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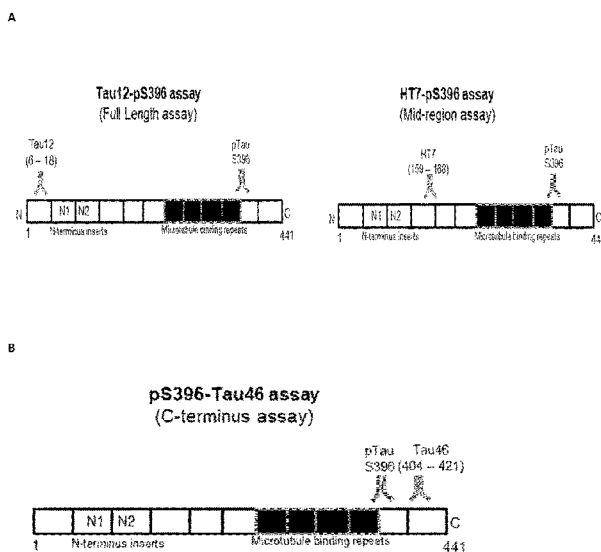

Fig 1

(57) **Abstract:** The present invention relates to an in vitro assay for measuring phosphorylated tau in a sample, said assay comprises the use of 2 antibodies i) a capture antibody specific for pS396 on tau and ii) a detection antibody binding tau on a different epitope that the capture antibody.

WO 2020/193500 A1

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USE OF A pS396 ASSAY TO DIAGNOSE TAUOPHATIES

The present invention relates to the use of anti-tau antibodies in an assay to
5 differentiate tau species in different tau pathologies. The assays according to the invention can be used *i.e.* to diagnose patients with tauopathies such as Alzheimer's disease, Pick's disease, corticobasal degeneration, progressive supranuclear palsy and globular glial tauopathy.

10 **BACKGROUND OF THE INVENTION**

The term tauopathy defines a group of pathological diseases characterized by deposition of the microtubule-associated protein tau. The deposited tau is phosphorylated abnormally and accumulates as intracellular inclusions. There are a number of specific tauopathies, each of which vary by the distribution and
15 morphological appearances of the protein-containing inclusions, as well as the relative burden of pathology affecting neurons and neuronal processes versus glial and glial processes (Dickson et al., 2011). The most common tauopathies are progressive supranuclear palsy (PSP), corticobasal degeneration (CBD), Pick's disease, and globular glial tauphathy (GGT) and chronic traumatic
20 encephalopathy (CTE). All but Pick's disease are commonly associated with movement disorders (Keith A. Josephs, Chapter IX, in Movement Disorders (Second Edition), 2015). Tauopathies are also denoted Fronto Temporal Dementia (FTD), Fronto Temporal Lobar Degeneration (FTLD). Most cases of FTD are associated with genetic mutations in MAPT (tau) or GRN (granulin). FTD
25 is associated with accumulation of tau protein.
In order to diagnose and find the right treatment for these patients it is important to have a method that can diagnose these patients and differentiate tau pathology that is characteristic for each disease. The inventors of the present invention have provided a number of assays that can help in the diagnosis of
30 these diseases.

SUMMARY OF THE INVENTION

The present invention relates to an *in vitro* assay for measuring phosphorylated tau in a sample, said assay comprises the use of 2 antibodies i) a capture

antibody specific for the phosphorylated(p) serine(S) residue 396 (pS396) on tau and ii) a detection antibody binding tau on a different epitope than the capture antibody. The detector antibody may bind a non-phosphorylated residue on tau as disclosed further herein.

5

In a second aspect the invention relates to a method for measuring phosphorylated tau in a sample, which method comprises the steps of

a. Mixing capture antibodies specific for pS396 attached to paramagnetic beads with biotinylated detection antibodies and a sample,
10 b. Incubating the mixture at a sufficient time to allow the antibodies to bind to tau in the sample (e.g. 1, 2, 3 or 5 minutes or more),
c. Optionally, washing the mixture in step b) after incubation,
d. Adding streptavidin-conjugated beta-galactosidase and allowing said streptavidin-conjugate and the biotinylated detector antibody to react
15 (e.g. 1, 2, 3 or 5 minutes or more),
e. Optionally washing the obtained mixture in step d),
f. Adding resorufin beta-D-galactopyranoside to the mixture in step d) or e) and allowing the hydrolysis of resorufin beta-D-galactopyranoside (e.g. 1, 2, 3 or 5 minutes or more),
20 g. Reading the fluorescence signal and comparing the signal with a standard

FIGURE

Figure 1 Schematic illustration of the pS396 tau assays.

