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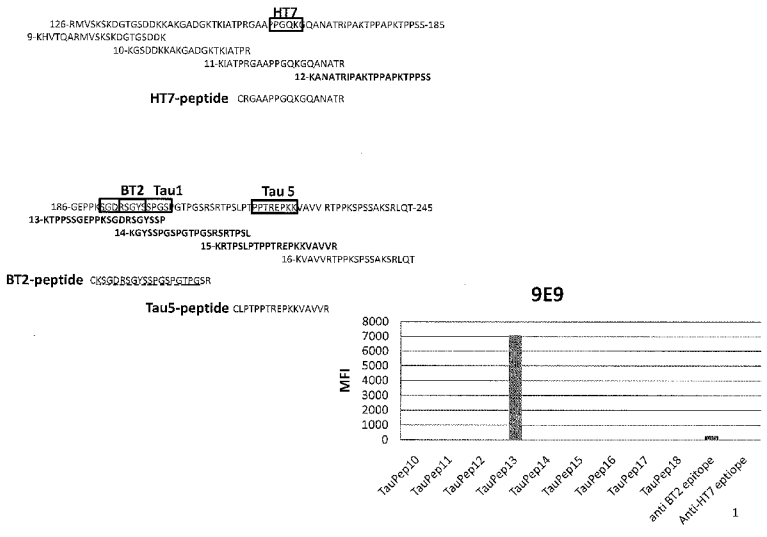
Figure 8: Screening Summary-Peptide overlay



(57) Abstract: The present invention provides novel peptides, compositions, and methods for creating quantitative novel compositions, as well as methods for creating quantitative standards to calibrate analytes. These peptides, compositions, and methods enable the creation of standards and calibrators for analyzing analytes and measuring clinical biomarkers (e.g., Tau). Also provided are kits comprising the peptides or compositions described herein, for use in assays (e.g., sandwich immunoassays).

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IMMUNOASSAY STANDARDS AND MEASUREMENT OF TAU USING INTRA-ASSAY CALIBRATION STANDARDS

Cross-Reference to Related Applications

This application claims priority to and the benefit of U.S. provisional patent application number 61/745,177, filed December 21, 2012, the entire contents of which are specifically incorporated by reference herein.

Field of the Invention

The invention relates to peptides and modified peptides that can be used as reference standards and calibrators to measure clinical biomarkers (*e.g.,* Tau) in an immunoassay, as well as methods of measuring the quantity of Tau in a biological sample.

Background of the Invention

Microtubule associated protein Tau (Tau) is a structural protein found in neuronal cells. There are six isoforms of Tau in the human brain resulting from RNA splicing (Himmler, Drechsel et al., 1989). These isoforms differ from one another in having three or four microtubule binding repeats (R) of 31-32 amino acids each in the C-terminal domain, and two, one or none amino terminal inserts (N) of 29 amino acids each (Goedert, Spillantini et al., 1989). Tau can be modified by phosphorylation, glycosylation, and other modifications (Hampel, Blennow et al., 2010). Tau contains repeated C-terminal microtubule binding domains and variable N-terminal domains and differences in the number and type of repeating domains results in the six isoforms (Himmler, Drechsel et al., 1989; Avila, Lucas et al., 2004; Hampel, Blennow et al., 2010). The sequence for the core domain of Tau is identical between the six isoforms and the epitopes used in the design of the present invention are all contained in the core domain, allowing these peptides to represent all six isoforms of Tau (Figure 1). Tau is an intrinsically disordered protein consisting of random coils with a global hairpin fold (Friedhoff, von Bergen et al., 2000; Jeganathan, von Bergen et al., 2006; Mukrasch, Bibow et al., 2009).

Alzheimer's Disease (AD) is a progressive neurodegenerative disorder that causes dementia. Neurofibrillary tangles (NFT) are found in brain lesions of AD patients and consist of hyperphosphorylated forms of Tau (Hampel, Blennow et al. 2010). Tau exists at elevated levels in the cerebrospinal fluid (CSF) of AD patients and along with phosphorylated Tau (pTau) and $A\beta_{1-42}$, has been shown to be a marker for progression from

Mild Cognitive Impairment to Alzheimer's disease (see Avila, Lucas et al., 2004; Mattsson and Zetterberg, 2009; Mattsson, Zetterberg et al., 2009; Hampel, Blennow et al., 2010; and Hampel, Frank et al. 2010).

Tau exists as a heterogeneous mixture in CSF. Multiple isoforms are present and each can be modified by phosphorylation, glycosylation, and digestion (see Goedert, Spillantini et al.,1989; Portelius, Hansson et al., 2008; Hampel, Blennow, et al. 2010; and Hanisch, Soininen et al., 2010). The epitopes used to identify Tau as an AD biomarker in CSF exist in the central domain of Tau that is shared between all six Tau isoforms (see Hampel, Blennow et al., 2010; and Hampel, Frank et al., 2010) (Figure 1). The core domain of Tau has been shown to exist in CSF as a part of proteolytically cleaved fragments that are extensively modified (see Johnson, Seubert et al., 1997; Portelius, Hansson et al., 2008; and Hanisch, Soininen et al., 2010).

Brief Summary of the Invention

Provided herein are isolated peptides and modified peptides which can be used (e.g., as reference standards) to identify and/or measure levels of Tau in a biological sample.

Representative peptides of the invention include peptides having the amino acid sequence set forth in SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, or SEQ ID NO:10.

Representative modified peptides of the invention include modified peptides which comprise an N-terminal immunoreactive region, a C-terminal immunoreactive region and a linker region, wherein the immunoreactive regions are defined by particular amino acid sequences. In one embodiment, the modified peptide comprises an N-terminal immunoreactive region, a C-terminal immunoreactive region, and a linker region, wherein:

(a) the N-terminal immunoreactive region comprises or has an amino acid sequence selected from the group consisting of SEQ ID NOs: 7, 8, 9, and 10; and/or

(b) the C-terminal immunoreactive region comprises or has an amino acid sequence selected from the group consisting of SEQ ID NOs: 7, 8, 9, and 10,

and wherein the N-terminal and C-terminal immunoreactive regions are different amino acid sequences.

In another embodiment, the modified peptide comprises N-terminal and C-terminal immunoreactive regions comprising or having amino acid sequences selected from the group consisting of SEQ ID NOs:7 and 9, SEQ ID NOs:7 and 8, SEQ ID NOs:8 and 9, and SEQ ID NOs:9 and 10, respectively. In another embodiment, the N-terminal and C-terminal immunoreactive regions comprise or have the amino acid sequences set forth in SEQ ID

NOs:7 and 9, respectively. In a particular embodiment, the modified peptide is: KSGDRSGYSSPGSPGTPGSR-Generic Linker-LPTPPTREPKKVAVVR (SEQ ID NO: 16). In another embodiment, the modified peptide is: RGAAPPGQKGQANATR-Generic Linker-KSGDRSGYSSPGSPGTPGSR (SEQ ID NO:17), RGAAPPGQKGQANATR-Generic Linker –LPTPPTREPKKVAVVR (SEQ ID NO:18), or KTPPSSGEPPKSGDRS-Generic Linker-LPTPPTREPKKVAVVR (SEQ ID NO:19).

In another embodiment, the modified peptide comprises or has an N-terminal immunoreactive region, a C-terminal immunoreactive region, and a linker region, wherein:

(a) the N-terminal immunoreactive region comprises or has the amino acid sequence set forth in SEQ ID NO:7 and the C-terminal immunoreactive region comprises or has the amino acid sequence set forth in SEQ ID NO:9

(b) the N-terminal immunoreactive region comprises or has the amino acid sequence set forth in SEQ ID NO:8 and the C-terminal immunoreactive region comprises or has the amino acid sequence set forth in SEQ ID NO:7;

(c) the N-terminal immunoreactive region comprises or has the amino acid sequence set forth in SEQ ID NO:8 and the C-terminal immunoreactive region comprises or has the amino acid sequence set forth in SEQ ID NO:9;

(d) the N-terminal immunoreactive region comprises or has the amino acid sequence set forth in SEQ ID NO:10 and the C-terminal immunoreactive region comprises or has the amino acid sequence set forth in SEQ ID NO:9;

(e) the N-terminal immunoreactive region comprises or has the amino acid sequence set forth in SEQ ID NO:7 and the C-terminal immunoreactive region comprises the amino acid sequence set forth in SEQ ID NO:8;

(f) the N-terminal immunoreactive region comprises or has the amino acid sequence set forth in SEQ ID NO:9 and the C-terminal immunoreactive region comprises or has the amino acid sequence set forth in SEQ ID NO:7;

(g) the N-terminal immunoreactive region comprises or has the amino acid sequence set forth in SEQ ID NO:9 and the C-terminal immunoreactive region comprises the amino acid sequence set forth in SEQ ID NO:8;

(h) the N-terminal immunoreactive region comprises or has the amino acid sequence set forth in SEQ ID NO:12 and the C-terminal immunoreactive region comprises the amino acid sequence set forth in SEQ ID NO:14;

(i) the N-terminal immunoreactive region comprises or has the amino acid sequence set forth in SEQ ID NO:12 and the C-terminal immunoreactive region comprises or has the amino acid sequence set forth in SEQ ID NO:11;

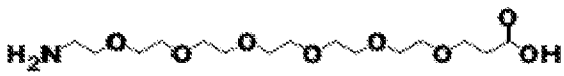
(j) the N-terminal immunoreactive region comprises or has the amino acid sequence set forth in SEQ ID NO:13 and the C-terminal immunoreactive region comprises or has the amino acid sequence set forth in SEQ ID NO:15; or

(k) the N-terminal immunoreactive region comprises or has the amino acid sequence set forth in SEQ ID NO:15 and the C-terminal immunoreactive region comprises or has the amino acid sequence set forth in SEQ ID NO:14.

In a particular embodiment, the N-terminal and C-terminal immunoreactive regions comprise or have the amino acid sequences set forth in SEQ ID NOs: 7 and 9, respectively.

Also provided are compositions (*e.g.*, pharmaceutical compositions) comprising the peptides or modified peptides described herein, optionally with a carrier.

Any suitable linker region can be included in the modified peptides described herein. Exemplary linkers include, but are not limited to, polyethylene glycol, a glutamic acid residue, a glycine residue, a serine residue, an alanine residue, a lysine residue, a lipid, a globular protein, a nucleic acid (including but not limited to DNA, RNA, and PNA), and an alkyl chain. In one embodiment, the linker region comprises a PEG6 linker, e.g., having the following structure:



In another embodiment, the linker region comprises or has the amino acid sequence set forth in SEQ ID NO:40 (GGSGGS). In another embodiment, the linker region comprises a non-immunoreactive domain.

Also provided are methods of measuring the quantity of Tau (*e.g.*, Tau 352, Tau 381, Tau 383, Tau 410, Tau 412, or Tau 441) in a biological sample. In one embodiment, the method comprises:

a) attaching a reference standard to at least two beads thereby forming a first bead set and a second bead set, wherein the reference standard comprises an epitope recognized by a first detection antibody and wherein each bead set comprises a different concentration of the reference standard;

b) attaching a capture antibody specific to Tau to a third bead set;

c) combining the bead sets together to form a suspension array;

d) applying the biological sample to the suspension array whereby Tau binds to the capture antibody on the third bead set;

e) adding a first detection antibody to the suspension array, wherein the first detection antibody binds the reference standard and Tau bound to the capture antibody;

f) measuring a first signal from the first detection antibody bound to the reference standard in the first bead set;

g) measuring a second signal from the first detection antibody bound to the reference standard in the second bead set;

h) generating a standard curve based upon the first and second signals; and

i) quantitating the amount of Tau in the third bead set by measuring a third signal from the first detection antibody and comparing the third signal to the first and second signal measurements on the standard curve,

wherein the reference standard comprises any one of the modified peptides described herein.

