermo Scientific	MN1050	pT181	Mouse	[57]
IgG	Abcam	ab81032	NA	Mouse	NA

¹ Amino acid numbering based on human Tau 441 sequence
NA: not applicable

20

A range of bands were detected with HT7, an antibody specific for a mid-domain epitope (amino acids (aa) 159-163; amino acid numbering based on human

Tau 441) (Fig. 1A). The majority of bands present in fractions 3 to 7 were specific for HT7, as these were not detected with the IgG1 isotype control antibody (Fig. 1D). These bands exhibited a range of apparent molecular weights (MWs), from < 20 kDa to ~40 kDa, suggesting the presence of Tau protein fragments. Interestingly, Tau-specific bands that comigrated with the Tau441 standard (~65 kDa) were not detected, suggesting that full length Tau is not present. A subset of the HT7-immunoreactive bands in fractions 6 to 8 was also detected with the N-terminal antibody Tau12 (epitope aa 9-18), indicating that these fragments span both N-terminal and mid-domain regions of Tau (Fig. 1B). In addition, bands only detected with Tau12 were also observed in fractions 6 and 7, providing evidence that truncated N-terminal Tau fragments are also present. Consistent with the HT7 findings, bands of ~65 kDa were not detected with Tau12, confirming the lack of full-length Tau in CSF. Interestingly, the overall Tau fragment pattern observed with both HT7 and Tau12 was similar in AD compared to control.

Fractions were also analyzed for the presence of C-terminal fragments using the rabbit polyclonal antibody K9JA, which binds to the microtubule repeat and C-terminal flanking region of Tau (aa 243-441). K9JA exhibited robust staining of full length Tau441 standard (Fig. 1C); however, unlike HT7 and Tau12, K9JA-specific Tau bands were not detected in the CSF samples (Fig. 1C). A set of bands of 60-65 kDa were detected at various levels in all of the fractions (Fig. 1C), though these are likely due to nonspecific immunoreactivity as they were detected to a lesser degree with the isotype control antibody (Fig. 1D), and appear to be due to cross-reactivity with contaminating keratin (data not shown); the lack of full length Tau is also consistent with ELISA data discussed below. Taken together, these results indicate that Tau in both control and AD CSF is present as a set of primarily N-terminal and mid-domain fragments.

2. Tau fragment and pTau assays

To more accurately quantify CSF Tau levels, a set of novel Tau and pTau ELISAs were developed (Fig. 2). Assays were designed to measure overlapping regions of Tau using different combinations of Tau and pTau antibodies (Table 1). Each assay is specific for different minimal regions of the Tau protein, as defined by the epitopes of the antibodies used, and thus may measure different subsets of

fragments in CSF. The minimal regions of the Tau assays are aa 9-163 (Tau12-HT7), aa 9-198 (Tau12-BT2), aa 159-198 (HT7-BT2), aa 159-225 (HT7-Tau5), aa 159-335 (HT7-77G7); the minimal regions of the pTau assays are aa 9-p181 (Tau12-AT270), aa 159-p181 (HT7-AT270) and aa 159-p231 (HT7-PHF6) (Fig 2).

5 The HT7-BT2, HT7-Tau5, Tau12-BT2 and Tau12-HT7 Tau assays demonstrated ~100-fold dynamic range of quantitation using the Tau 441 standard, with the LLQ ranging from 1.6 pg/ml to 7.8 pg/ml (Fig. 3). Signal from pooled AD and control CSF samples were within the dynamic range of the HT7-BT2 assay when diluted 2- to 64-fold (left panel, Fig. 3A). Consistent dilution-corrected Tau levels
10 were observed with CSF dilutions ranging from 16- to 64-fold; similar results were observed for both AD and control samples (right panel, Fig. 3A). A dilution of 30-fold was identified as optimal for CSF sample analysis. Similar results were observed for HT7-Tau5, Tau12-BT2 and Tau12-HT7 (Fig. 3B, C, D, respectively) with optimal CSF dilutions of 10-fold, 25-fold and 20-fold, respectively. Tau specificity in each
15 assay was verified based on immuno-depletion (Fig. 9) and spike recovery (Fig. 10). Taken together, these results confirm the ability of these assays to accurately measure Tau in CSF.

 The HT7-77G7 assay is specific for Tau species containing more C-terminal sequences (aa 159-335). The dynamic range and LLQ observed were similar to the
20 other Tau ELISAs (Fig. 4); however, a HT7-77G7 signal was not detected in either the pooled control or pooled AD samples, regardless of sample dilution (Fig. 4). The lack of signal was not an artifact of matrix interference as robust recovery of a 100 pg/ml Tau 441 spike was observed in both control and AD CSF over a range of dilutions (Fig. 4). These results indicate that Tau species containing the region aa
25 159-335 are not present in these pooled CSF samples.

 All three pTau assays, HT7-AT270, Tau12-AT270 and HT7-PHF6, demonstrated ~100-fold dynamic range of quantitation using synthetic pTau standards, with LLQs ranging from 2 pg/ml to 7.8 pg/ml (Fig. 5). In the HT7-AT270 assay, consistent dilution-corrected pTau levels were observed in AD and control CSF
30 with dilutions ranging from 2- to 16-fold (right panel, Fig. 5A). In the Tau12-AT270 assay (Fig. 5B) and HT7-PHF6 assays (Fig. 5C), dilution linearity was observed when samples were measured neat or diluted up to 4-fold. CSF pTau signal specificity was

verified by a combination of immuno-depletion and peptide competition (Fig. 11) and spike recovery (Fig. 12). Taken together, these results confirm specificity of the pTau signal measured in these assays.

3. Evaluation of CSF Tau and pTau in a cohort of control and AD CSF samples

- 5 To evaluate the discriminatory power of the assays, Tau and pTau levels were measured in a cohort of 20 AD and 20 age-matched control CSF samples (20x20 sample set); demographic information included in Table 2. The relative ability of each assay to detect differences between AD and control samples was assessed using Student's t-test comparison of log-transformed data and receiver operating
- 10 characteristic (ROC) area under the curve (AUC) evaluations. Differences were deemed significant for $p < 0.01$. Samples were also benchmarked for A β 42, Tau, and p181Tau using INNO-BIA AlzBio3. Levels of CSF A β 42 were significantly reduced while levels of Tau and pTau were significantly increased in AD samples compared to the age-matched controls when measured using INNO-BIA AlzBio3 (Fig. 13; Table
- 15 3). ROC AUC values were significant for all three measures, ranging from 0.78 to 0.81, consistent with expectations for a typical AD vs control sample set.

Table 2. Demographics of 20x20 CSF sample set

	Control	AD
n	20	20
Age at LP (SD), yr	68 (6)	72 (6)
Gender F/M	10/10	10/10
MMSE (SD)	30 (0.5)	21 (4)

Table 3. Summary of analysis of 20x20 CSF sample set

		Raw data, pg/ml ¹		Log transformed data ¹			ROC analysis		
Assay		Control	AD	Control	AD	Fold-diff	p-value ²	AUC	p-value
AlzBio 3	Aβ42	262 (57)	184 (78)	2.409 (0.096)	2.225 (0.192)	0.65	0.0005	0.78	0.0029
	Tau	48 (17)	99 (66)	1.662 (0.143)	1.920 (0.257)	1.8	0.0003	0.81	0.0008
	pTau	23 (7)	46 (28)	1.330 (0.147)	1.591 (0.249)	1.8	0.0003	0.80	0.0011
Tau	Tau12-HT7	(aa 9-163)	714 (497)	2.474 (0.137)	2.773 (0.267)	2.0	<0.0001	0.85	0.0001
	Tau12-BT2	(aa 9-198)	1162 (639)	2.747 (0.155)	3.011 (0.223)	1.8	0.0001	0.86	0.0001
	HT7-BT2	(aa 159-198)	2546 (1914)	3.112 (0.153)	3.330 (0.268)	1.7	0.0031	0.77	0.0031
	HT7-Tau5	(aa 159-225)	379 (185)	2.630 (0.159)	2.943 (0.308)	2.1	0.0003	0.82	0.0005
	HT7-77G7	(aa 159-335)	<LLQ						
pTau	HT7-AT270	(aa 159-p181)	46 (15)	1.646 (0.132)	1.856 (0.208)	1.6	0.0005	0.81	0.0008
	HT7-PHF6	(aa 159-p231)	18 (18)	1.122 (0.331)	1.501 (0.382)	2.4	0.0018	0.77	0.0040
	Tau12-AT270	(aa 9-p181)	21 (7)	1.306 (0.162)	1.439 (0.148)	1.4	0.0100	0.71	0.0266

¹Data based on n = 20 control, n = 20 AD samples. Values represent mean (SD)²p-values based on unpaired, 2-tailed Student's t test comparison of log transformed control and AD data

LLQ: Lower limit of quantitation

In the Tau ELISAs, the highest CSF Tau levels were detected using the HT7-BT2 assay, specific for Tau species containing aa 159-198. In comparison, levels of Tau species containing additional N-terminal sequence (aa 9-198, Tau12-BT2; aa 9-163, Tau12-HT7) were 2- to 4-fold lower, while Tau species containing additional C-terminal sequence (aa 159-225, HT7-Tau5) were 3-fold lower (Table 4). Levels measured in these assays were highly correlated ($r^2 = 0.87-0.95$) (Table 5). On the other hand, Tau containing additional C-terminal sequence aa 159-335 (HT7-77G7) could not be detected (Fig. 6), consistent with results from the pooled samples (Fig. 4). A signal above background was detected in 11 of the 20 AD samples and 3 of the 20 control samples though additional work will be needed to quantify and verify the specificity of this HT7-77G7 signal.