25 (A) The Tau12-pS396 assay, also known as the full-length pS396 (or FL pS396) assay, uses the anti-pS396 antibody as the capture antibody and Tau12 (epitope at amino acids 6-18) as the detection antibody.

(B) The HT7-pS396 (mid-region pS396 or MR pS396) assay measures pS396 on tau species that stretch from the mid-region (epitope 159-168). This assay uses
30 the pS396 antibody as the capture and HT7 the detection antibody.

(C) The pS396-Tau46 (C-terminus pS396 or CT pS396) assay, which uses the anti-pS396 antibody as capture and Tau46 (epitope 404-441) as detection antibody, is specific for pS396 phosphorylated tau that contains the extreme C-terminus region (amino acids 404-441).

Figure 2 pS396 phosphorylated tau species differentiate neurodegenerative diseases with tau pathology. TBS-soluble fractions of frontal grey matter brain isolates from individuals with clinically confirmed tauopathies were tested with the FL, MR and CT pS396 assays. Five hundred-fold dilutions of equimolar concentrations (0.46 mg/ml) of each specimen (n = 24 total) prepared with the assay diluent were analysed with each pS396 assay. Dilution corrected data (mean ± standard error of the mean [SEM]) have been shown here. The samples consisted of Alzheimer's disease (AD, n=5), Pick's disease (PiD, n=5), corticobasal degeneration (CBD, n=5), progressive supranuclear palsy (PSP, n=5), globular glial tauopathy (GGT, n=2), and healthy controls (Ctrl, n=2 [n=1 for the MR assay]).

(A) Concentration of FL pS396 in the tauopathy brain samples. Mean concentrations ± SEM: AD = 5769 ± 621 pg/ml, PiD = 4737 ± 960 pg/ml, CBD = 10716 ± 2452 pg/ml, PSP = 8888 ± 1002 pg/ml, GGT = 10122 ± 3955 pg/ml, Ctrl = 3326 ± 73 pg/ml. No statistically significant difference was recorded between the groups (Kruskal-Wallis test followed by Dunn's multiple comparison test).

(B) MR pS396 concentrations in brain specimen from different tauopathies. Mean levels ± SEM: AD = 23432 ± 4773 pg/ml, PiD = 29949 ± 6938 pg/ml, CBD = 17434 ± 9359 pg/ml, PSP = 5563 ± 1047 pg/ml, GGT = 9830 ± 3933 pg/ml, Ctrl = 1862 pg/ml. No statistically significant difference was recorded between the groups (one-way Analysis of variance [ANOVA]).

(C) Levels of CT pS396 in brain samples from five different tauopathy groups compared to controls. Mean concentration ± SEM: AD = 17692 ± 2060 pg/ml, PiD = 12549 ± 1701 pg/ml, CBD = 11203 ± 5698 pg/ml, PSP = 4766 ± 706 pg/ml, GGT = 7857 ± 3097 pg/ml, Ctrl = 397 ± 54 pg/ml. CT pS396 concentrations in AD were significantly higher compared to same in PSP ($p < 0.05$) and Ctrl ($p < 0.01$). Similarly, CT pS396 levels in PiD were significantly higher than same in controls (Ctrls) ($p < 0.05$; Kruskal-Wallis test followed by Dunn's multiple comparison test).

(D) The ratio of MR to FL pS396 levels significantly separated the different tauopathies. The MR/FL ratio for AD was significantly different compared to those in PiD, CBD, PSP, GGT and controls ($p < 0.01$ each). Moreover, the ratio of MR to FL in the PiD patients was significantly different from

CBD, PSP, GGT and controls (p=0.0001 each; one-way ANOVA followed by the Dunnett's multiple comparison test).