Any suitable biological sample can be used in the methods described herein. In one embodiment, the biological sample is blood, serum, plasma, peripheral blood mononuclear cells, peripheral blood lymphocytes, tissue, or cerebrospinal fluid or cell culture supernatants from primary cell culture, tissue slices, or genetically engineered cell line samples.

In one embodiment, the method is performed in a multi-well plate, nitrocellulose filter, glass fiber or on a glass slide. In another embodiment, the first signal and second signal is a signal selected from the group consisting of phycoerytherin, Alexa 532, streptavidin-phycoerythrin, and streptavidin-Alexa 532. In another embodiment, the reference standard is covalently attached to the bead. In another embodiment, the capture antibody is covalently attached to the bead. In another embodiment, the covalent attachment is a carbodiimide bond.

In another aspect, the present invention provides a kit for conducting an immunoassay to detect Tau, the kit comprising any one or more of the peptides or modified peptides described herein, optionally with instructions for use in the methods described herein.

Brief Description of the Drawings

Figure 1 shows the six isoforms of Tau found in CSF.

Figure 2 shows the structure of an exemplary linker.

Figure 3 is a schematic of a sandwich immunoassay.

Figure 4 is a schematic of a VITROS® immunoassay.

Figure 5 is a schematic of a Luminex assay.

Figure 6 shows a typical standard curve obtained using a modified peptide calibrator, (native Tau amino acids (190-209)-(PEG)6-native Tau amino acids (215-230); SEQ ID NO:16)).

Figure 7 shows the stability of a modified peptide calibrator (native Tau amino acids (190-209)-(PEG)6-native Tau amino acids (215-230); SEQ ID NO:16)) at 2-8°C over a period of six months.

Figure 8A depicts the amino acid sequence of the middle region of the Tau protein (i.e., amino acids 126-245), as well as Peptides 9-16, which were created from this region to map the epitope of antibody 9E9. Figure 8B shows the epitope mapping data for antibody 9E9, as assessed by Luminex.

Figure 9 shows the epitope mapping data for antibody 9E9, as assessed by Biacore.

Detailed Description of the Invention

The invention relates to peptides, modified peptides, and compositions that can be used as reference standards and calibrators to measure clinical biomarkers (*e.g.*, Tau) in an immunoassay, as well as methods of measuring the quantity of Tau (*e.g.*, Tau352, Tau381, Tau 383, Tau 410, Tau 412, or Tau 441) in a biological sample. Specifically, the present invention is aimed at creating non-aggregating, non-precipitating, and non-surface-adhering peptide reference standards for Tau for use in immunoassay formats.

I. Definitions

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

As used herein, the term "Tau" refers to a microtubule associated protein found in neuronal cells. There are six isoforms of Tau in the human brain resulting from RNA splicing (Himmler, Drechsel et al. 1989). These isoforms differ from one another in having three or four microtubule binding repeats (R) of 31-32 amino acids each in the C-terminal domain, and two, one or none amino terminal inserts (N) of 29 amino acids each (Goedert, Spillantini et al. 1989). Tau can be modified by phosphorylation, glycosylation, and other modifications (Hampel, Blennow et al. 2010). Tau contains repeated C-terminal microtubule binding domains and variable N-terminal domains and differences in the number and type of repeating domains results in the six isoforms (Himmler, Drechsel et al. 1989; Avila, Lucas et

al. 2004; Hampel, Blennow et al. 2010). The sequence for the core domain of Tau is identical between the six isoforms and the epitopes used in the design of the present invention are all contained in the core domain, allowing these peptides to represent all six isoforms of Tau (Figure 1). The amino acid sequences of the six isoforms (Tau 441, Tau 352, Tau 381, Tau 383, Tau 410, and Tau 412) are set forth below in Table 1.

Table 1: Tau Peptide Sequences

Amino Acid Sequence	Description of Peptide/Modified Peptide Sequence
(SEQ ID NO: 1) MAEPRQEFEV MEDHAGTYGL GDRKDQGGYT MHQDQEGDTD AGLKESPLQT PTEDGSEEPG SETSDAKSTP TAEDVTAPLV DEGAPGKQAA AQPHTEIPEG TTAEEAGIGD TPSLEDEAAG HVTQARMVSK SKDGTGSDDK KAKGADGKTK IATPRGAAPP GQKGQANATR IPAKTPPAPK TPPSSGEPPK SGDRSGYSSP GSPGTPGSRS RTPSLPTPPT REPKKVAVVR TPPKSPSSAK SRLQTAPVPM PDLKNVKSKI GSTENLKHQP GGGKVQIINK KLDLSNVQSK CGSKDNIKHV PGGGSVQIVY KPVDLSKVTS KCGSLGNIHH KPGGGQVEVK SEKLDFKDRV QSKIGSLDNI THVPGGGNKK IETHKLTFRE NAKAKTDHGA EIVYKSPVVS GDTSPRHLSN VSSTGSIDMV DSPQLATLAD EVSASLAKQG L	Native Tau 441 amino acid sequence
(SEQ ID NO: 2) MAEPRQEFEV MEDHAGTYGL GDRKDQGGYT MHQDQEGDTD AGLKESPLQT PTEDGSEEPG SETSDAKSTP TAEDVTAPLV DEGAPGKQAA AQPHTEIPEG TTAEEAGIGD TPSLEDEAAG HVTQARMVSK SKDGTGSDDK KAKGADGKTK IATPRGAAPP GQKGQANATR IPAKTPPAPK TPPSSGEPPK SGDRSGYSSP GSPGTPGSRS RTPSLPTPPT REPKKVAVVR TPPKSPSSAK SRLQTAPVPM PDLKNVKSKI GSTENLKHQP GGGKVQIVYK PVDLSKVTSK CGSLGNIHHK PGGGQVEVKS EKLDFKDRVQ SKIGSLDNIT HVPGGGNKKI ETHKLTFREN AKAKTDHGAE IVYKSPVVSG DTSPRHLSNV SSTGSIDMVD SPQLATLADE VSASLAKQGL	Native Tau 410 amino acid sequence
(SEQ ID NO: 3) MAEPRQEFEV MEDHAGTYGL GDRKDQGGYT MHQDQEGDTD AGLKESPLQT PTEDGSEEPG SETSDAKSTP TAEAEEAGIG DTPSLEDEAA GHVTQARMVS KSKDGTGSDD KKAKGADGKT KIATPRGAAP PGQKGQANAT RIPAKTPPAP KTPPSSGEPP KSGDRSGYSS PGSPGTPGSR SRTPSLPTPP TREPKKVAVV RTPPKSPSSA KSRLQTAPVP MPDLKNVKSK IGSTENLKHQ PGGGKVQIIN KKLDLSNVQS KCGSKDNIKH	Native Tau 412 amino acid sequence

VPGGGSVQIV YKPVDLSKVT SKCGSLGNIH HKPGGGQVEV KSEKLDFKDR VQSKIGSLDN ITHVPGGGNK KIETHKLTFR ENAKAKTDHG AEIVYKSPVV SGDTSPRHLS NVSSTGSIDM VDSPQLATLA DEVSASLAKQ GL	
(SEQ ID NO: 4) MAEPRQEFEV MEDHAGTYGL GDRKDQGGYT MHQDQEGDTD AGLKESPLQT PTEDGSEEPG SETSDAKSTP TAEAEEAGIG DTPSLEDEAA GHVTQARMVS KSKDGTGSDD KKAKGADGKT KIATPRGAAP PGQKGQANAT RIPAKTPPAP KTPPSSGEPP KSGDRSGYSS PGSPGTPGSR SRTPSLPTPP TREPKKVAVV RTPPKSPSSA KSRLQTAPVP MPDLKNVKSK IGSTENLKHQ PGGGKVQIVY KPVDLSKVTS KCGSLGNIHH KPGGGQVEVK SEKLDFKDRV QSKIGSLDNI THVPGGGNKK IETHKLTFRE NAKAKTDHGA EIVYKSPVVS GDTSPRHLSN VSSTGSIDMV DSPQLATLAD EVSASLAKQG L	Native Tau 381 amino acid sequence
(SEQ ID NO: 5) MAEPRQEFEV MEDHAGTYGL GDRKDQGGYT MHQDQEGDTD AGLKAEEAGI GDTPSLEDEA AGHVTQARMV SKSKDGTGSD DKKAKGADGK TKIATPRGAA PPGQKGQANA TRIPAKTPPA PKTPPSSGEP PKSGDRSGYS SPGSPGTPGS RSRTPSLPTP PTREPKKVAV VRTPPKSPSS AKSRLQTAPV PMPDLKNVKS KIGSTENLKH QPGGGKVQII NKKLDLSNVQ SKCGSKDNIK HVPGGGSVQI VYKPVDLSKV TSKCGSLGNI HHKPGGGQVE VKSEKLDFKD RVQSKIGSLD NITHVPGGGN KKIETHKLTF RENAKAKTDH GAEIVYKSPV VSGDTSPRHL SNVSSTGSID MVDSPQLATL ADEVSASLAK QGL	Native Tau 383 amino acid sequence
(SEQ ID NO: 6) MAEPRQEFEV MEDHAGTYGL GDRKDQGGYT MHQDQEGDTD AGLKAEEAGI GDTPSLEDEA AGHVTQARMV SKSKDGTGSD DKKAKGADGK TKIATPRGAA PPGQKGQANA TRIPAKTPPA PKTPPSSGEP PKSGDRSGYS SPGSPGTPGS RSRTPSLPTP PTREPKKVAV VRTPPKSPSS AKSRLQTAPV PMPDLKNVKS KIGSTENLKH QPGGGKVQIV YKPVDLSKVT SKCGSLGNIH HKPGGGQVEV KSEKLDFKDR VQSKIGSLDN ITHVPGGGNK KIETHKLTFR ENAKAKTDHG AEIVYKSPVV SGDTSPRHLS NVSSTGSIDM VDSPQLATLA DEVSASLAKQ GL	Native Tau 352 amino acid sequence

As used herein, the term "antibody" is used in the broadest sense, and specifically covers monoclonal antibodies (including full length monoclonal antibodies or fragments thereof, such as Fab fragments and single chain antibodies), polyclonal antibodies, multispecific antibodies (*i.e.*, bispecific antibodies), and genetically engineered antibodies, including affinity matured antibodies. "Antibody" can also refer to an antibody or antibody

fragments fused to carrier proteins/organisms, such as phage or other display carriers, that have the same properties as isolated antibodies.

As used herein, the term "isolated", as used herein with reference to peptides, refers to a preparation of protein or protein complex (*e.g.*, modified peptide molecule) that is essentially free from contaminating proteins normally present with the protein or complex (*i.e.*, in the cellular milieu in which the protein or complex is found endogenously). Thus, an isolated protein complex is isolated from cellular components that normally would "contaminate" or interfere with the study of the peptide or complex in isolation. It is to be understood, however, that such an "isolated" complex may incorporate other proteins the modulation of which, by the protein or protein complex, is being investigated.