Table 4. Analysis of Tau assay ratios in 20x20 sample set

Assay Ratio		Control	AD	p-value ^a	ROC AUC	p-value ^b
HT7-BT2	HT7-Tau5	3.08 (0.59)	2.47 (0.38)	0.0004	0.84	0.0002
HT7-BT2	Tau12-HT7	4.39 (0.63)	3.65 (0.61)	0.0006	0.83	0.0003
Tau12-HT7	Tau12-BT2	0.536 (0.063)	0.584 (0.087)	0.0515	0.67	0.0659
HT7-BT2	Tau12-BT2	2.35 (0.37)	2.12 (0.36)	0.0544	0.67	0.0743
HT7-Tau5	Tau12-BT2	0.787 (0.193)	0.885 (0.238)	0.1608	0.61	0.2340
HT7-Tau5	Tau12-HT7	1.47 (0.36)	1.50 (0.27)	0.7770	0.56	0.5162
Values represent mean (SD) from 20 control and 20 AD samples						
^a p-values based on unpaired, 2-tailed Student's t test comparison of control and AD data						
^b p-values based on ROC AUC analysis of control vs AD						

Table 5. Tau ELISA correlations

	HT7- BT2	HT7- Tau5	Tau12- BT2	Tau12- HT7	AlzBio3 Tau	HT7- AT270	HT7- PHF6	T12- AT270*	AlzBio3 pTau
HT7- BT2		0.93	0.91	0.91	0.92	0.67	0.18	0.30	0.57
HT7- Tau5			0.87	0.90	0.91	0.67	0.27	0.30	0.62
Tau12- BT2				0.95	0.89	0.70	NS	0.32	0.62
Tau12- HT7					0.93	0.79	0.22	0.37	0.70
AlzBio3 Tau						0.72	0.25	0.33	0.70
HT7- AT270							0.35	0.24	0.60
HT7- PHF6								NS	0.20
T12- AT270*									0.41
AlzBio3 pTau									
Analysis based on log-transformed data									
NS = p > 0.01									

Overall, CSF Tau levels were found to be significantly higher in AD samples compared to controls in all but the HT7-77G7 assay (Fig. 6; Table 3). ROC AUC values were also significant, ranging from 0.77 to 0.86 (Table 3). The highest ROC AUCs (0.85, 0.86) were observed using assays specific for Tau containing aa 9-163 (Tau12-HT7) and aa 9-198 (Tau12-BT2), respectively, while a significantly lower AUC (0.77) was detected using the HT7-BT2 assay, specific for Tau species containing aa 159-198. The ROC AUC for the INNO-BIA AlzBio3 Tau assay (0.81) was nearly identical to the AUC measured for the HT7-Tau5 assay (0.82), consistent with the fact that both assays are specific for the same Tau region (aa 159-225). Taken together, these results indicate that the discriminatory power of CSF Tau is dependent on the Tau species measured.

The 20x20 samples were also analyzed using the pTau ELISAs. All three pTau ELISAs exhibited significantly higher levels in AD compared to control samples, with increases ranging from 1.4-fold to 2.4-fold (Fig 6; Table 3). Of these, HT7-AT270, specific for Tau species containing aa 159-p181, exhibited the highest ROC AUC (0.81), similar to the AUC generated using the comparable INNO-BIA AlzBio3 pTau assay (0.80, Table 3). Interestingly, p181 failed to exhibit a significant ROC AUC when measured in the context of Tau species containing additional N-

terminal sequence aa 9-p181 (Tau12-AT270) (0.71, $p = 0.0266$, Table 3). Thus, similar to results for the Tau ELISAs, the discriminatory power of CSF p181 is dependent on the pTau species measured. Significant discrimination of AD from control samples was also observed using HT7-PHF6, specific for aa 159-p231, though
 5 the AUC (0.77) was lower than observed in the HT7-AT270 assay. In comparison to the Tau ELISA results, a weaker degree of correlation was observed using the pTau measures (Table 4). Of the 3 assays, HT7-AT270 demonstrated the highest degree of correlation with the Tau assays ($r^2 = 0.67-0.72$). By comparison, the HT7-PHF6 and Tau12-AT270 correlations were less robust ($r^2 = 0.18-0.37$) and in some cases not
 10 significant.

To further compare the discriminatory power of the different Tau and pTau assays, all possible combinations of Tau and pTau ratios were calculated for each individual CSF sample and then these values used to evaluate differences and discrimination between AD and control samples (Tables 3, 4, 6). This analysis of
 15 ratios enables comparison of the relative disease-associated changes between different assays. Of all the ratios analyzed, only two, HT7-BT2/HT7-Tau5 and HT7-BT2/Tau12-HT7, exhibited significant differences in levels and significant discrimination between AD and control (Table 4). In the case of HT7-BT2/HT7-Tau5, the ROC AUC observed (0.84) was higher than either assay alone (0.82,
 20 HT7+Tau5; 0.77, BT2+HT7). These results further support the idea that discrimination of AD from controls is dependent on the subset of CSF Tau species measured.

Table 6. Analysis of pTau assay ratios in 20 x 20 sample set

Assay Ratio		Control	AD	p-value ^a	ROC AUC	p-value ^b
HT7-AT270	Tau12-HT7	0.156 (0.051)	0.124 (0.030)	0.0206	0.76	0.0058
Tau12-AT270	Tau12-HT7	0.0756 (0.0367)	0.0501 (0.0207)	0.0101	0.73	0.0139
HT7-PHF6	Tau12-AT270	1.18 (1.96)	1.50 (1.02)	0.5282	0.70	0.0285
Tau12-AT270	HT7-Tau5	0.0541 (0.0287)	0.0354 (0.0180)	0.0184	0.70	0.0295
HT7-AT270	HT7-Tau5	0.114 (0.061)	0.0858 (0.0285)	0.0655	0.68	0.0484
Tau12-AT270	Tau12-BT2	0.0409 (0.0215)	0.0284 (0.0099)	0.0227	0.68	0.0515
HT7-PHF6	HT7-AT270	0.413 (0.423)	0.565 (0.449)	0.2778	0.67	0.0620
HT7-PHF6	HT7-BT2	0.0164 (0.0202)	0.0210 (0.0242)	0.5125	0.66	0.0765
HT7-PHF6	Tau12-BT2	0.0401 (0.0530)	0.0434 (0.0448)	0.8340	0.65	0.1167
HT7-AT270	Tau12-AT270	2.52 (1.47)	2.74 (0.85)	0.5721	0.63	0.1678
HT7-PHF6	Tau12-HT7	0.0711 (0.0902)	0.0762 (0.0886)	0.8578	0.62	0.1851
Tau12-AT270	HT7-BT2	0.0174 (0.0085)	0.0140 (0.0061)	0.1558	0.60	0.3040
HT7-PHF6	HT7-Tau5	0.0469 (0.0513)	0.0521 (0.0667)	0.7839	0.59	0.3235
HT7-AT270	Tau12-BT2	0.0843 (0.0331)	0.0715 (0.0145)	0.1213	0.58	0.3721
HT7-AT270	HT7-BT2	0.0371 (0.0167)	0.0344 (0.0083)	0.5139	0.51	0.9031
Values represent mean (SD) from 20 control and 20 AD samples						
^a p-values based on unpaired, 2-tailed Student's t test comparison of control and AD data						
^b p-values based on ROC AUC analysis of control vs AD						

Discussion

5 In this study, Tau profiles and the relative differences in Tau and pTau levels between AD and age-matched control CSF samples were investigated using a combination of qualitative and quantitative biochemical assays. A sensitive western-blotting method was used to demonstrate that Tau is present in CSF as a series of N-terminal and mid-domain fragments. Results from a set of novel ELISAs specific for

10 different overlapping regions of Tau demonstrated that the ability of CSF Tau and pTau to differentiate AD from control is dependent on the Tau species measured. These endpoints provide novel tools to investigate CSF Tau in AD and other neurodegenerative disorders.