(E) Significant difference in the ratio of CT to FL pS396 concentrations in the disease groups. The CT/FL pS396 levels were significantly different in AD compared to CBD, PSP, GGT and controls (p <0.01 each), and PiD compared to CBD, PSP, GGT and controls (p <0.01 each; one-way ANOVA followed by the Dunnett's multiple comparison test).

All three assays measure tau pS396 in human tauopathy brain samples. The results indicate that the populations of pS396 tau in different tauopathies significantly vary with respect to the tau species present, suggesting that the combined measurement of pS396 and tau fragmentation is a promising approach to separating between these diseases.

Figure 3 High levels of pS396-Tau46 (CT pS396) in the rTg4510 transgenic tau mouse model of Alzheimer's disease. (A) CSF samples from different animals were analysed with the CT pS396 assay at 50 or 75 fold dilutions. The measured and dilution-adjusted concentrations are both shown. (B) CT pS396 concentrations in 10 or 20 fold dilutions of plasma samples from six different animals, including those whose CSF levels of CT pS396 are shown in (A). Assay Limit of detection = 1.50 pg/ml

CT pS396 is present in the CSF and plasma of rTg4510 transgenic tau mouse animals. The CT pS396 assay is an important tool for studying molecular changes in CT pS396 processing in this model and for preclinical evaluation of drug efficacy.

Figure 4 Measurement of CT pS396 in human CSF. CT pS396 signal in 15 ml human CSF was enriched by spin filtration (using the Ultracel®-YM3 device; conditions shown in Table 1) followed by size exclusion chromatography (SEC) with the S200 10/300 GL column in 50 mM Tris pH 7.5, 10% glycerol running buffer. The eluted fractions were analysed directly with the Simoa CT pS396.

(A) Elution profile of the retentate of spin-filtered human CSF showing elution volume on the horizontal axis and UV absorbance on the vertical axis. The gridlines show the elution volumes of molecular weight markers: blue dextran (void

volume, 2000 kDa; 7.76 ml), albumin (66 kDa; 13.45 ml), carbonic anhydrase (29 kDa; 16.28 ml), cytochrome (12.4 kDa; 20.39 ml), aprotinin (6.5 kDa; 24.36 ml).

(B) Chromatogram of spin-concentrated human CSF, showing the elution fraction IDs on the horizontal axis and UV absorbance on the vertical axis. Gridlines refer to the same molecular weight markers shown in (A).

(C) Concentration of CT pS396 in neat (untreated) CSF, spin-filtered CSF (retentate), and the eluted SEC fractions. No CT pS396 signal was detected in the neat CSF, which explains the need for pre-processing to enrich the signal. CT pS396 was not detected in the filtration product either. However, CT pS396 could be measured after SEC fractionation of the filtration product. The highest concentrations of CT pS396 were found in fractions C1, C2 and C3 (6.1, 9.9, and 6.1 pg/ml respectively in this sample) corresponding to elution volumes 12.0 – 13.5 ml and molecular weight ~ 66 kDa. These properties suggest that the fractions eluting at 12.0 – 13.5 ml are enriched in tau monomers containing both the pS396 and Tau46 epitopes.

CT pS396 is not measurable in untreated human CSF. However, pre-analytical processing by spin filtration and SEC enriches CT pS396 signal, with the highest concentrations eluting at 12.0 – 13.5 ml. Consistent results have been recorded using spin filters of different capacities and models (**Table 1**), by changing the CSF starting volume (**Table 1**), and by varying the SEC elution volumes collected per fraction.

The sequential treatment of CSF samples by spin filtration followed by SEC fractionation is necessary for the CT pS396 signal enrichment because omitting either step leads to much reduced or undetectable amounts.