As used herein, the term "isolated" as also used herein with respect to nucleic acids, such as DNA or RNA, refers to molecules in a form which does not occur in nature. Moreover, an "isolated nucleic acid" is meant to include nucleic acid fragments which are not naturally occurring as fragments and would not be found in the natural state.

As used herein, the term "nucleic acid" refers to polynucleotide, such as deoxyribonucleic acid (DNA), and, where appropriate, ribonucleic acid (RNA). The term should also be understood to include, as equivalents, analogs of either RNA or DNA made from nucleotide analogs, and, as applicable to the embodiment being described, single-stranded (such as sense or antisense) and double-stranded polynucleotide. "Nucleic acid" can also refer to a peptide nucleic acid "PNA" or an artificially synthesized DNA or RNA.

As used herein, the term "peptides", "proteins", and "polypeptides" are used interchangeably herein. The term "purified protein" refers to a preparation of protein or proteins that are preferably isolated from or otherwise substantially free of other proteins normally associated with the protein(s) in a cell or cell lysate.

As used herein, the term "modified peptide" refers to a peptide that has been modified relative to the native sequence of that peptide. For example, a modification may include the removal of a deleterious domain or the addition of a linker within the native peptide sequence.

As used herein, the term "binding" refers to direct association between two molecules, due to, for example, covalent, electrostatic, hydrophobic, ionic, and/or hydrogen-bond interactions under physiological conditions. Likewise, "complex formation", between two or more polypeptides, refers to a direct association between polypeptides, due to, for example, covalent, electrostatic, hydrophobic, ionic, and/or hydrogen-bond interactions under physiological conditions.

As used herein, the term "domain" refers to a region of protein that comprises a particular structure and/or performs a particular function (e.g., microtubule binding domain, phosphorylation domain, *etc.*).

As used herein, the term "immunoreactive domain" refers to a region of protein that comprises a particular amino acid sequence that can be recognized by an antibody. This region includes amino acid sequences that contain modifications such as glycosylation, methylation, phosphorylation, or any other post-translational modifications known to one of ordinary skill in the art. Examples of amino acids that can be phosphorylated are tyrosine, serine, or threonine amino acids. An "immunoreactive domain" that includes amino acids that are phosphorylated would also be characterized as a phosphorylation domain. An "immunoreactive domain" also includes two or more regions of a protein that are in close proximity to one another in the protein's native folded state, which together comprise an antibody binding site.

As used herein, the term "immunoassay" refers to a biochemical test that utilizes one or more antibodies to measure the presence or concentration of an analyte (e.g., Tau) in a biological matrix. This assay can produce a measurable signal in response to a specific binding if an antibody to an immunoreactive domain of a specific protein or peptide.

II. Reference Standards

Provided herein are isolated peptides and modified peptides that can be used as reference standards and calibrators to measure clinical biomarkers (*e.g.*, Tau). In one embodiment, the reference standard comprises an isolated peptide (*e.g.*, an immunoreactive region). In a particular embodiment, the peptide has the amino acid sequence set forth in SEQ ID NO: 7, 8, 9, 10, 11, 12, or 13, as set forth below in Table 2. Phosphorylated regions of Tau can also be used to generate modified peptides for use as reference standards, e.g., peptides having the amino acid sequence set forth in SEQ ID NO: 14 or 15.

Table 2: Peptides/ Immunoreactive Regions

Amino Acid Sequence	Description
KSGDRSGYSSPGSPGTPGSR (SEQ ID NO:7)	native Tau amino acids (190-209)
RGAAPPGQKGQANATR (SEQ ID NO:8)	native Tau amino acids (155-170)
LPTPPTREPKKVAVVR	native Tau amino acids (215-230)

(SEQ ID NO:9)	
KTPPSSGEPPKSGDRS (SEQ ID NO:10)	native Tau amino acids (180-195)
SGDRSGYSSP (SEQ ID NO:11)	native Tau amino acids (191-200)
GAAPPGQKGQAN (SEQ ID NO:12)	native Tau amino acids (156-167)
IPAKTPPAPKT(PO_4)PPSSGEPPK (SEQ ID NO:13)	native Tau amino acids (171-190); amino acid T181 is phosphorylated
REPKKVAVVRT($P0_4$)PPKSPSSAK (SEQ ID NO:14)	native Tau amino acids (221-240); amino acid T231 is phosphorylated

*All amino acid numbers for the peptides correspond to the Tau 441 sequence.

In another embodiment, the reference standard is a modified peptide, *e.g.*, comprising a linker, a deletion, or substitution in an immunoreactive and/or non-immunoreactive domain. For example, in one embodiment, the modified peptide comprises an N-terminal immunoreactive region, a C-terminal immunoreactive region and a linker region, wherein the immunoreactive regions are defined by particular amino acid sequences. In another embodiment, the modified peptide comprises an N-terminal immunoreactive region, a C-terminal immunoreactive region, and a linker region, wherein:

(a) the N-terminal immunoreactive region comprises or has an amino acid sequence selected from the group consisting of SEQ ID NOs: 7, 8, 9, and 10; and/or

(b) the C-terminal immunoreactive region comprises or has an amino acid sequence selected from the group consisting of SEQ ID NOs: 7, 8, 9, and 10,

and wherein the N-terminal and C-terminal immunoreactive regions are different amino acid sequences.

In another embodiment, the modified peptide comprises N-terminal and C-terminal immunoreactive regions comprising or having amino acid sequences selected from the group consisting of SEQ ID NOs:7 and 9, SEQ ID NOs:7 and 8, SEQ ID NOs:8 and 9, and SEQ ID NOs:9 and 10, respectively. In another embodiment, the N-terminal and C-terminal immunoreactive regions comprise or have the amino acid sequences set forth in SEQ ID NOs:7 and 9, respectively. In a particular embodiment, the modified peptide is: KSGDRSGYSSPGSPGTPGSR-Generic Linker-LPTPPTREPKKVAVVR (SEQ ID NO: 16). In another embodiment, the modified peptide is: RGAAPPGQKGQANATR-Generic Linker-KSGDRSGYSSPGSPGTPGSR (SEQ ID NO:17), RGAAPPGQKGQANATR-Generic

Linker –LPTPPTREPKKVAVVR (SEQ ID NO:18), or KTPPSSGEPPKSGDRS-Generic Linker-LPTPPTREPKKVAVVR (SEQ ID NO:19).

In another embodiment, the modified peptide comprises an N-terminal immunoreactive region, a C-terminal immunoreactive region, and a linker region, wherein:

(a) the N-terminal immunoreactive region comprises or has the amino acid sequence set forth in SEQ ID NO:7 and the C-terminal immunoreactive region comprises or has the amino acid sequence set forth in SEQ ID NO:9;

(b) the N-terminal immunoreactive region comprises or has the amino acid sequence set forth in SEQ ID NO:8 and the C-terminal immunoreactive region comprises or has the amino acid sequence set forth in SEQ ID NO:7;

(c) the N-terminal immunoreactive region comprises or has the amino acid sequence set forth in SEQ ID NO:8 and the C-terminal immunoreactive region comprises or has the amino acid sequence set forth in SEQ ID NO:9;

(d) the N-terminal immunoreactive region comprises or has the amino acid sequence set forth in SEQ ID NO:10 and the C-terminal immunoreactive region comprises or has the amino acid sequence set forth in SEQ ID NO:9;

(e) the N-terminal immunoreactive region comprises or has the amino acid sequence set forth in SEQ ID NO:7 and the C-terminal immunoreactive region comprises or has the amino acid sequence set forth in SEQ ID NO:8;

(f) the N-terminal immunoreactive region comprises or has the amino acid sequence set forth in SEQ ID NO:9 and the C-terminal immunoreactive region comprises or has the amino acid sequence set forth in SEQ ID NO:7;

(g) the N-terminal immunoreactive region comprises or has the amino acid sequence set forth in SEQ ID NO:9 and the C-terminal immunoreactive region comprises or has the amino acid sequence set forth in SEQ ID NO:8;

(h) the N-terminal immunoreactive region comprises or has the amino acid sequence set forth in SEQ ID NO:12 and the C-terminal immunoreactive region comprises or has the amino acid sequence set forth in SEQ ID NO:14;

(i) the N-terminal immunoreactive region comprises or has the amino acid sequence set forth in SEQ ID NO:12 and the C-terminal immunoreactive region comprises or has the amino acid sequence set forth in SEQ ID NO:11;

(j) the N-terminal immunoreactive region comprises or has the amino acid sequence set forth in SEQ ID NO:13 and the C-terminal immunoreactive region comprises or has the amino acid sequence set forth in SEQ ID NO:15; or

(k) the N-terminal immunoreactive region comprises or has the amino acid sequence set forth in SEQ ID NO:15 and the C-terminal immunoreactive region comprises or has the amino acid sequence set forth in SEQ ID NO:14.

In a particular embodiment, the N-terminal and C-terminal immunoreactive regions comprise or have the amino acid sequences set forth in SEQ ID NOs: 7 and 9, respectively.

In another embodiment, the modified peptide is shown below in Table 3:

Table 3: Modified Peptides/ Constructs

Amino Acid Sequence	Description of Peptide/Modified Peptide Sequence
KSGDRSGYSSPGSPGTPGSR-Generic Linker-LPTPPTREPKKVAVVR (SEQ ID NO:16)	native Tau amino acids (190-209)-(PEG)6-native Tau amino acids (215-230)
RGAAPPGQKGQANATR-Generic Linker-KSGDRSGYSSPGSPGTPGSR (SEQ ID NO:17)	native Tau amino acids (155-170)-(PEG)6-native Tau amino acids (190-209)
RGAAPPGQKGQANATR-Generic Linker -LPTPPTREPKKVAVVR (SEQ ID NO:18)	native Tau amino acids (155-170)-(PEG)6-native Tau amino acids (215-230)
KTPPSSGEPPKSGDRS-Generic Linker-LPTPPTREPKKVAVVR (SEQ ID NO:19)	native Tau amino acids (180-195) -(PEG)6-native Tau amino acids (215-230)
RGAAPPGQKGQANATR-(PEG)6-KSGDRSGYSSPGSPGTPGSR (SEQ ID NO:20)	native Tau amino acids (155-170)-(PEG)6-native Tau amino acids (190-209)
RGAAPPGQKGQANATRGGSGGSKSGDRSGYSSPGSPGTPGSR (SEQ ID NO:21)	native Tau amino acids (155-170)-GGSGGS-native Tau amino acids (190-209)
KSGDRSGYSSPGSPGTPGSR-(PEG)6-RGAAPPGQKGQANATR (SEQ ID NO:22)	native Tau amino acids (190-209)-(PEG)6-native Tau amino acids (155-170)
KSGDRSGYSSPGSPGTPGSR-GGSGGS-RGAAPPGQKGQANATR (SEQ ID NO:23)	native Tau amino acids (190-209)-GGSGGS-native Tau amino acids (155-170)
KSGDRSGYSSPGSPGTPGSR-(PEG)6-LPTPPTREPKKVAVVR (SEQ ID NO:24)	native Tau amino acids (190-209)-(PEG)6-native Tau amino acids (215-230)
KSGDRSGYSSPGSPGTPGSR-GGSGGS-LPTPPTREPKKVAVVR (SEQ ID NO:25)	native Tau amino acids (190-209)-GGSGGS-native Tau amino acids (215-230)
LPTPPTREPKKVAVVR-(PEG)6-KSGDRSGYSSPGSPGTPGSR (SEQ ID NO:26)	native Tau amino acids (215-230)-(PEG)6-native Tau amino acids (190-209)
LPTPPTREPKKVAVVR-GGSGGS-	native Tau amino acids (215-