A number of previous studies have reported on the characterization of Tau in

15 CSF using western-blotting-based techniques; however, results from these studies are mixed and incongruent [7,22,27,32,33,34,35,36,37,40,41]. Many studies report the presence of Tau fragments in CSF, though the size, number and prevalence of these fragments vary. Many also report the presence of full-length Tau and in two studies only full-length Tau was detected [40,41]. Some of the discrepancies between studies

20 may be due to nonspecific binding artifacts commonly observed with western-blotting. Applicants have identified a number of nonspecific binding activities in

fractionated samples with various Tau-specific antibodies (data not shown). Indeed, such nonspecific artifacts may account for some of the findings reported ([35,36]; see [42]). In some studies, incorporation of an immunoprecipitation step was used to enrich Tau prior to western-blotting, potentially limiting the Tau fragments detected
5 [7,33,40]. And use of postmortem CSF [27,37] may have compromised analysis as CSF Tau levels are highly sensitive to the integrity of brain tissue [43].

To address these limitations, RP-HPLC was utilized to enrich and concentrate Tau from a large volume of pooled, denatured CSF thereby enabling analysis of the relatively low levels of Tau by western-blotting. N-terminal and mid-domain Tau
10 fragments were detected in both AD and control CSF, ranging in size from <20 kDa to ~40 kDa. In contrast, C-terminal-containing fragments were not detected using the K9JA polyclonal antibody. Furthermore, full-length Tau was not detected with any of the antibodies tested. The lack of detectable C-terminal fragments was surprising given reports indicating that MTBR-containing fragments are present in CSF
15 [22,44,45]. Applicants confirmed the ability to fractionate and detect full-length Tau and MTBR-containing fragments using purified standards (data not shown). The lack of detectable C-terminal fragments could be due to limited sensitivity of K9JA to detect these by western-blot (~30-100 pg) or the inability to resolve higher order oligomers or aggregates using the RP-HPLC system. Thus, additional enrichment
20 methods and C-terminal-specific monoclonal antibody reagents will be needed to help resolve these issues. Nevertheless, these results demonstrate that CSF Tau is composed of a complex mixture of fragments.

Tau is a putative substrate for various proteases such as calpain, caspases, cathepsins and thrombin (reviewed in [46,47]). Tau fragments observed in CSF could
25 be a direct result of processing by these proteases. Indeed, cleavages at many of the known sites may partly explain differences in absolute levels detected in the different Tau ELISAs [46,47,48]. However, technical issues related to the range of Tau species present and the relative affinity of antibodies for those species could also contribute to assay differences. Thus, comparisons of absolute levels between ELISAs must be
30 interpreted with caution.

In order to investigate discriminatory potential, each ELISA was evaluated for its ability to differentiate between 20 AD and 20 matched control CSF samples. In

general, all of the Tau ELISAs, with the exception of HT7-77G7, behaved in a similar manner exhibiting significant differences in levels and significant discrimination between AD and control. These findings are consistent with the high degree of correlation between assays. Despite the similarities, however, subtle differences in
5 Tau assay performance were also noted. Most interesting was the fact that the two assays specific for Tau species containing N-terminal sequences, aa 9-163 (Tau12-HT7) and aa 9-198 (Tau12-BT2), exhibited the highest ROC AUCs of any measured. In contrast to these, a significantly lower ROC AUC was measured using HT7-BT2, specific for aa 159-198. This difference could be partly linked to the sensitivity of
10 BT2 to phosphorylation at S199 [49] as levels of p199 are reported to be increased in AD CSF [11]. However BT2 sensitivity cannot account for differences between Tau12-BT2 and HT7-BT2. Taken together these findings suggest that N-terminal-containing Tau species may be more sensitive biomarkers of AD. Additional N-terminal assays and larger sample cohorts will be needed to confirm and fully explore
15 this finding.

Another interesting finding was the lack of a quantifiable CSF signal in the HT7-77G7 assay. This was not an assay artifact since Applicants observed complete recovery of a Tau spike in CSF matrix. Given the relatively robust signal measured in the HT7-Tau5 assay (aa 159-225), the lack of signal in the HT7-77G7 assay (aa 159-
20 335) suggests extensive cleavage of Tau between the Tau5 (aa 218-225) and 77G7 (aa 316-335) epitopes, further supporting the idea that CSF Tau is fragmented. The HT7-77G7 result is also consistent with the inability to detect full-length Tau by western-blotting. This was further confirmed using another ELISA, Tau12-DC39, specific for Tau containing aa 9-441 [43]. As comparable to HT7-77G7, a Tau signal could not
25 be detected in CSF although a Tau441 spike was readily measured with this assay (data not shown). The lack of a quantifiable HT7-77G7 signal in CSF, however, does not exclude the possible presence of fragments containing MTBR sequences only or more C-terminal regions as these would be undetectable by the HT7-77G7 assay.

Of the pTau ELISAs evaluated, the HT7-AT270 (aa 159-p181) assay
30 exhibited the highest level of discrimination, though only slightly better than HT7-PHF6 (aa 159-p231). This finding is consistent with data reporting that p181, p231 and p199 were equivalent in their ability to discriminate AD from controls [11].

Interestingly, significant discrimination of AD from control was lost when p181 was measured in the context of Tau species containing additional N-terminal sequence aa 9-p181 (Tau12-AT270). This finding is surprising given that the Tau ELISAs dependent on the same N-terminal regions exhibited the highest ROC AUCs measured. These results suggest that there may be distinct pathways leading to increased CSF levels of these Tau and pTau species.

In summary, our results indicate that Tau is present in control and AD CSF as a mixture of fragments, primarily composed of N-terminal and mid-domain regions. Results from our novel Tau and pTau assays provide evidence that the discrimination of AD from control is dependent on the subset of Tau species measured and that development of more robust AD biomarkers may be possible. One limitation of the study is the relatively small sample set used. Ultimate confirmation of these findings will require analysis of larger cohorts of AD and control samples to ensure robust statistical analysis. Additional assays will also be needed to fully investigate any C-terminal fragments or aggregates present but not detected by the methods employed here. Finally, these results could have implications in the development of CSF Tau and pTau biomarkers for other neurodegenerative diseases, including other tauopathies where disease-associated changes in Tau and pTau have not been consistently observed.

All references cited in this specification, including without limitation all papers, publications, patents, patent applications, presentations, texts, reports, manuscripts, brochures, books, internet postings, journal articles, periodicals, product fact sheets, and the like, are hereby incorporated by reference into this specification in their entireties. The discussion of the references herein is intended to merely summarize the assertions made by their authors and no admission is made that any reference constitutes prior art and Applicants' reserve the right to challenge the accuracy and pertinence of the cited references.

EQUIVALENTS

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be readily apparent to those of ordinary skill in the art in light of the teachings of this invention

that certain changes and modifications may be made thereto without departing from the spirit or scope of the dependant claims.

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WHAT IS CLAIMED IS:

1. A method of detecting the level of at least one Tau protein in a biological sample, comprising:
 - (a) contacting a biological sample from a subject with a first antibody which
5 binds to an N-terminal portion of Tau 441 (SEQ ID NO: 1), and a second antibody which binds to a middle portion of Tau 441 (SEQ ID NO: 1), wherein the first antibody and the second antibody both bind to the Tau protein to form a complex; and
 - (b) detecting the level of the Tau protein in the complex.
2. The method of claim 1, wherein the first antibody binds to an N-
10 terminal portion of amino acid residues 9-158 of Tau 441 (SEQ ID NO: 1).
3. The method of claim 2, wherein the first antibody binds to amino acid residues 9-18 of Tau 441 (SEQ ID NO: 1).
4. The method of claim 1, wherein the second antibody binds to a middle portion of amino acid residues 159-231 of Tau 441 (SEQ ID NO: 1).
- 15 5. The method of claim 4, wherein the second antibody binds to amino acid residues 194-198, residues 159-163, the phosphorylated threonine residue 181, or the phosphorylated threonine residue 231 of Tau 441 (SEQ ID NO: 1).
6. The method of claim 1, wherein the first antibody is Tau12.
7. The method of claim 1, wherein the second antibody is selected from:
20 BT2, HT7, Tau5, AT270, and PHF6.
8. A method of detecting the level of at least one Tau protein in a biological sample, comprising:
 - (a) contacting a biological sample from a subject with a first antibody which
binds to amino acid residues 159-163 of Tau 441 (SEQ ID NO: 1), and a second
25 antibody which binds to amino acid residues 194-198, residues 218-225, or the phosphorylated threonine residue 231 of Tau 441 (SEQ ID NO: 1), wherein the first antibody and the second antibody both bind to the Tau protein to form a complex; and
 - (b) detecting the level of the Tau protein in the complex.

9. The method of claim 8, wherein the first antibody is HT7, and the second antibody is selected from BT2, Tau5, and PHF6.

10. The method of claim 1 or 8, wherein the Tau protein is phosphorylated, and the level of the phosphorylated Tau protein is detected.

5 11. The method of claim 1 or 8, wherein the first antibody or the second antibody is immobilized on a solid substrate.

12. The method of claim 1 or 8, wherein the first antibody or the second antibody comprises a label.

10 13. The method of claim 1 or 8, wherein the biological sample is cerebrospinal fluid (CSF), blood, serum or plasma.