DETAILED DESCRIPTION OF THE INVENTION

Traditional ELISA has been used for many years in analysing molecules of different kinds in samples. The present invention is directed to the use of the single molecule array assay (Simoa) ELISA technology to detect tau species in samples from patients diagnosed with tauopathies such as Alzheimer’s disease, Pick’s disease, corticobasal degeneration, progressive supranuclear palsy, globular glial tauopathy and chronic traumatic encephalopathy

In the present invention tau is human tau of the following sequence, Methionine being number 1
MAEPRQEFEVMEDHAGTYGLGDRKDQGGYTMHQDQEGDTDAGLKESPLQT
PTEDGSEEPGSETSDAKSTPTAEDVTAPLVDEGAPGKQAAAQPHTEIPEG
TTAEEAGIGDTPSLEDEAAGHVTQARMVSKSKDGTGSDDKKAKGADGKTK
IATPRGAAPPGQKGQANATRIPAKTPPAPKTPPSSGEPPKSGDRSGYSSP
GSPGTPGSRSRTPSLPTPPTREPKKVAVVRTPPKSPSSAKSRLQTAPVPM
PDLKNVKSKIGSTENLKHQPGGGKVQIINKKLDLSNVQSKCGSKDNIKHV
PGGGSVQIVYKPVDLSKVTSKCGSLGNIHHKPGGGQVEVKSEKLDFKDRV
QSKIGSLDNITHVPGGGNKKIETHKLTFRENAKAKTDHGAEIVYKSPVVS
GDTSPRHLSNVSSTGSIDMVDSPQLATLADEVSASLAKQGL **(SEQ ID NO.: 1)**

In the first step of the single-molecule immunoassay capture antibodies are attached to the surface of paramagnetic beads (~ 2.7 um diameter) that will be used to concentrate a dilute solution of tau molecules in a sample. A biotinylated detection antibody is added to the mixture, and the capture and detector antibodies are allowed to react to the tau in the sample. To remove non-specific protein binding the mixture may be washed. Subsequently beta-galactosidase–labelled streptavidin is added, followed by an optional washing step, and resorufin beta-D-galctopyranoside is added. The reaction mixture is allowed to react and generate a fluorescent product which may be read and analysed in an appropriate machine.
The assay developed by the inventors of the present invention is based on two antibodies, a capture antibody and a biotinylated detector antibody. The capture antibody is conjugated to a paramagnetic bead as described in Example 1. These are specific for the phosphorylated (p) S396 of tau. The generation of such antibodies are disclosed in for example WO2017/009308 and these antibodies are further described in Table I below. In the Examples of the present invention the pS396 specific antibody used is the antibody designated “C10-2 Humanized” from patent WO2018/011073 and described in Table II below

Table I: pS396 antibodies disclosed in WO2017/009308

D1.2			
Light Chain	CDR1 (SEQ ID NO.: 2)	CDR2 (SEQ ID NO.: 3)	CDR3 (SEQ ID NO.: 4)
	RSSQSLVHSN GNTYLH	KVSNRFS	SQSTHVP
VL (SEQ ID NO.: 5)	DVMMTQTPLS LPVSLGDQAS ISCRSSQSLV HSNGNTYLHW HLQKPGQSPK FLIYKVSNRF SGVPDRFSGS GSGTDFTLKI SRVEAEDLGV YFCSQSTHVP FTFGSGTKLE IKRADAAPTV SIFPPSSEQL TSGGASVVCF LNNFYPKDIN VKWKIDGSER QNGVLNSWTD QDSKDSTYSM SSTLTLTKDE YERHNSYTCE ATHKTSTSPI VKSFNRNEC		
Heavy Chain	CDR1 (SEQ ID NO.: 6)	CDR2 (SEQ ID NO.: 7)	CDR3 (SEQ ID NO.: 8)
	KASGNTFTDY EIH	AIDPETGNTA YNQKFKG	SRGFDY
VH (SEQ ID NO.: 9)	QVQLQQSGAE LVRPGASVTL SCKASGNTFT DYEIHWVKQT PVHGLEWIGA IDPETGNTAY NQKFKGKARL TADKSSSTAY MELRSLTSED SAVYYCTRSR GFDYWGQGTT LTVSSAKTTP PSVYPLAPGC GDTTGSSVTL GCLVKGYFPE SVTVTWNSGS LSSSVHTFPA LLQSGLYTMS SSVTVPSSTW PSQTVTCSVA HPASSTTVDK KLEPSGPIST INPCPPCKEC HKCPAPNLEG GPSVFIFPPN IKDVLMISLT PKVTCVVVDV SEDDPDVRIS WFVNNVEVHT AQTQTHREDY NSTIRVVSAL PIQHQDWMSG KEFKCKVNNK DLPSPIERTI SKIKGLVRAP QVYILPPPAE QLSRKDVSLT CLVVGFNPGD ISVEWTSNGH TEENYKDTAP VLDSDGSYFI YSKLDIKTSK WEKTDSFSCN VRHEGLKNYY LKKTISRSPG K		
C10.2			
Light Chain	CDR1 (SEQ ID NO.: 10)	CDR2 (SEQ ID NO.: 11)	CDR3 (SEQ ID NO.: 12)