Amino Acid Sequence	Description of Peptide/Modified Peptide Sequence
KSGDRSGYSSPGSPGTPGSR (SEQ ID NO:27)	230)-GGSGGS-native Tau amino acids (190-209)
RGAAPPGQKGQANATR-GGSGGS-LPTPPTREPKKVAVVR (SEQ ID NO:28)	native Tau amino acids (155-170)-GGSGGS-native Tau amino acids (215-230)
LPTPPTREPKKVAVVR-(PEG)6-RGAAPPGQKGQANATR (SEQ ID NO:29)	native Tau amino acids (215-230)-(PEG)6-native Tau amino acids (155-170)
LPTPPTREPKKVAVVR-GGSGGS-RGAAPPGQKGQANATR (SEQ ID NO:30)	native Tau amino acids (215-230)-GGSGGS-native Tau amino acids (155-170)
KSGDRSGYSSPGSPGTPGSR-Generic Linker-RGAAPPGQKGQANATR (SEQ ID NO:31)	native Tau amino acids (190-209)-(PEG)6-native Tau amino acids (155-170)
LPTPPTREPKKVAVVR-Generic Linker-KSGDRSGYSSPGSPGTPGSR (SEQ ID NO:32)	native Tau amino acids (215-230)-(PEG)6-native Tau amino acids (190-209)
RGAAPPGQKGQANATR-Generic Linker-LPTPPTREPKKVAVVR (SEQ ID NO:33)	native Tau amino acids (155-170)-(PEG)6-native Tau amino acids (215-230)
LPTPPTREPKKVAVVR-Generic Linker-RGAAPPGQKGQANATR (SEQ ID NO:34)	native Tau amino acids (215-230)-(PEG)6-native Tau amino acids (155-170)
GAAPPGQKGQAN-Generic Linker-REPKKVAVVRT(PO_4)PPKSPSSAK (SEQ ID NO:35)	native Tau amino acids (156-167)-generic linker-native Tau amino acids (221-240); amino acidT231 is phosphorylated
GAAPPGQKGQAN-Generic Linker-SGDRSGYSSP (SEQ ID NO:36)	native Tau amino acids (156-167)-generic linker-native Tau amino acids (191-200)
IPAKTPPAPKT(PO_4)PPSSGEPPK-Generic Linker- SGDRSGYSSP (SEQ ID NO:37)	native Tau amino acids (171-190)-generic linker-native Tau amino acids (191-200); amino acid T180 is phosphorylated
SGDRSGYSSP-Generic Linker-REPKKVAVVRT(PO_4)PPKSPSSAK (SEQ ID NO:38)	native Tau amino acids (191-200)-generic linker-native Tau amino acids (221-240); amino acid T231 is phosphorylated

*All amino acid numbers for the modified peptides correspond to the Tau 441 sequence.

The performance of the reference standards (e.g., immunoassay reference standards) or calibrators described herein should have comparable performance to native Tau in an immunoassay. Recombinant full length Tau proteins can be purchased commercially from a number of vendors as a catalog item (Signal Chem and rPeptide). Standard methods can be used to verify the abundance of the full length protein (Smith, Krohn et al., 1985). Additionally, standard methods can be used to verify the abundance of the full length construction from truncated species using mass spectrometry techniques, such as amino acid analysis, that are well known in the field (Kanu et al., 2008).

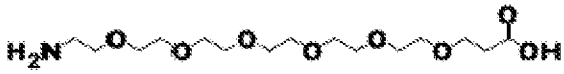
III. Linkers

Any suitable linker region can be used in the modified peptides described herein (e.g., to link two peptides or immunoreactive regions). In one embodiment, amino acids that do not aggregate are contemplated as the linker. In another embodiment, the amino acids that do not aggregate are in the form of a hydrophilic spacer. In another embodiment, a series of charged residues are used as a linker between the two immunoreactive regions. In another embodiment, any polymer with chemistry able to couple to amino acid residues is used as a linker or spacer. This polymer includes linear polymers, as well as those of known branched topology, such as dendrimers and branched co-polymers.

In one embodiment, the bond between the peptide backbone and linker comprises a covalent bond, avidin-biotin complex or any other stable bond. In another embodiment, the construct does not lead to self-aggregation or nonspecific absorption to laboratory plastics, in particular polypropylene, polystyrene, polycarbonate and other laboratory plastic resins of which pipette tips, tubes, plates and other vessels that hold fluids in which the analyte of interest can be measured.

Exemplary linkers include, but are not limited to, polyethylene glycol, a glutamic acid residue, a glycine residue, a serine residue, an alanine residue, a lysine residue, a lipid, a globular protein, a nucleic acid (including but not limited to DNA, RNA, and PNA), an alkyl chain, or any other linkage that adds to the stability of the two peptides of interest in the immunoassay.

In one embodiment, various forms of polyethylene glycol (PEG) are used as a linker. In a particular embodiment, PEG6 is used to join two peptides. In one embodiment, the linker region comprises a PEG6 linker, e.g., having the following structure:



In another embodiment, the linker region comprises or has the amino acid sequence set forth in SEQ ID NO:40 (GGSGGS). In another embodiment, the linker region is a non-immunoreactive domain.

IV. Methods

Provided herein are methods for generating a peptide, modified peptide, or compositions thereof, as well as methods of using the same as a reference standard or calibrator (e.g., in an immunoassay) to measure the abundance of an analyte (e.g., Tau 352, Tau 381, Tau 383, Tau 410, Tau 412, or Tau 441) in a biological sample (e.g., blood, serum, plasma, peripheral blood mononuclear cells, peripheral blood lymphocytes tissue, cerebrospinal fluid or cells). In one aspect, the invention relates to methods of measuring the clinical markers (e.g., Tau) with the reference standards of the invention using, for example, an immunoassay.

An immunoassay often requires biologically specific capture reagents, such as antibodies, to capture the analytes or biomarkers of interest (e.g., Tau). Antibodies can be produced by methods well known in the art, e.g., by immunizing animals with the biomarkers as antigens. Biomarkers can be isolated from samples based on their binding characteristics. Alternatively, if the amino acid sequence of a polypeptide biomarker is known, the polypeptide can be synthesized and used to generate antibodies by methods well known in the art. Examples of biomarkers include, but are not limited to Tau peptides and phosphorylated Tau peptides.

Exemplary immunoassays include, for example, sandwich immunoassays, such as ELISAs (Enzyme-Linked ImmunoSorbent Assays) or fluorescence-based immunoassays, as well as other enzyme immunoassays. In one embodiment, the immunoassay is a SELDI-based immunoassay, wherein 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.

In another embodiment, the immunoassay is a single antibody immunoassay, often run in a competitive or "competition" mode for immunoreactive binding sites to measure Tau in a biological sample. For example, a single antibody specific to Tau is immobilized to a solid surface, such as a well in a microtiter plate, a bead, or other immunoassay relevant surface. The antibody can be covalently linked via many different methods, such as EDC mediated linkage of carboxyl and amine groups, or via passive absorbance or through a

Protein A or Protein G interface. A Tau competitor is then generated from a Tau standard or calibrator containing the full length Tau peptide or a modified version that retains the epitope of the capture antibody. The Tau competitor is used to generate competition between the native Tau in the biological sample and the binding site (paratope) on the immobilized antibody. A paratope is a term used to describe the binding region of the antibody that recognizes the epitope or immunoreactive domain on the analyte. The Tau competitor is tagged for detection purposes. In one embodiment, the tag is an enzyme, such as horseradish peroxidase or alkaline phosphatase. In another embodiment, the tag is a fluorescein such as phycoerythrin. In yet another embodiment, the tag is another tag, such as biotin or ruthenium. In yet another embodiment, the tag is a nucleic acid such as DNA, RNA or PNA, where by detection of the antibody is quantitated using sensitive technology to detect the nucleic acid flag, such as Polymerase Chain Reaction (PCR). A single concentration of the Tau competitor is used in the assay and would be determined based on the ability to compete with the natural levels of Tau found in biological samples.

In another embodiment, the immunoassay is a double sandwich immunoassay or ELISA (see, *e.g.*, Figure 3). For example, the immunoassay is a sandwich immunoassay, wherein the capture antibody is biotinylated and the reporter (detection) antibody is conjugated to horseradish peroxidase (HRP). These two antibodies are mixed with Tau peptides or CSF and incubated. The well is washed and detection occurs through enhanced chemiluminescence. Traditional assay formats for these assays include ELISA techniques that provide quantitation suitable for the analysis of clinical samples. However, they are often limited to one biomarker assay per well. Newer technologies have been developed that allow multiple biomarkers to be analyzed in a single well or reaction vessel. Some of the multiplexed technologies utilize antibodies spotted onto a solid surface such as glass slides or specialized microtiter plates.

In another aspect, the immunoassay is an intra-assay calibration system. In this approach, an immunoassay format, such as the Ortho Clinical Diagnostics VITROS® system (see, *e.g.*, Figure 4), the Luminex bead based system (see, *e.g.*, Figure 5), or the Meso-Scale Discovery ECL plate based system is used. Peptides containing amino acid residues that encompass the antibody binding epitope of the detection antibody are generated. In one embodiment, these peptides include modifications that enable them to be covalently coupled to a solid phase or modifications that increase their solubility and use in aqueous immunoassays. These peptides are immobilized at different concentrations to the relevant

solid phase as defined by multiplexed immunoassay systems, to create a set of well-defined standards from which to create a standard curve.

In one embodiment, the suspension array technology is the Luminex *xMAP* technology. Luminex *xMAP* technology uses latex beads that contain a ratio of two fluorescent dyes. Different bead "sets" are created by altering the ratio of these two dyes. The beads are mixed together to form a suspension array. The bead mixture is analyzed by an instrument that identifies each bead by the fluorescence ratio as it passes in front of a laser. These bead sets have different modifications on their surface that are used for the covalent attachment of molecules such as proteins, peptides, antibodies, etc. This allows the assay to be performed on the surface of these beads. Assays are quantitated through the incorporation of a third fluorescent label, such as phycoerythrin to a reporter antibody directed at the analyte of interest (e.g., Tau). A second laser in the instrument measures the fluorescence of this reporter label as the beads move through the instrument.

In one embodiment, the method comprises:

a) attaching a reference standard to at least two beads thereby forming a first bead set and a second bead set, wherein the reference standard comprises an epitope recognized by a first detection antibody and wherein each bead set comprises a different concentration of the reference standard;
b) attaching a capture antibody specific to Tau to a third bead set;
c) combining the bead sets together to form a suspension array;
d) applying the biological sample to the suspension array whereby Tau binds to the capture antibody on the third bead set;
e) adding a first detection antibody to the suspension array, wherein the first detection antibody binds the reference standard and Tau bound to the capture antibody;
f) measuring a first signal from the first detection antibody bound to the reference standard in the first bead set;
g) measuring a second signal from the first detection antibody bound to the reference standard in the second bead set;
h) generating a standard curve based upon the first and second signals; and
i) quantitating the amount of Tau in the third bead set by measuring a third signal from the first detection antibody and comparing the third signal to the first and second signal measurements on the standard curve,

wherein the reference standard comprises any one of the compositions described herein.