14. The method of claim 1 or 8, wherein the subject has a Tau-related neurological disease.

15. The method of claim 14, wherein the Tau-related neurological disease is Alzheimer's disease.

15 16. A method of identifying a subject having or at risk of having a Tau-related neurological disease, comprising:

(a) isolating a biological sample from the subject;

(b) detecting the level of the Tau protein in the biological sample according to the method of claim 1 or 8; and

20 (c) comparing the level of the Tau protein to a reference; and

(d) if the level of the Tau protein is increased compared to the reference, identifying the subject to have or at risk of having a Tau-related neurological disease.

17. The method of claim 16, wherein the Tau-related neurological disease is Alzheimer's disease.

25 18. A method of identifying a subject having or at risk of having a Tau-related neurological disease, comprising:

(a) isolating a biological sample from the subject;

(b) detecting a first level of the Tau protein in the biological sample according to the method of claim 8, wherein the first antibody is HT7 and the second antibody is BT2;

(c) detecting a second level of the Tau protein in the biological sample
5 according to the method of claim 8, wherein the first antibody is HT7 and the second antibody is Tau5;

(d) determining the ratio of the first level from (b) versus the second level from (c);

(e) comparing the ratio to a reference; and

10 (f) if the ratio is decreased compared to the reference, identifying the subject to have or at risk of having a Tau-related neurological disease.

19. A method of identifying a subject having or at risk of having a Tau-related neurological disease, comprising:

(a) isolating a biological sample from the subject;

15 (b) detecting a first level of the Tau protein in the biological sample according to the method of claim 8, wherein the first antibody is HT7 and the second antibody is BT2;

(c) detecting a second level of the Tau protein in the biological sample according to the method of claim 1, wherein the first antibody is Tau12 and the
20 second antibody is HT7;

(d) determining the ratio of the first level from (b) versus the second level from (c);

(e) comparing the ratio to a reference; and

25 (f) if the ratio is decreased compared to the reference, identifying the subject to have or at risk of having a Tau-related neurological disease.

20. The method of claim 18 or 19, wherein the Tau-related neurological disease is Alzheimer's disease.

21. A kit comprising: (1) a first antibody which binds to an N-terminal portion of a Tau protein; (2) a second antibody which binds to a middle portion of a

Tau protein; and (3) reagents necessary for facilitating an antibody-antigen complex formation.

22. The kit of claim 21, wherein the first antibody is Tau12, and the second antibody is selected from: BT2, HT7, Tau5, AT270, and PHF6.

5 23. A kit comprising: (1) a first antibody which binds to amino acid residues 159-163 of Tau 441 (SEQ ID NO: 1); (2) a second antibody which binds to amino acid residues 194-198, residues 218-225, or the phosphorylated threonine residue 231 of Tau 441 (SEQ ID NO: 1); and (3) reagents necessary for facilitating an antibody-antigen complex formation.

10 24. The kit of claim 21, wherein the first antibody is HT7, and the second antibody is selected from BT2, Tau5, and PHF6.

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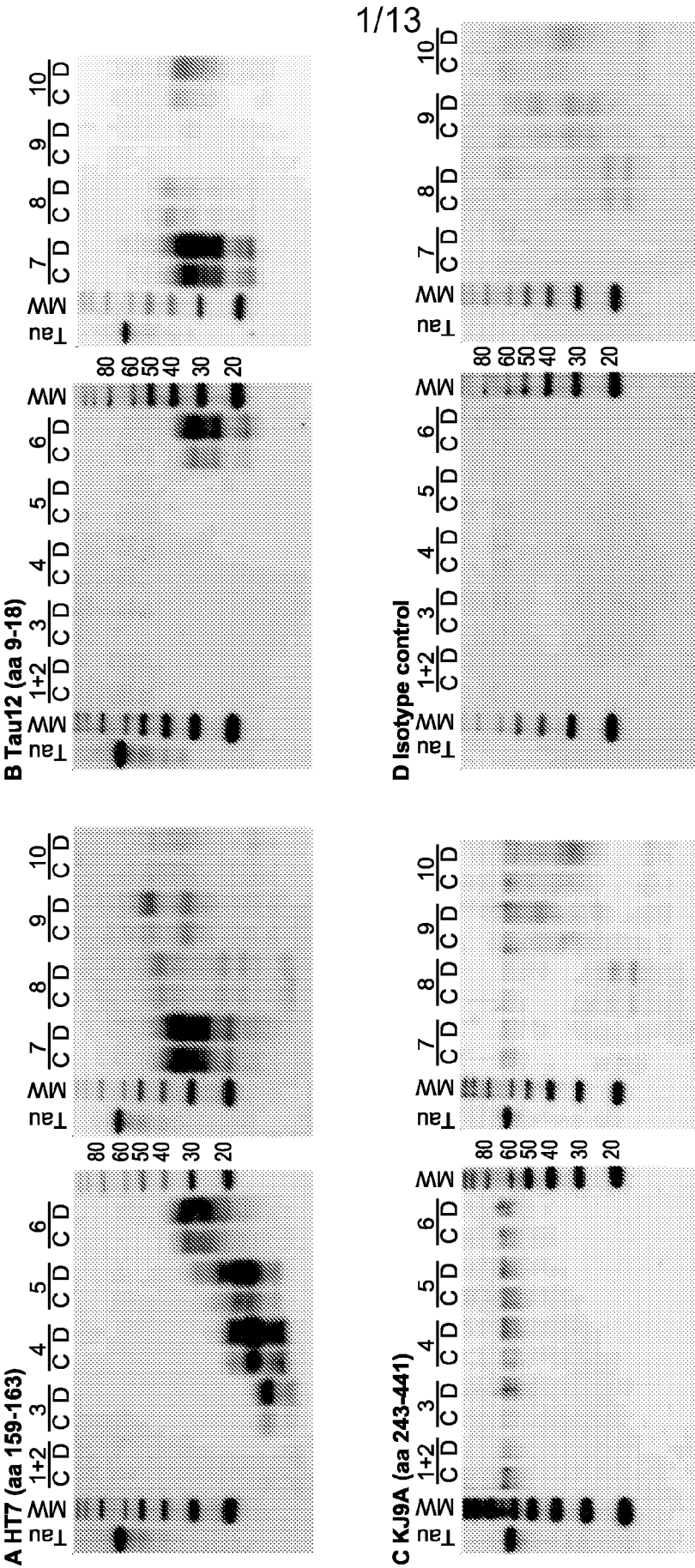
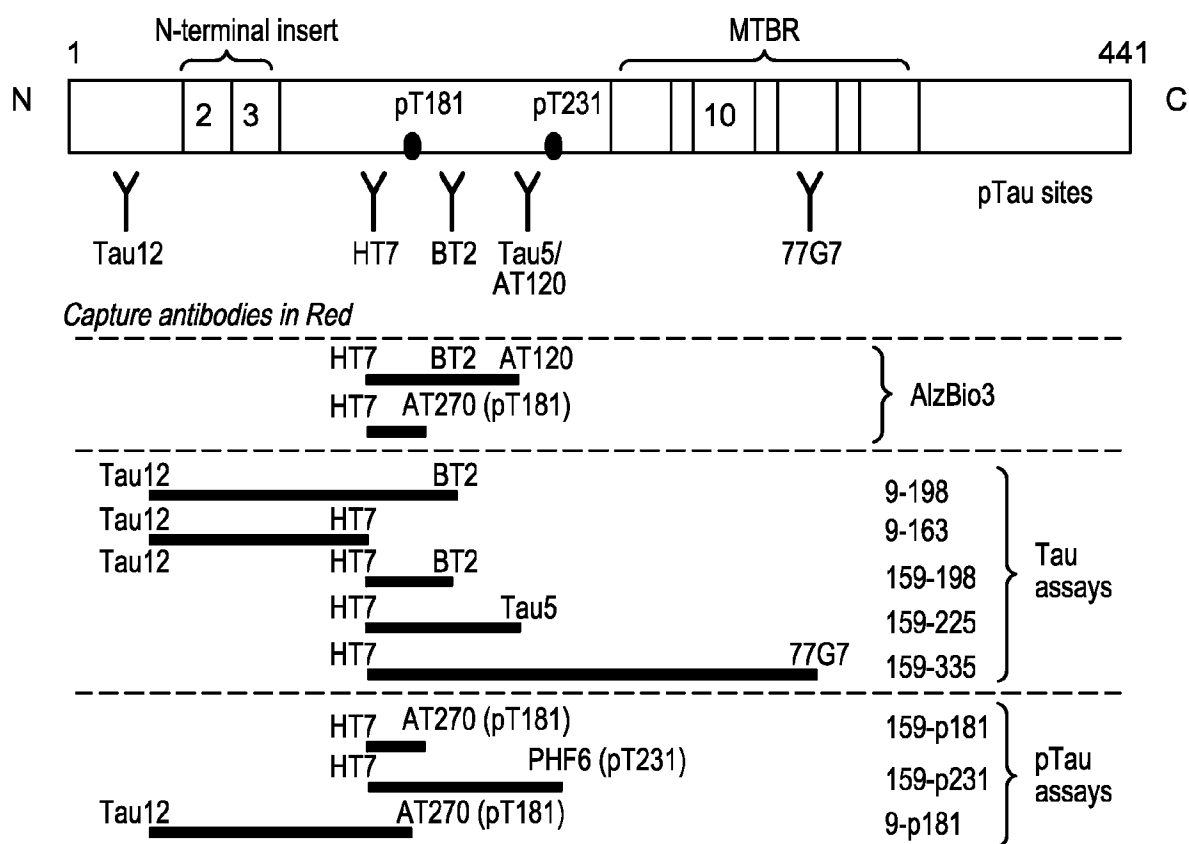
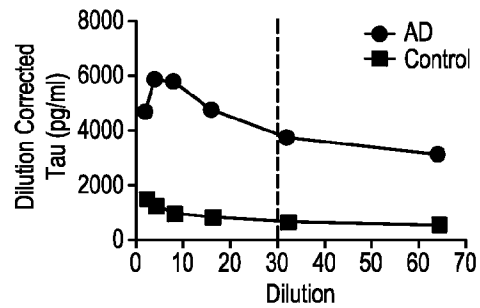
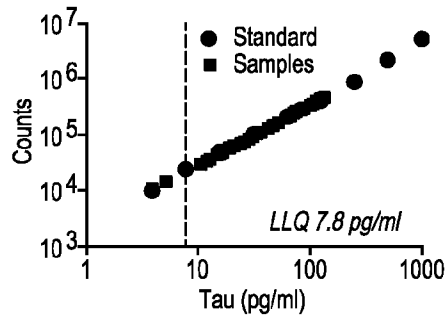
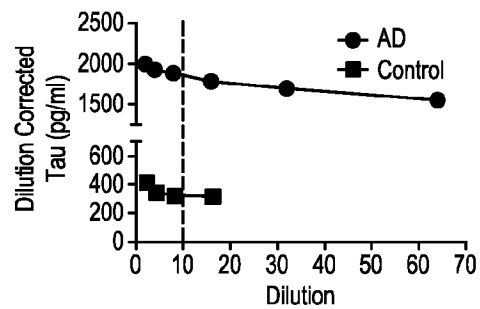
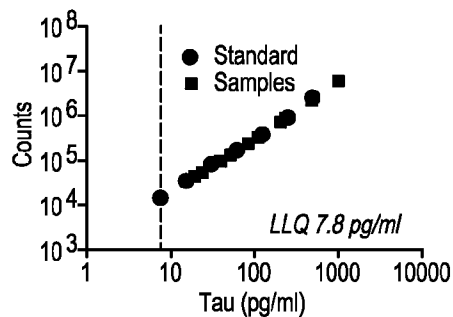
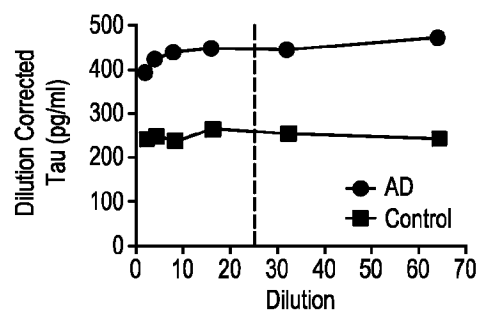
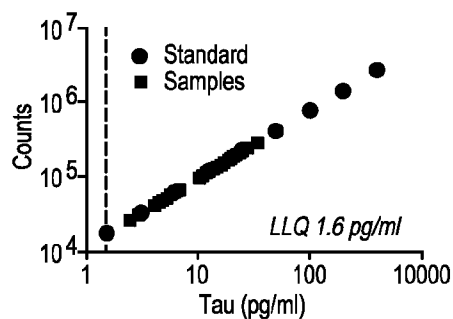
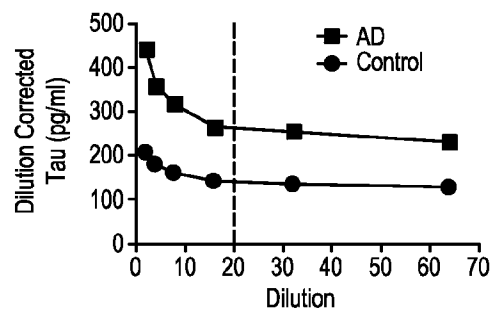
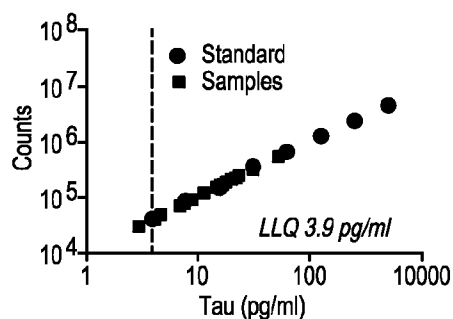