	QASQGTSINL N	GASNLED	LQHTYLP
VL (SEQ ID NO.: 13)	DVQMIQSPSS LSASLGDIVT MTCQASQGTS INLNWFQQKP GKAPKLLIYG ASNLEDGVPS RFSGSRYGTD FTLTISSLED EDMATYFCLQ HTYLPFTFGS GTKLEIKRAD AAPTVSIFPP SSEQLTSGGA SVVCFLNNFY PKDINVKWKI DGSERQNGVL NSWTDQDSKD STYSMSSTLT LTKDEYERHN SYTCEATHKT STSPIVKSFN RNEC		
Heavy Chain	CDR1 (SEQ ID NO.: 14)	CDR2 (SEQ ID NO.: 15)	CDR3 (SEQ ID NO.: 16)
	KASGYTFTDR TIH	YIYPGDGSTK YNENFKG	RGAMDY
VH (SEQ ID NO.: 17)	QVQLQQSDAE LVKPGASVKI SCKASGYTFT DRTIHWVKQR PEQGLEWIGY IYPGDGSTKY NENFKGKATL TADKSSSTAY MQLNSLTSED SAVYFCARRG AMDYWGQGTS VTVSSAKTTP PSVYPLAPGS AAQTNSMVTL GCLVKGYFPE PVTVTWNSGS LSSGVHTFPA VLQSDLYTLS SSVTVPSSTW PSETVTCNVA HPASSTKVDK KIVPRDCGCK PCICTVPEVS SVFIFPPKPK DVLTITLTPK VTCVVVDISK DDPEVQFSWF VDDVEVHTAQ TQPREEQFNS TFRSVSELPI MHQDWLNGKE FKCRVNSAAF PAPIEKTISK TKGRPKAPQV YTIPPPKEQM AKDKVSLTCM ITDFFPEDIT VEWQWNGQPA ENYKNTQPIM DTDGSYFVYS KLNVQKSNWE AGNTFTCSVL HEGLHNHHTE KSLSHSPGK		
C5.2			
Light Chain	CDR1 (SEQ ID NO.: 18)	CDR2 (SEQ ID NO.: 19)	CDR3 (SEQ ID NO.: 20)
	QASQDTSINL N	GASNLED	LQHTYLP
VL (SEQ ID NO.: 21)	DVQMIQSPSS LSASLGDIVT MTCQASQDTS INLNWFQQKP GKAPKLLIYG ASNLEDGVPS RFSGSRYGTD FTLTISSLED EDMATYFCLQ HTYLPFTFGS GTKLEIKRAD AAPTVSIFPP SSEQLTSGGA SVVCFLNNFY PKDINVKWKI DGSERQNGVL NSWTDQDSKD STYSMSSTLT LTKDEYERHN SYTCEATHKT STSPIVKSFN RNEC		