In another embodiment, the assay is made quantitative by establishing a calibration curve. In another embodiment, quantitation is performed by making a set of Tau standards or calibrators that are either the full length Tau peptide or a modified version that retains the epitope of the capture antibody. These untagged standards or calibrators are prepared in either buffer or a biological matrix that does not contain Tau. In one embodiment, the calibration curve is established by mixing one of the concentrations of the untagged standards or calibrators with the tagged Tau competitor to the immobilized antibody. The resulting signal value from each tested concentration of untagged standard or calibrator is used to generate a standard curve; plotting the concentration of the untagged Tau standards or calibrators versus the resulting signal values. Once a standard quantitative curve is established, an assay is used to determine the levels of Tau in biological samples by mixing the tagged Tau competitor at the same fixed concentration with the biological sample. The resulting signal value is plotted on the standard curve to determine the level of Tau in the biological sample.

The analyte and/or reference standard may be bound to a variety of surfaces. A surface can be any solid phase surface to which an antibody or reference standard can be immobilized by covalent linkage, passive absorbance, biotin-streptavidin or any other linkage known to one of ordinary skill in the art. For example, the surface may be a bead, plate, slides, fiber, surface plasmon resonance sensors or any solid surface.

In another embodiment, the method is performed in a multi-well plate, nitrocellulose filter or on a glass slide. In another embodiment, the first and second signals are detected by fluorescence. For example, the first signal and second signal may be a signal selected from the group consisting of phycoerythrin, alexa 532, streptavidin-phycoerythrin and streptavidin-Alexa 532. In another embodiment, the signal is detected by enzymatic activity *(i.e.,* horseradish peroxidase or alkaline phosphatase), chemiluminescence, radioactivity, infra-red emission, fluorescence resonance energy transfer (FRET) or any other method known to one of ordinary skill in the art.

V. Kits

In another aspect, the invention provides a kit for conducting an immunoassay to detect a Tau peptide, wherein the kit comprise a reference standard (*e.g.*, peptide, modified peptide, or composition) of the invention. The kits may include a label indicating the intended use of the contents of the kit (*e.g.*, in the methods described herein). The term label

includes any writing, marketing materials or recorded material supplied on or with the kit, or which otherwise accompanies the kit.

The present invention is further illustrated by the following examples which should not be construed as limiting. The entire contents of Sequence Listing, Figures and all references, patents and published patent applications cited throughout this application are expressly incorporated herein by reference.

EXAMPLES

Materials

All full length recombinant Tau proteins were obtained from SignalChem (SC, cat # T08-54N)) as a 0.2 mg/mL stock in Phosphate Buffered Saline (PBS), pH 7.4 or 50 mM Tris, pH 7.5, containing 150 mM NaCl, 0.25 mM DTT, o.1 mM PMSF, 25% glycerol. All modified peptides were received as lyophilized powder and were obtained from The American Peptide Company (AP), BaChem (BC), and Anaspec (AN). These peptides were synthesized using solid phase methods known to those skilled in the art (see, for example, Barany, G. et al., Gross, E. et al., (1980); and Stewart, et al. (1984).

Stability of a Tau peptide, native Tau amino acids (190-209)-(PEG)6-native Tau amino acids (215-230); (SEQ ID NO:16) was measured by storing the dissolved peptides at 2-8°C and -20°C and periodically measuring the signal given by the peptides in a sandwich immunoassay. As shown in Figure 7, the peptide is stable for at least 6 months when stored at 2-8°C. In this particular assay, the antibody reagents lose activity over time. In order to account for this, the signal for the peptides stored at 2-8°C was normalized to the signal for the peptides stored at -20°C.

Mouse anti-Tau antibodies (1A4.C3 and F11-1-1) were obtained via protein-G purification of culture supernatants produced by the relevant hybridoma cell lines. Phycoerythrin-streptavidin conjugate was obtained from Jackson Immunoresearch (West Grove, PA). Tween-20, l-ethyl-3-[3 dimethylaminopropyl] carbodiimide hydrochloride (EDC), sodium azide, IgG free Bovine Serum Albumin 10 (BSA), and sodium phosphate were purchased from Sigma-Aldrich Corporation (St. Louis, MO). Phosphate buffered saline (PBS) was obtained from Mediatech Incorporated (Herndon, VA). Carboxylated Luminex beads were purchased from Bio-Rad Incorporated (Hercules, CA). Phycoerythrin label goat anti-mouse IgG antibody was obtained from Jackson Immunoresearch (West Grove, PA).

Example 1: VITROS® Immunoassay

A schematic of a VITROS® immunoassay is shown in Figure 4. Briefly, a biological sample (human CSF) was added to a VITROS® microwell coated with streptavidin (Ortho Clinical Diagnostics, Rochester, NY (OCD). A biotin labeled anti-Tau antibody (mouse monoclonal anti-Tau) was added next, followed by a second horseradish peroxidase tagged (HRP)-labeled antibody (mouse monoclonal anti-Tau), completing the sandwich. Both

antibodies are specific to a different sequence within the core domain that is common between all six isoforms of Tau. Additionally, a reference standard Tau peptide (e.g., reference standard Tau 441 (SEQ ID NO. 1) or a modified Tau reference standard (e.g., as set forth in Table 3) was added to a VITROS® microwell coated with streptavidin (Ortho Clinical Diagnostics, Rochester, NY (OCD)). A biotin labeled anti-Tau antibody was added next, followed by a second horseradish peroxidase tagged (HRP) antibody, completing the sandwich. The wells were then incubated 30 minutes at 37°C to allow Tau 441, Tau reference peptides, or Tau in CSF to be bound by the antibodies, forming a sandwich on the surface of the microwell (e.g., the Tau-antibody complex is captured by the streptavidin on the microwell). Unbound materials were removed by washing.

The bound HRP conjugate was then measured by a luminescent reaction. Specifically, a reagent containing luminogenic substrates (a luminol derivative and a peracid salt) and an electron transfer agent were added to the wells. The HRP in the bound conjugate catalyzes the oxidation of the luminol derivative, producing light. The electron transfer agent (a substituted acetanilide) increases the level of light produced and prolongs its emission. The light signals were then detected on a Vitros ECiQ clinical analyzer using an enhanced chemiluminescent substrate. The raw luminescence units (ALU) measured by the instrument were fit to a 4-parameter logistic model to create a standard curve, as described below in Example 3. The amount of HRP conjugate bound is directly proportional to the concentration of Tau present. The measured concentrations of Tau in human CSF samples are shown in Table 4, as set forth below.

Table 4: Tau Levels Measured in Human CSF

CSF	Diagnosis	Vitros Tau (pg/mL)
1	AD	1440
2	AD	1057
3	AD	861
4	AD	1010
5	AD	977
6	Normal	351
7	Normal	354
8	Normal	217
9	Normal	196
10	Normal	419

In a specific example, a modified Tau peptide, native Tau amino acids (190-209)-(PEG)6-native Tau amino acids (215-230); (SEQ ID NO:16)) was used as a reference standard. The standard curve generated using is described in further detail below in Example 3 and is shown in Figure 6.

Example 2: Intra-Assay Bead Approach

Figure 5 illustrates the Tau intra-assay bead approach (*i.e.*, a Luminex assay). Bead sets coupled with different concentrations of Tau standard peptides (or other native or modified Tau peptides) were combined with a bead set coupled with an anti-Tau specific capture antibody to form a suspension array. The array was incubated with a biological sample, where the Tau peptide in the biological sample was captured by the bead coupled with anti-Tau capture antibody. A tagged detection antibody specific to a separate sequence within the core domain of the Tau peptide was added to the suspension array, thereby binding to the captured Tau peptide and also to the beads that have Tau peptides coupled to their surface. The MFI values obtained from the beads with Tau peptides coupled to their surface were used to generate an intra-assay calibration curve. The amount of Tau in the biological sample was determined from the amount of captured Tau on the bead coupled with the anti-Tau capture using an intra-assay standard curve.

Example 3: Calibration Curve

To determine the amount of Tau in a biological sample using the sandwich based assay described in Example 1, a calibration curve was created. Specifically, a calibration curve was generated by harmonizing the signal given by the modified peptide (Table 3, native Tau amino acids (190-209)-(PEG)6-native Tau amino acids (215-230)) with that given by native Tau 441 (Table 1, SEQ ID NO: 1) in a buffer matrix. Briefly, a standard curve was generated by first diluting the modified peptide into calibrator matrix at specific concentration levels within the linear range of the assay. The modified peptides are diluted to a corresponding molar equivalent of native Tau 441 while taking the percent peptide, purity, and molecular mass of the modified peptide and the purity of native Tau 441 into account. The levels of the modified peptide were then run in the immunoassay as described in Example 1, along with a separate set of native calibrator standards made with recombinant Tau 441 (Table 1, SEQ ID 1). The raw ALU values generated from the synthetic tau peptides were fit to the native Tau 441 standard curve and a concentration equivalent to Tau 441 (pg/mL) was assigned for each modified peptide level. These assigned values for the

modified peptides were then plotted against ALU to generate the calibration curve. This calibration curve was then used to measure the levels of Tau in a biological fluid. A modified peptide calibration curve generated as described in Example 1 is shown in Figure 6.

Example 4: Epitope Mapping for Anti-Tau Antibody 9E9 by Luminex Method

Figure 8A shows the amino acid sequence of the middle region of the Tau protein (i.e., from amino acids 126-245). Peptides 9-16 were created from this region of Tau. These peptides were 20 amino acids in length and overlapped by 5 amino acids down the length of the Tau sequence.

Peptides 9-16, as well as peptides consisting of known epitopes for commercially available antibodies (BT2, HT7, Tau 5, and Tau1), were fused to luminex beads using standard amine couple chemistries. For epitope mapping, purified antibodies were incubated with the luminex bead sets and run on a Bioplex 100 instrument.

As shown in Figure 8B, antibody 9E9 bound only to peptide 13 and not the BT2 peptide or peptide 14.

Example 5: Epitope Mapping of Anti-Tau Antibody 9E9 by Biacore

Antibody 9E9 also was characterized using Biacore T100 (immobilized to the CM5 gold surface by amino coupling). 9E9 (10µg/mL in 10Mm Sodium Acetate Buffer) was injected onto active CM5 at 1-ul/min for seven minutes to a final immobilization of 10000RU. The surface was then deactivated by passage of 1 M ethanolamine. Short 8 AA long peptides spanning the region from AA 186-199 (peptide overlay) were flowed over the chip for binding activity. Specifically, recombinant Tau 441 (positive control) (final conc. 25nM) followed by IL-23 (negative control), and peptides 15-17 at two concentrations, 25 µg/mL and 125 µg/mL. The surface was regenerated by a 30ul/min of Glycine pH 1.75. Dilutions were prepared using 1X HBS-EP buffer. Data was analyzed using Biacore Evaluation software 1.0.

As shown in Figure 9, Peptide 16 was the only peptide that generated a response with two different concentrations of 9E9 antibody, thereby narrowing down the binding epitope to amino acids 189-196.