FIG. 1

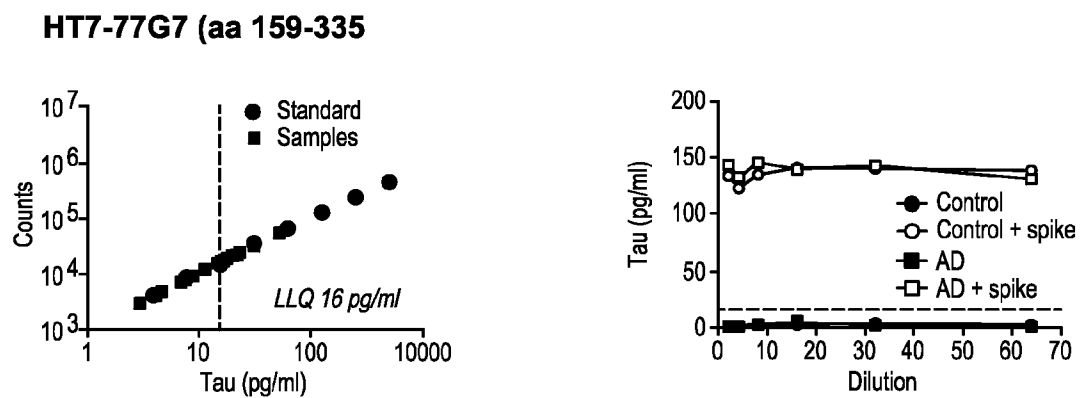
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**FIG. 2**

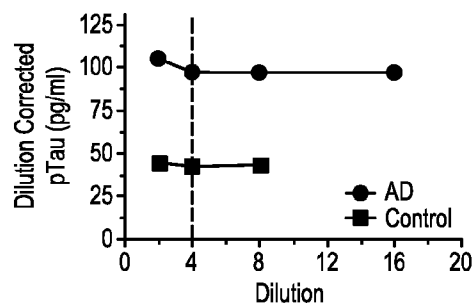
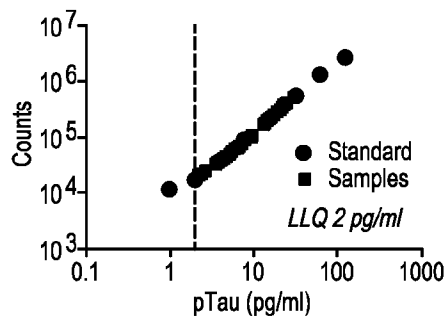
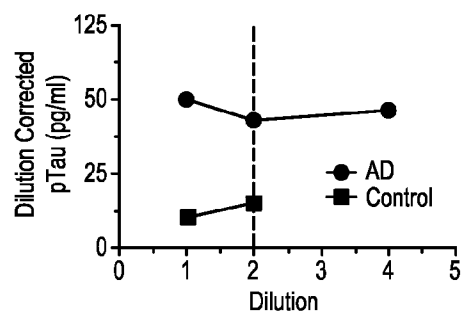
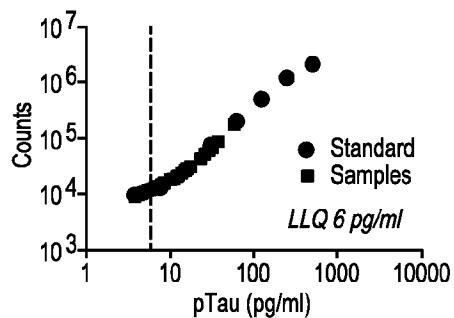
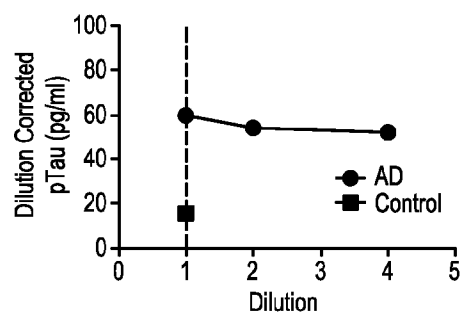
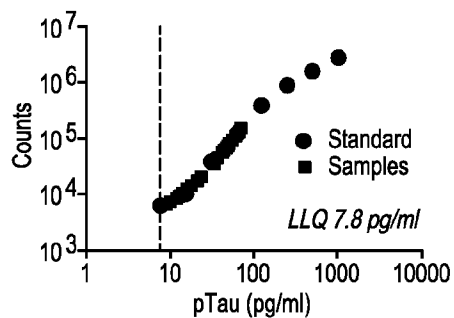
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A HT7-BT2 (aa 159-198)**B HT7-Tau5 (aa 159-225)****C Tau12-BT2 (aa 9-198)****D Tau12-HT7 (aa 9-163)****FIG. 3**

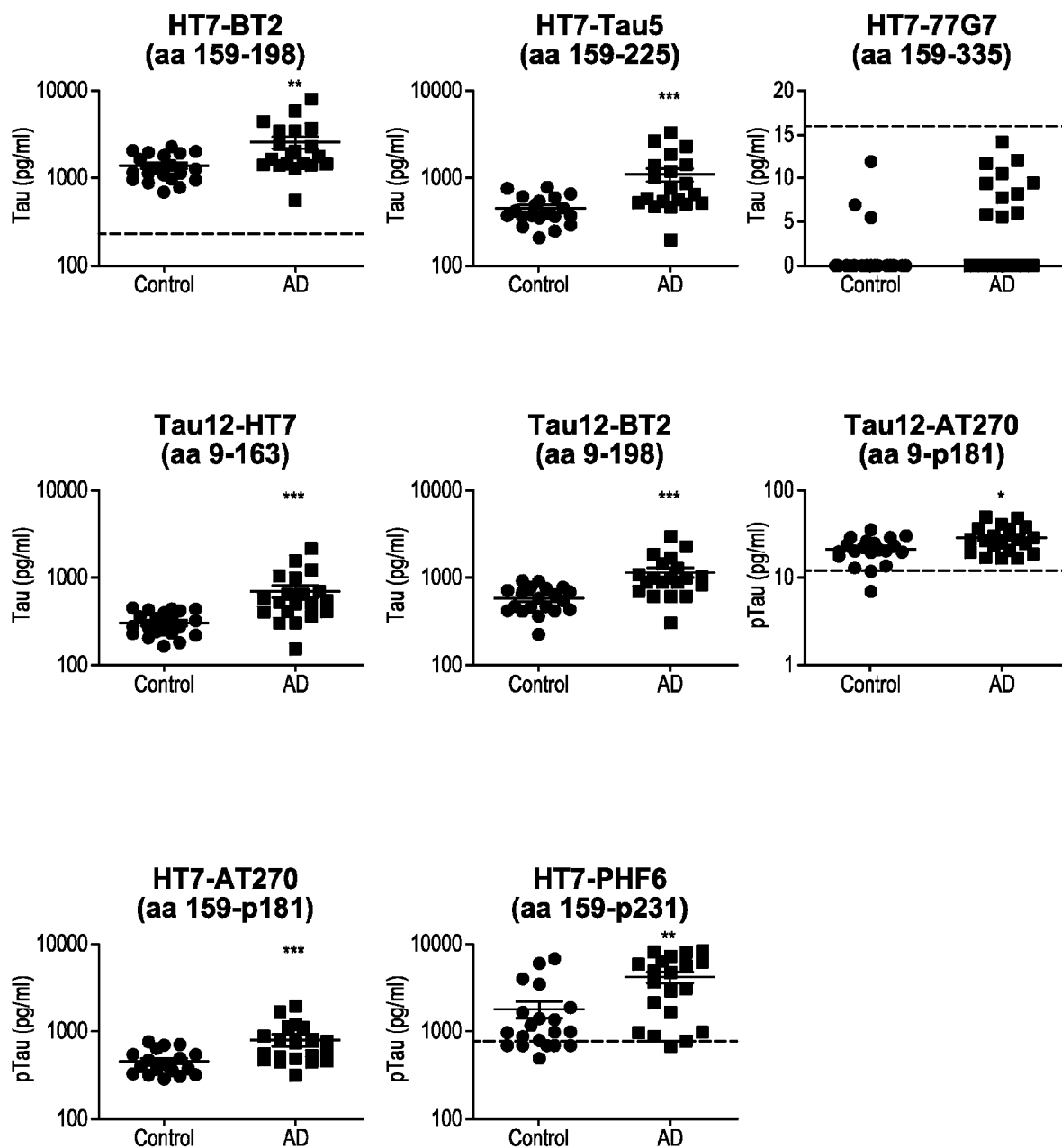
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**FIG. 4**

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A HT7-AT270 (aa 159-p181)**B Tau12-AT270 (aa 9-p181)****C Ht7-PHF (aa 159-p231)****FIG. 5**

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**FIG. 6**

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1-MAEPRQEFVMEDHAGTYGLGDRKDQGGYTMHQDQEGDTDAGLKESPLQTPTEDGSEEPGSETSD-651-KMAEPRQEFVMEDHAGTYGL