SUMMARY OF SEQUENCE LISTING

Amino Acid Sequence	Description of Peptide/Modified Peptide Sequence
(SEQ ID NO: 1)	Native Tau 441 amino acid sequence
(SEQ ID NO: 2)	Native Tau 410 amino acid sequence
(SEQ ID NO: 3)	Native Tau 412 amino acid sequence
(SEQ ID NO: 4)	Native Tau 381 amino acid sequence
(SEQ ID NO: 5)	Native Tau 383 amino acid sequence
(SEQ ID NO: 6)	Native Tau 352 amino acid sequence
(SEQ ID NO:7)	Native Tau amino acids (190-209)
(SEQ ID NO:8)	Native Tau amino acids (155-170)
(SEQ ID NO:9)	Native Tau amino acids (215-230)
(SEQ ID NO:10)	Native Tau amino acids (180-195)
(SEQ ID NO:11)	Native Tau amino acids (191-200)
(SEQ ID NO:12)	Native Tau amino acids (156-167)
(SEQ ID NO:13)	Native Tau amino acids (171-190)
(SEQ ID NO:14)	Native Tau amino acids (221-240); amino acid T231 is phosphorylated
(SEQ ID NO:15)	Native Tau amino acids (191-200); amino acid T180 is phosphorylated
(SEQ ID NO:16)	Native Tau amino acids (190-209)-(PEG)6-native Tau amino acids (215-230)
(SEQ ID NO:17)	Native Tau amino acids (155-170)-(PEG)6-native Tau amino acids (190-209)
(SEQ ID NO:18)	Native Tau amino acids (155-170)-(PEG)6-native Tau amino acids (215-230)
(SEQ ID NO:19)	Native Tau amino acids (180-195) - (PEG)6-native Tau amino acids (215-230)
(SEQ ID NO:20)	Native Tau amino acids (155-170)-(PEG)6-native Tau amino acids (190-209)
(SEQ ID NO:21)	Native Tau amino acids (155-170)-GGSGGS-native Tau amino acids (190-209)
(SEQ ID NO:22)	Native Tau amino acids (190-209)-(PEG)6-native Tau amino acids (155-170)
(SEQ ID NO:23)	Native Tau amino acids (190-209)-GGSGGS-native Tau amino acids (155-170)
(SEQ ID NO:24)	Native Tau amino acids (190-209)-(PEG)6-native Tau amino acids (215-230)
(SEQ ID NO:25)	Native Tau amino acids (190-209)-GGSGGS-native Tau amino acids (215-230)
(SEQ ID NO:26)	Native Tau amino acids (215-230)-(PEG)6-native Tau amino acids (190-209)
(SEQ ID NO:27)	Native Tau amino acids (215-230)-GGSGGS-native Tau amino acids (190-209)
(SEQ ID NO:28)	Native Tau amino acids (155-170)-GGSGGS-native Tau amino

	acids (215-230)
(SEQ ID NO:29)	Native Tau amino acids (215-230)-(PEG)6-native Tau amino acids (155-170)
(SEQ ID NO:30)	Native Tau amino acids (215-230)-GGSGGS-native Tau amino acids (155-170)
(SEQ ID NO:31)	Native Tau amino acids (190-209)-(PEG)6-native Tau amino acids (155-170)
(SEQ ID NO:32)	Native Tau amino acids (215-230)-(PEG)6-native Tau amino acids (190-209)
(SEQ ID NO:33)	Native Tau amino acids (155-170)-(PEG)6-native Tau amino acids (215-230)
(SEQ ID NO:34)	Native Tau amino acids (215-230)-(PEG)6-native Tau amino acids (155-170)
(SEQ ID NO:35)	Native Tau amino acids (156-167)-generic linker-native Tau amino acids (221-240); amino acid T231 is phosphorylated
(SEQ ID NO:36)	Native Tau amino acids (156-167)-generic linker-native Tau amino acids (191-200)
(SEQ ID NO:37)	Native Tau amino acids (171-190)-generic linker-native Tau amino acids (191-200); amino acid T180 is phosphorylated
(SEQ ID NO:38)	Native Tau amino acids (191-200)-generic linker-native Tau amino acids (221-240); amino acid T231 is phosphorylated
(SEQ ID NO: 39)	A-B)-Generic Linker-(X-Y) Generic A, B, X, Y, Linker to be specified
(SEQ ID NO: 40)	Peptide Linker
(SEQ ID NO:41)	PEG6 Linker

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What Is Claimed:

1. An isolated peptide having an amino acid sequence selected from the group consisting of SEQ ID NOs: 7, 8, 9 and 10.

2. A modified peptide comprising an N-terminal immunoreactive region, a C-terminal immunoreactive region, and a linker region, wherein:

(a) the N-terminal immunoreactive region comprises an amino acid sequence selected from the group consisting of SEQ ID NOs: 7, 8, 9, and 10; and/or

(b) the C-terminal immunoreactive region comprises an amino acid sequence selected from the group consisting of SEQ ID NOs: 7, 8, 9, and 10,

and wherein the N-terminal and C-terminal immunoreactive regions are different amino acid sequences.

3. The modified peptide of claim 2, wherein the N-terminal and C-terminal immunoreactive regions comprise the amino acid sequences selected from the group consisting of SEQ ID NOs:7 and 9, SEQ ID NOs:7 and 8, SEQ ID NOs:8 and 9, and SEQ ID NOs:9 and 10, respectively.

4. The modified peptide of claim 3, wherein the N-terminal and C-terminal immunoreactive regions comprise the amino acid sequences set forth in SEQ ID NOs: 7 and 9, respectively.

5. A modified composition comprising an N-terminal immunoreactive region, a C-terminal immunoreactive region, and a linker region, wherein:

(a) the N-terminal immunoreactive region comprises the amino acid sequence set forth in SEQ ID NO:7 and the C-terminal immunoreactive region comprises the amino acid sequence set forth in SEQ ID NO:9;

(b) the N-terminal immunoreactive region comprises the amino acid sequence set forth in SEQ ID NO:8 and the C-terminal immunoreactive region comprises the amino acid sequence set forth in SEQ ID NO:7;

(c) the N-terminal immunoreactive region comprises the amino acid sequence set forth in SEQ ID NO:8 and the C-terminal immunoreactive region comprises the amino acid sequence set forth in SEQ ID NO:9;

(d) the N-terminal immunoreactive region comprises the amino acid sequence set forth in SEQ ID NO:10 and the C-terminal immunoreactive region comprises the amino acid sequence set forth in SEQ ID NO:9;

(e) the N-terminal immunoreactive region comprises the amino acid sequence set forth in SEQ ID NO:7 and the C-terminal immunoreactive region comprises the amino acid sequence set forth in SEQ ID NO:8;

(f) the N-terminal immunoreactive region comprises the amino acid sequence set forth in SEQ ID NO:9 and the C-terminal immunoreactive region comprises the amino acid sequence set forth in SEQ ID NO:7;

(g) the N-terminal immunoreactive region comprises the amino acid sequence set forth in SEQ ID NO:9 and the C-terminal immunoreactive region comprises the amino acid sequence set forth in SEQ ID NO:8;

(h) the N-terminal immunoreactive region comprises the amino acid sequence set forth in SEQ ID NO:12 and the C-terminal immunoreactive region comprises the amino acid sequence set forth in SEQ ID NO:14;

(i) the N-terminal immunoreactive region comprises the amino acid sequence set forth in SEQ ID NO:12 and the C-terminal immunoreactive region comprises the amino acid sequence set forth in SEQ ID NO:11;

(j) the N-terminal immunoreactive region comprises the amino acid sequence set forth in SEQ ID NO:13 and the C-terminal immunoreactive region comprises the amino acid sequence set forth in SEQ ID NO:15; or

(k) the N-terminal immunoreactive region comprises the amino acid sequence set forth in SEQ ID NO:15 and the C-terminal immunoreactive region comprises the amino acid sequence set forth in SEQ ID NO:14.

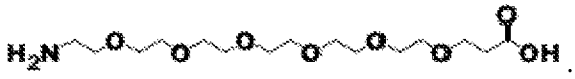
6. The modified peptide of claim 5, wherein the N-terminal and C-terminal immunoreactive regions comprise the amino acid sequences set forth in SEQ ID NOs: 7 and 9, respectively.

7. The modified peptide of any one of claims 2-6, wherein the linker region comprises a linker selected from the group consisting of polyethylene glycol, a glutamic acid

residue, a glycine residue, a serine residue, an alanine residue, a lysine residue, a lipid, a globular protein, a nucleic acid, and an alkyl chain.

8. The modified peptide of any one of claims 2-6, wherein the linker region comprises a PEG6 linker.

9. The modified peptide of claim 8, wherein the PEG6 linker has the following structure:



10. The modified peptide of any one of claims 2-6, wherein the linker region comprises the amino acid sequence set forth in SEQ ID NO:40.

11. The modified peptide of any one of claims 2-6, wherein the linker region is a non-immunoreactive domain.

12. A composition comprising the peptide of claim 1 or the modified peptide of any one of claims 2-11 and a carrier.

13. Use of the modified peptide of any one of claims 2-11 as a reference standard in an immunoassay.

14. The use of claim 13, wherein the immunoassay is selected from the group consisting of a sandwich immunoassay, a single antibody assay, a double sandwich immunoassay, a competition assay, and a suspension array assay.

15. A method of measuring the quantity of Tau in a biological sample, the method comprising:

a) attaching a reference standard to at least two beads thereby forming a first bead set and a second bead set, wherein the reference standard comprises an epitope recognized by a first detection antibody and wherein each bead set comprises a different concentration of the reference standard;

b) attaching a capture antibody specific to Tau to a third bead set;

c) combining the bead sets together to form a suspension array;

d) applying the biological sample to the suspension array whereby Tau binds to the capture antibody on the third bead set;

e) adding a first detection antibody to the suspension array, wherein the first detection antibody binds the reference standard and Tau bound to the capture antibody;

f) measuring a first signal from the first detection antibody bound to the reference standard in the first bead set;

g) measuring a second signal from the first detection antibody bound to the reference standard in the second bead set;

h) generating a standard curve based upon the first and second signals; and

i) quantitating the amount of Tau in the third bead set by measuring a third signal from the first detection antibody and comparing the third signal to the first and second signal measurements on the standard curve,

wherein the reference standard comprises the composition of any one of claims 1-13.

16. The method of claim 15, wherein the method is performed in a multi-well plate, nitrocellulose filter, glass fiber or on a glass slide.

17. The method of claim 16, wherein the biological sample is selected from the group consisting of blood, serum, plasma, peripheral blood mononuclear cells, peripheral blood lymphocytes, tissue, cerebrospinal fluid and cells.

18. The method of claim 17, wherein Tau is selected from the group consisting of Tau352, Tau381, Tau 383, Tau 410, Tau 412, and Tau 441.