2-KGTYGLGDRKDQGGYTMHQDQ

3-KMHQDQEGDTDAGLKESPLQT

4-KSPLQTPTEDGSEEPGSETSD

66-AKSTPTAEDVTAPLVDEGAPGKQAAQPHTEIPEGTTAEEAGTGDTPSLEDEAAGHVTQA-125

5-KSETSDAKSTPTAEDVTAPLV

6-KTAPLVDEGAPGKQAAQPH

7-KAQPHTEIPEGTTAEEAGIGD

8-KAGIGDTPSLEDEAAGHVTQA

126-RMVSKSKDGTGSDDKKAKGADGKTKIATPRGAAPPGQKGQANATRIPAKTPPAPKTPPSS-185

9-KHVTQARMVSKSKDGTGSDDK

10-KGSDDKKAKGADGKTKIATPR

11-KIATPRGAAPPGQKGQANATR

12-KANATRIPAKTPPAPKTPPSS

186-GEPPKSGDRSGYSSPGSPGTPGSRRTPSLPTPTREPCKVAVV RTPPKSPSSAKSRLQT-245

13-KTPPSSGEPPKSGDRSGYSSP

14-KGYSSPGSPGTPGSRRTPSL

15-KRTPSLPTPTREPCKVAVVR

16-KVAVVRTPPKSPSSAKSRLQT

246-APVMPDLKNVSKIGSTENLKHQPGGGKVQIINKKLDLSNVQSKCGSKDNIKHVPGGSS-305

17-KSRLQTAPVMPDLKNVSKI

18-KVSKIGSTENLKHQPGGGKV

19-KGGGKVQIINKKLDLSNVQSK

20-KNVQSKCGSKDNIKHVPGGSS

306-VQIVYKPVDLISKVTSKCGSLGNIHHKPGGGQVEVKSEKLDFKDRVQSKIGSLDNITHVPG-365

21-KPGGGSVQIVYKPVDLISKVTS

22-KSKVTSKCGSLGNIHHKPGGG

23-KKPGGGQVEVKSEKLDFKDRV

24-KFKDRVQSKIGSLDNITHVPG

366-GGNKKIETHKLTFRENAKAKTDHGAEIVYKSPVVS~~GD~~TSRHLNSVSTGSIDMVDSPQLATLADEVASLAKQGL-441

25-KTHVPGGGNKKIETHKLTFR

26-KLTFRENAKAKTDHGAEIVYK

27-KEIVYKSPVVS~~GD~~TSRHLNS

28-KRHLNSVSTGSIDMVDSPQL

29-KDSPQLATLADEVASLAKQGL

FIG. 7

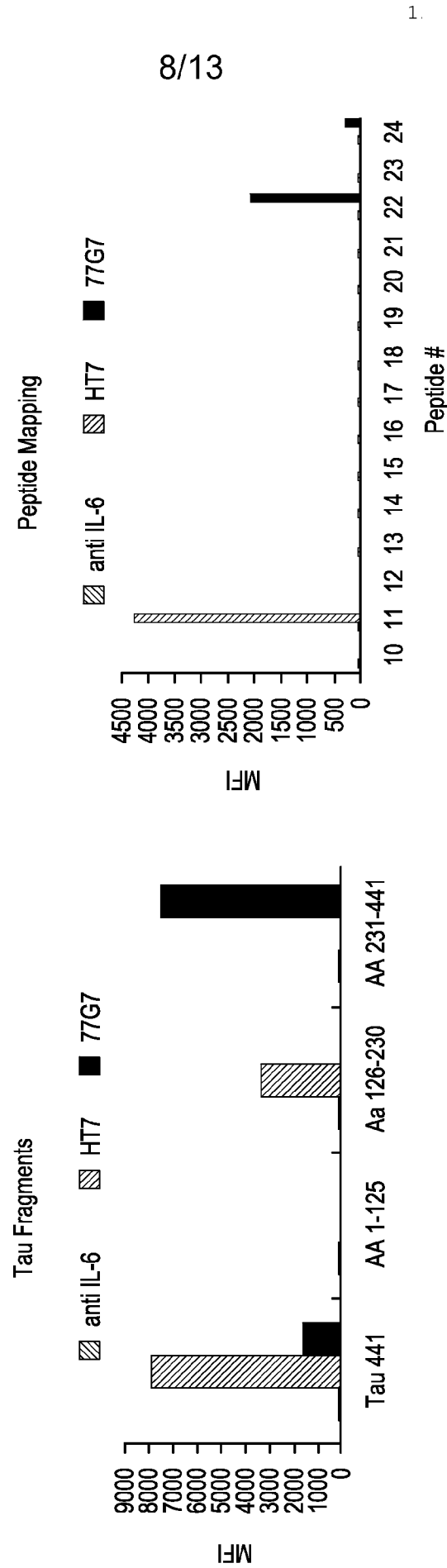
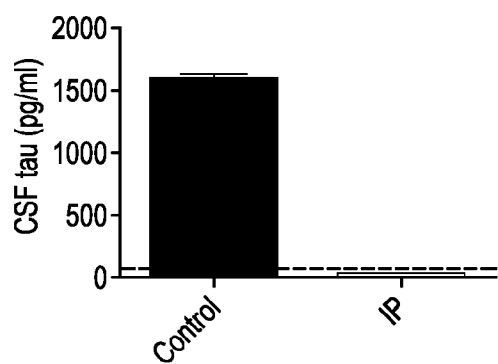
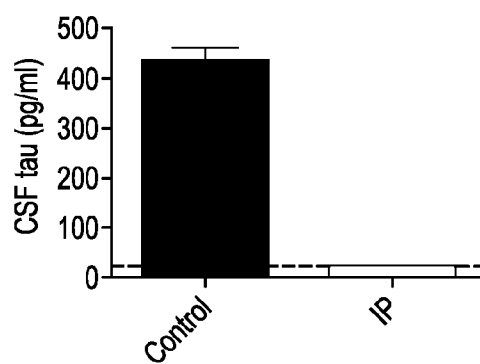
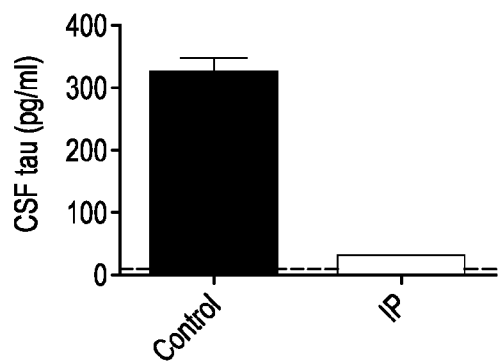
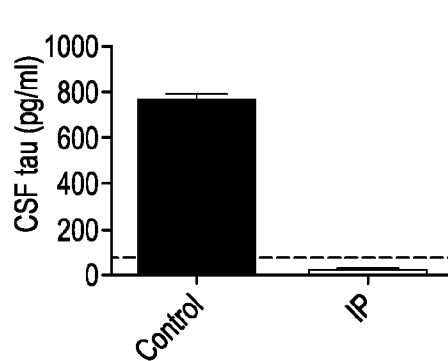
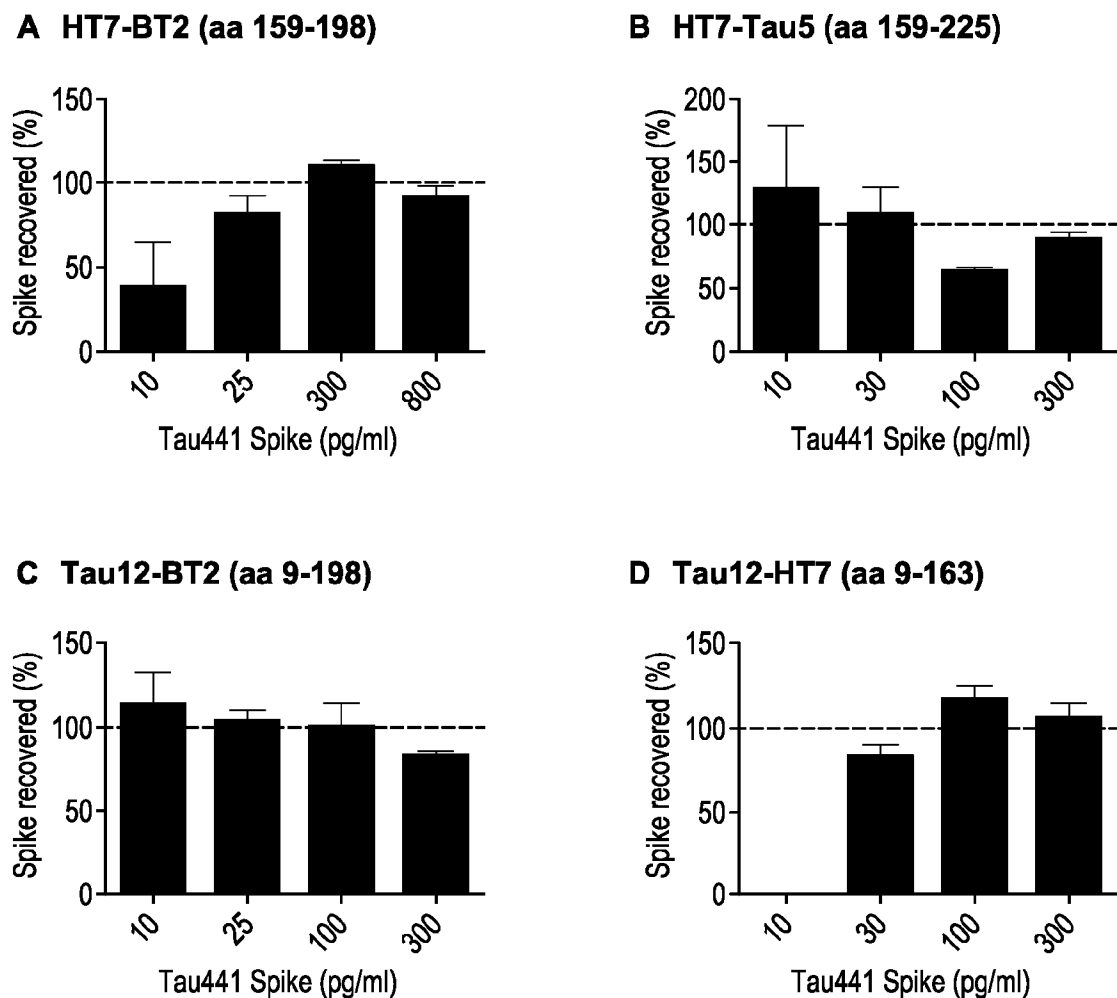


FIG. 8

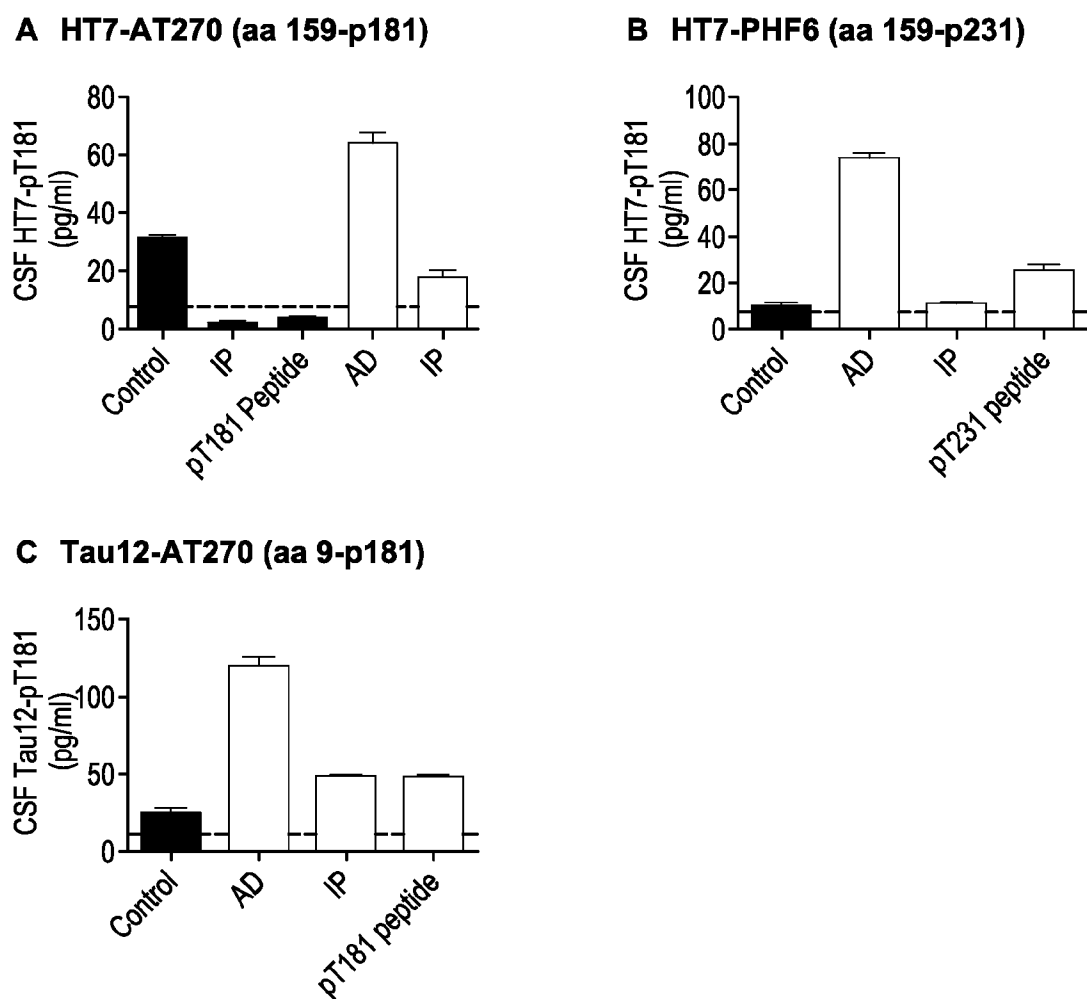
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A HT7-BT2 (aa 159-198)**B HT7-Tau5 (aa 159-225)****C Tau12-BT2 (aa 9-198)****D Tau12-HT7 (aa 9-163)****FIG. 9**