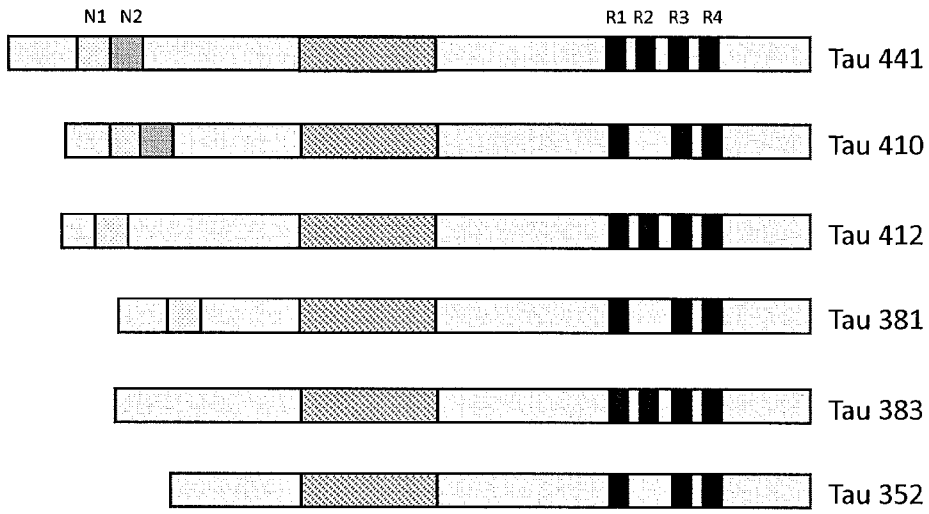


Figure 1: The six isoforms of tau are formed through RNA splicing. There are two possible N-terminal inserts (N1 and N2, gray bars) and four possible C-terminal repeats (R1–R4, black bars). The epitopes used in this invention are from the domain represented in this figure by the bar with the diagonal lines. This domain ranges from amino acid residue 155 to residue 230 of tau 441.

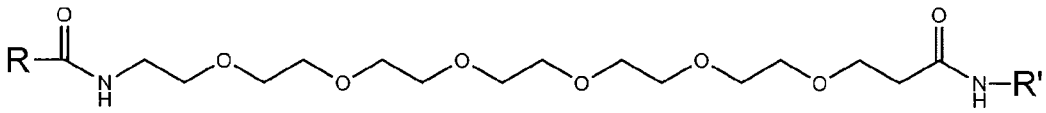


Figure 2: The chemical structure of the $(PEG)_6$ linker. R and R' represent peptides that can be used in this assay.

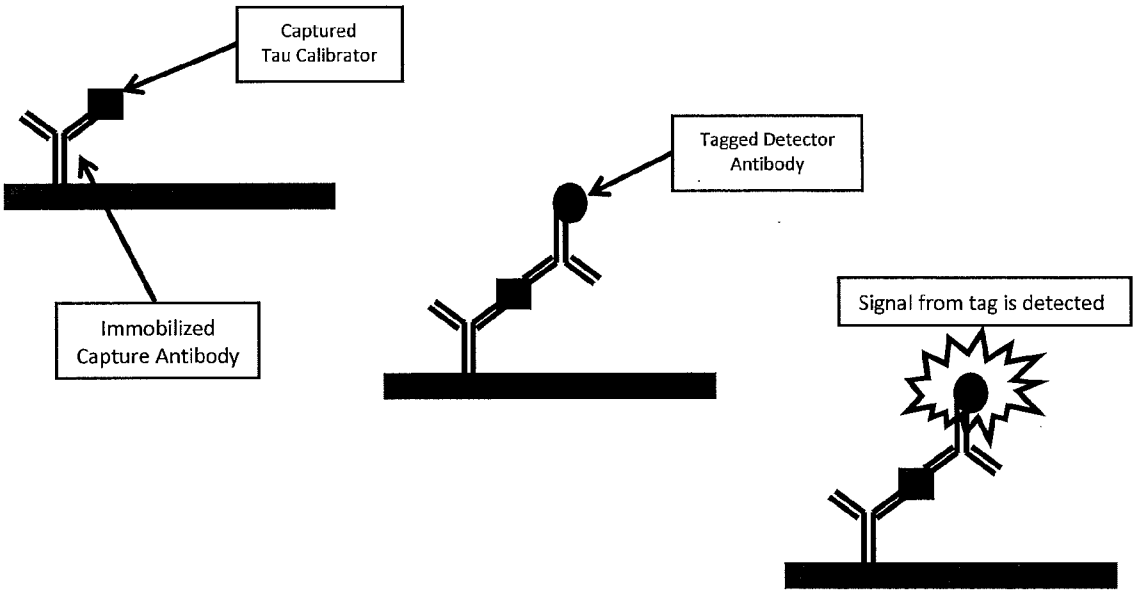


Figure 3: Schematic of a sandwich immunoassay.

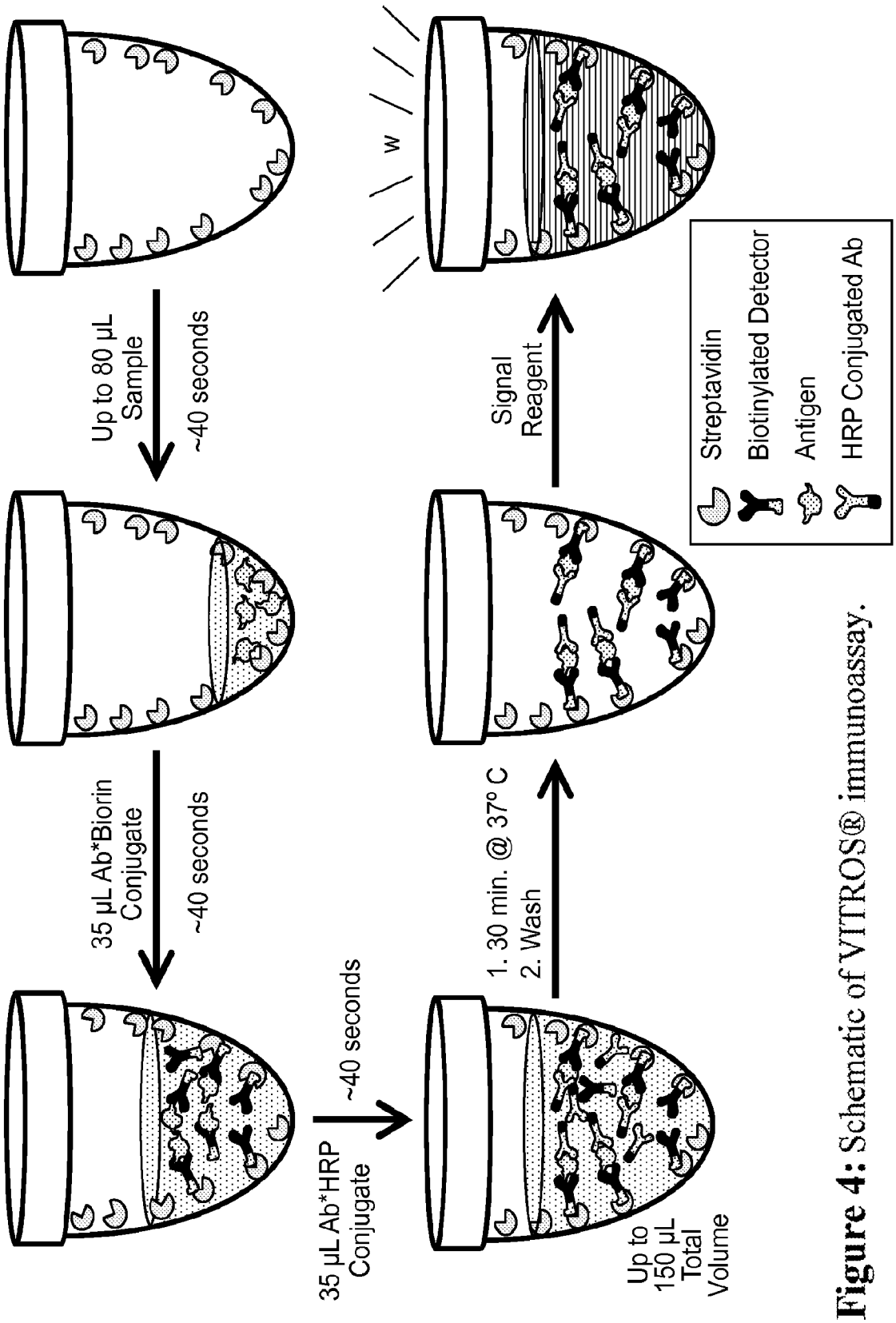


Figure 4: Schematic of VITROS® immunoassay.

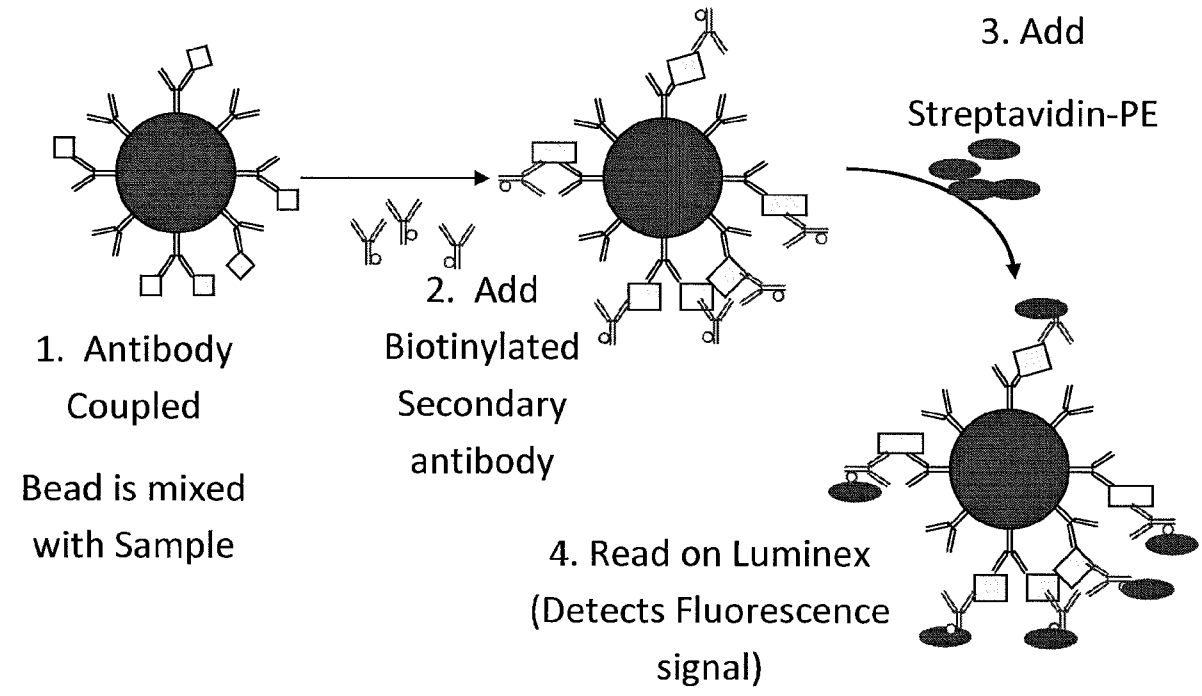


Figure 5: Schematic of Luminex Assay.