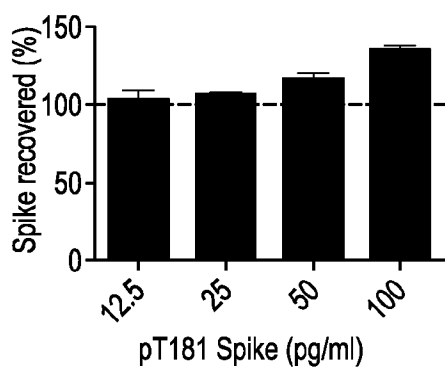
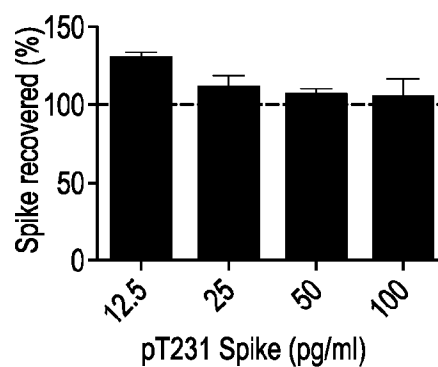
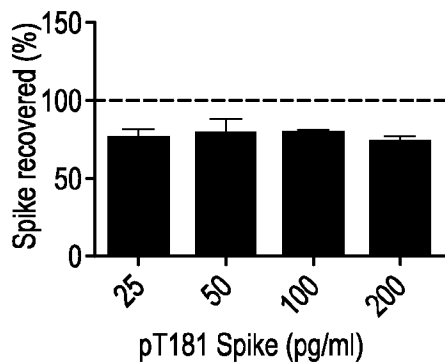
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**FIG. 10**

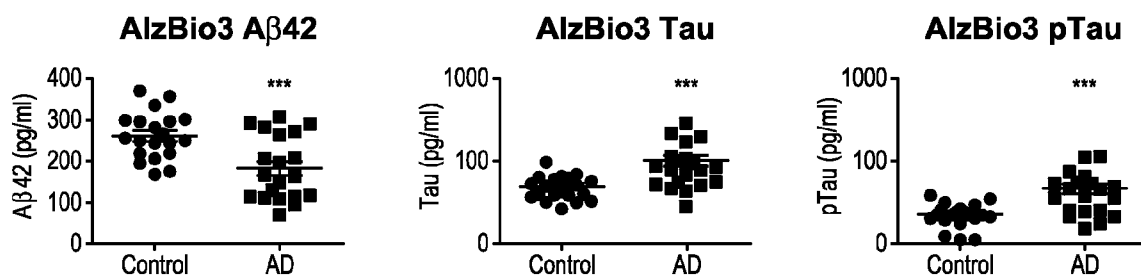
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**FIG. 11**

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A HT7-AT270 (aa 159-p181)**B HT7-PHF6 (aa 159-p231)****C Tau12-AT270 (aa 9-p181)****FIG. 12**

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**FIG. 13**

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2013/050242

A. CLASSIFICATION OF SUBJECT MATTER
INV. G01N33/68
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, BIOSIS, COMPENDEX, EMBASE, INSPEC, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	BARTEN DONNA M ET AL: "Tau Transgenic Mice as Models for Cerebrospinal Fluid Tau Biomarkers", JOURNAL OF ALZHEIMER'S DISEASE,, vol. 24, no. Suppl. 2, Sp. Iss. SI, 1 January 2011 (2011-01-01), pages 127-141, XP009174406, ISSN: 1387-2877	1-9, 11-17, 21-24
Y	abstract page 129, column 2, paragraph 2 - page 130, column 1, paragraph 1 figures 1,2 ----- -/-	10,18-20



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

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Date of the actual completion of the international search

21 November 2013

Date of mailing of the international search report

09/12/2013

Name and mailing address of the ISA/

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Authorized officer

Thumb, Werner

INTERNATIONAL SEARCH REPORT

International application No

PCT/US2013/050242

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 1 535 930 A1 (MITSUBISHI CHEM CORP [JP]; MITSUBISHI KAGAKU IATRON INC [JP] MITSUBISHI) 1 June 2005 (2005-06-01) abstract paragraphs [0011], [0012], [0016]; example 2 -----	1,2,4,5, 7,11-17, 21
X	S. SAMAN ET AL: "Exosome-associated Tau Is Secreted in Tauopathy Models and Is Selectively Phosphorylated in Cerebrospinal Fluid in Early Alzheimer Disease", JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 287, no. 6, 4 November 2011 (2011-11-04), pages 3842-3849, XP055089563, ISSN: 0021-9258, DOI: 10.1074/jbc.M111.277061	21,22
Y	abstract page 3843, column 2, paragraph 2 - page 3844, column 1, paragraph 1 figure 1a -----	1-7, 10-17, 19,20
Y	XIAO-SHU WANG ET AL: "Apolipoprotein E (ApoE) Peptide Regulates Tau Phosphorylation Via Two Different Signaling Pathways", JOURNAL OF NEUROSCIENCE RESEARCH, vol. 51, no. 5, 1 March 1998 (1998-03-01), pages 658-665, XP55008909, ISSN: 0360-4012, DOI: 10.1002/(SICI)1097-4547(19980301)51:5<658: AID-JNR13>3.0.CO;2-Z abstract page 659, column 2, paragraph 3 page 660, column 1, paragraph 2 -----	5,7-9, 21-24
Y	N GHOSHAL: "Tau Conformational Changes Correspond to Impairments of Episodic Memory in Mild Cognitive Impairment and Alzheimer's Disease", EXPERIMENTAL NEUROLOGY, vol. 177, no. 2, 1 October 2002 (2002-10-01), pages 475-493, XP055089574, ISSN: 0014-4886, DOI: 10.1006/exnr.2002.8014 abstract table 1 page 479, column 1, paragraph 4 page 480, column 2, paragraphs 3,4 ----- -/--	1-9, 11-24

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2013/050242

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5 811 310 A (GHANBARI HOSSEIN A [US] ET AL) 22 September 1998 (1998-09-22) abstract example 1; table A -----	1-24
Y	WO 2011/154321 A1 (CONSIGLIO NAZIONALE RICERCHE [IT]; AMADORO GIUSEPPINA [IT]; CALISSANO) 15 December 2011 (2011-12-15) abstract page 18, line 20 - page 19, line 28 page 5, line 22 - page 6, line 25 -----	1-24
Y	"KEY-CODE: INX49010 80317 INNOTEST PHOSPHO-TAU", 1 April 2010 (2010-04-01), XP055089613, Retrieved from the Internet: URL: http://www.bio-protech.com.tw/databank/DataSheet/PathoDiag/Innogenetics/user's manual/80317_Ptau_110704.pdf [retrieved on 2013-11-21] the whole document -----	8-10
A	BUEE L ET AL: "TAU PROTEIN ISOFORMS, PHOSPHORYLATION AND ROLE IN NEURODEGENERATIVE DISORDERS", BRAIN RESEARCH REVIEWS, ELSEVIER, NL, vol. 33, no. 1, 1 August 2000 (2000-08-01), pages 95-130, XP000974598, ISSN: 0165-0173, DOI: 10.1016/S0165-0173(00)00019-9 the whole document -----	1-24

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2013/050242

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☒ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 1-7, 21, 22(completely); 10-17, 19, 20(partially)

A method for detecting the level of at least one Tau protein comprising contacting the sample with antibody specific for the N-terminal portion of Tau 441 and an antibody specific for the middle portion of Tau 441. Kit comprising said antibodies.

2. claims: 8, 9, 18, 23, 24(completely); 10-17, 19, 20(partially)

A method for detecting the level of at least one Tau protein comprising contacting the sample with two antibodies specific for the middle portion of Tau 441. Kit comprising said antibodies.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/US2013/050242

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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			EP 1535930 A1 01-06-2005
			ES 2352668 T3 22-02-2011
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