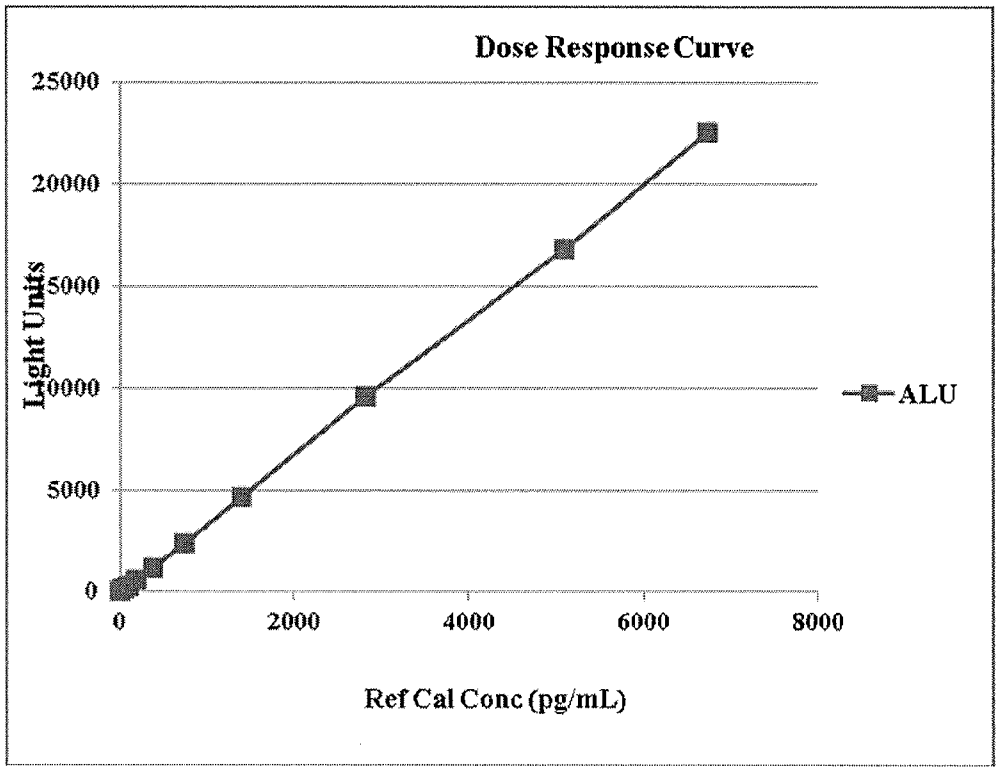


Figure 6: A dose response curve using the modified Tau peptide consisting of native Tau amino acids (190-209)-(PEG)6-native Tau amino acids (215-230) (SEQ ID NO:16) shows that the peptide gives a linear response within the range of the assay.

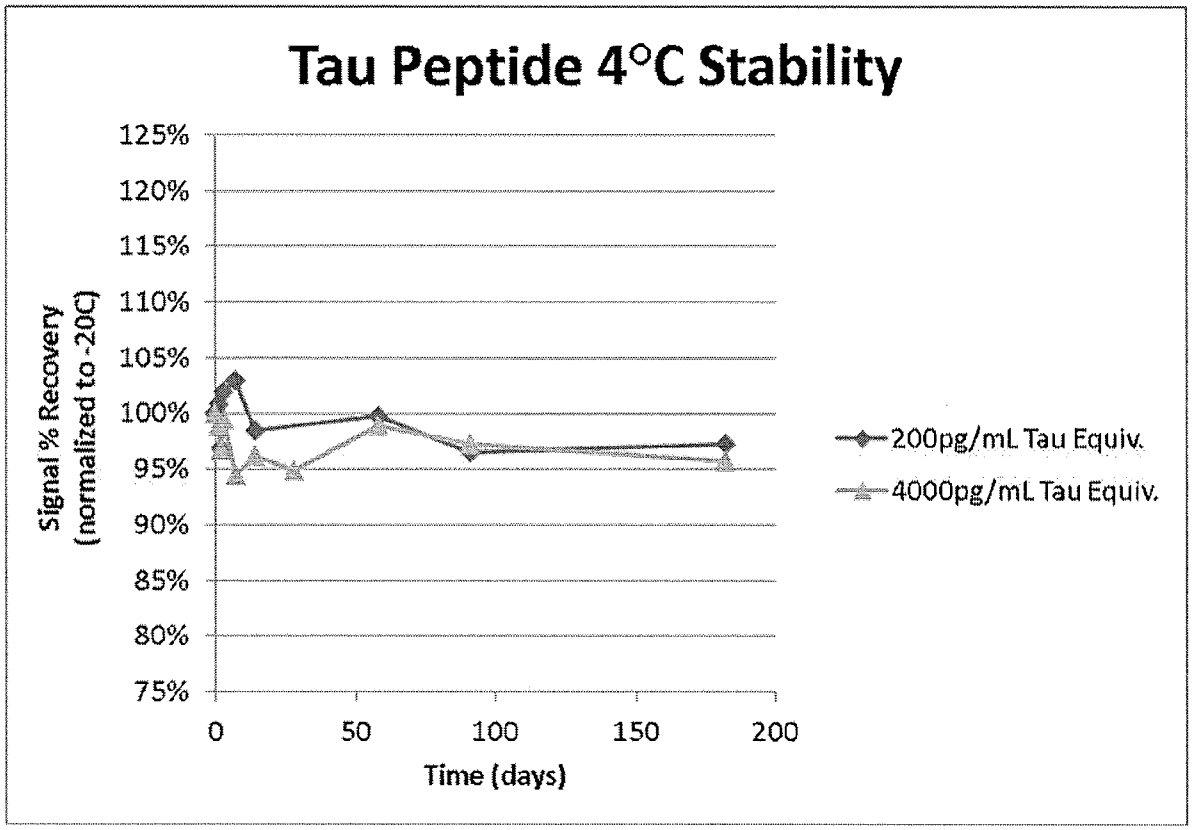


Figure 7: Stability of Tau Peptide consisting of native Tau amino acids (190-209)-(PEG)6-native Tau amino acids (215-230) (SEQ ID NO:16)). This peptide is stable in solution for at least 6 months when stored at 2-8°C. The data for each time point and concentration is normalized to an equivalent sample stored at -20C.

Figure 8: Screening Summary-Peptide overlay

HT7

126-RMVSKSKDGTGSDDKKAKGADGKTKIATPRGAAPPGQKGQANATRIPAKTPPAPKTPPSS-185

9-KHVTQARMVSKSKDGTGSDDK

10-KGSDDKKAKGADGKTKIATPR

11-KIATPRGAAPPGQKGQANATR

12-KANATRIPAKTPPAPKTPPSS

HT7-peptide CRGAAPPGQKGQANATR

BT2 Tau1 Tau 5

186-GEPPKSGDRSGYSSPGSPGTPGSRSRTPSLPTPPTREPKKVAVV RTPPKSPSSAKSRLQT-245

13-KTPPSSGEPPKSGDRSGYSSP

14-KGYSSPGSPGTPGSRSRTPSL

15-KRTPSLPTPPTREPKKVAVVR

16-KVAVVRTPPKSPSSAKSRLQT

BT2-peptide CKSGDRSGYSSPGSPGTPGSR

Tau5-peptide CLPTPPTREPKKVAVVR

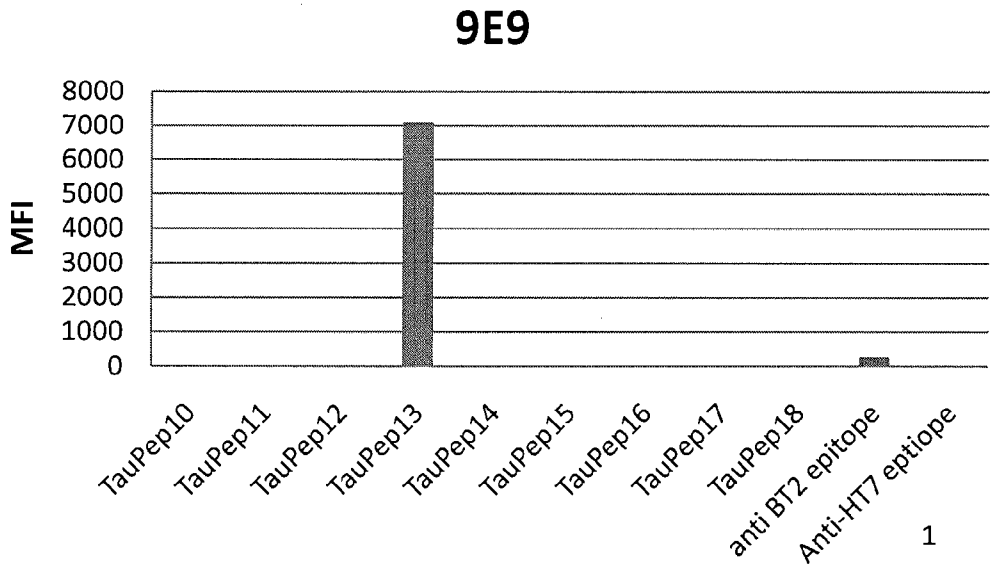


Figure 9: Biacore Analysis of 9E9 Antibody

Peptide	RU	RU
	25ug/ml (amt of antibody)	125ug/ml
Buffer	0.1	
Tau 441 (pos control)	962	
IL23 (neg control)	-0.5	
peptide 15 (8 aa)	-0.2	
peptide 16	2.9	13.3
peptide 17	-0.4	

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2013/076046

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07K14/435 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)
C07K 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, Sequence Search, EMBASE, FSTA, 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	WO 2011/100292 A1 (SQUIBB BRISTOL MYERS CO [US]; RHYNE PAUL [US]; MAPELLI CLAUDIO [US]; W) 18 August 2011 (2011-08-18) paragraphs [0062], [0085]; claim 12; table 3	1-18
X	VANMECHELEN E ET AL: "QUANTIFICATION OF TAU PHOSPHORYLATED AT THREONINE 181 IN HUMAN CEREBROSPINAL FLUID: A SANDWICH ELISA WITH A SYNTHETIC PHOSPHOPEPTIDE FOR STANDARDIZATION", NEUROSCIENCE LETTERS, LIMERICK, IE, vol. 285, no. 1, 5 May 2000 (2000-05-05), pages 49-52, XP000972339, ISSN: 0304-3940, DOI: 10.1016/S0304-3940(00)01036-3 the whole document	1-18

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[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

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"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

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
13 March 2014	27/03/2014

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Voigt-Ritzer, Heike

1

Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DATABASE Geneseq [Online] 17 February 2011 (2011-02-17), "Human tau protein epitope fragment sequence, SEQ ID 92.", XP002721658, retrieved from EBI accession no. GSP:AYN10524 Database accession no. AYN10524 sequence ----- DATABASE Geneseq [Online] 27 October 2011 (2011-10-27), "Human first antibody-specific tau epitope, SEQ ID 6.", XP002721659, retrieved from EBI accession no. GSP:AZM74874 Database accession no. AZM74874 sequence ----- DATABASE Geneseq [Online] 8 September 2005 (2005-09-08), "Human tau long splice form protein, SEQ ID NO: 1.", XP002721660, retrieved from EBI accession no. GSP:AEB27979 Database accession no. AEB27979 sequence -----	1,2 1,2 1-7

1

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/US2013/076046

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2011100292 A1	18-08-2011	AU 2011215970 A1	20-09-2012
		CN 102859363 A	02-01-2013
		EP 2534490 A1	19-12-2012
		JP 2013518927 A	23-05-2013
		KR 20130043088 A	29-04-2013
		US 2012309028 A1	06-12-2012
		WO 2011100292 A1	18-08-2011