Heavy Chain	CDR1 (SEQ ID NO.: 22)	CDR2 (SEQ ID NO.: 23)	CDR3 (SEQ ID NO.: 24)
	KASGYTFTDR TIH	YIYPGDDSTK YNDNFKG	RGTMDY
VH (SEQ ID NO.: 25)	QVQLQQSDAE LVKPGASVKI SCKASGYTFT DRTIHWVKQR PEQGLEWIGY IYPGDDSTKY NDMFKAKATL TADKSSNTAY MQLNSLTSDD SAVYFCARRG TMDYWGQGTS VTVSSAKTTP PSVYPLAPGS AAQTNSMVTL GCLVKGYFPE PVTVTWNSGS LSSGVHTFPA VLQSDLYTLS SSVTVPSSTW PSETVTCNVA HPASSTKVDK KIVPRDCGCK PCICTVPEVS SVFIFPPKPK DVLTITLTPK VTCVVVDISK DDPEVQFSWF VDDVEVHTAQ TQPREEQFNS TFRSVSELPI MHQDWLNGKE FKCRVNSAAF PAPIEKTISK TKGRPKAPQV YTIPPPKEQM AKDKVSLTCM ITDFFPEDIT VEWQWNGQPA ENYKNTQPIM DTDGSYFVYS KLNVQKSNWE AGNTFTCSVL HEGLHNHHTE KSLSHSPGK		
C8.3			
Light Chain	CDR1 (SEQ ID NO.: 26)	CDR2 (SEQ ID NO.: 27)	CDR3 (SEQ ID NO.: 28)
	QASQGTSINL N	GSSNLED	LQHSYLP
VL (SEQ ID NO.: 29)	DVQMIQSPSS LSASLGDIVT MTCQASQGTS INLNWFQQKP GKAPKLLIYG SSNLEDGVPS RFSGSRYGTD FTLTISSLED EDMATYFCLQ HSYLPFTFGS GTKLEIKRAD AAPTVSIFPP SSEQLTSGGA SVVCFLNNFY PKDINVKWKI DGSERQNGVL NSWTDQDSKD STYSMSSTLT 180 LTKDEYERHN SYTCEATHKT STSPIVKSFN RNEC		
Heavy Chain	CDR1 (SEQ ID NO.: 30)	CDR2 (SEQ ID NO.: 31)	CDR3 (SEQ ID NO.: 32)
	KASGYTFTDR TIH	YIYPGDGSTK YNENFKG	RGAMDY
VH (SEQ ID NO.: 33)	QVQLQQSDAE LVNPGASVKI SCKASGYTFT DRTIHWVKQR PEQGLEWIGY IYPGDGSTKY		

	NENFKGKATL TADKSSSTAY MQLNSLASED SAVYFCARRG AMDYWGQGTS VTVSSAKTTP PSVYPLAPGS AAQTNSMVTL GCLVKGYFPE PVTVTWNSGS LSSGVHTFPA VLQSDLYTLS SSVTVPSSTW PSETVTCNVA HPASSTKVDK KIVPRDCGCK PCICTVPEVS SVFIFPPKPK DVLTITLTPK VTCVVVDISK DDPEVQFSWF VDDVEVHTAQ TQPREEQFNS TFRSVSELPI MHQDWLNGKE FKCRVNSAAF PAPIEKTISK TKGRPKAPQV YTIPPPKEQM AKDKVSLTCM ITDFFPEDIT VEWQWNGQPA ENYKNTQPIM DTDGSYFVYS KLNVQKSNWE AGNTFTCSVL HEGLHNHHTE KSLSHSPGK

Table II: pS396 antibodies disclosed in WO2018/011073

The antibodies in the below scheme are all engineered versions of the antibody designated “C10-2 humanized antibody”. Differences compared to the C10-2 humanized antibody are shown specifically, otherwise grey boxes in the table are intended to indicate identical amino acids as the C10-2 humanized antibody. Thus, for example, D55E has the same CDR 1-3 of the light chain and CDR1 and 3 of the heavy chain as the C10-2 humanized antibody (grey boxes), whereas the CDR2 of the heavy chain differs (amino acid residues are given) and thus VH differs from the C10-2 humanized antibody (amino acid residues are given)

C10-2 Humanized			
Light Chain	CDR1 (SEQ ID NO.: 34)	CDR2 (SEQ ID NO.: 35)	CDR3 (SEQ ID NO.: 36)
	QASQDTSINL N	GASNLET	LQHTYLPFT
VL (SEQ ID NO.: 37)	DVQMTQSPSS LSASVGDRVT MTCQASQDTS INLNWFQQKP GKAPKLLIYG ASNLETGVPS RFSGSRSGTD FTLTISSLQP EDMATYYCLQ HTYLPFTFGS GTKLEIKRTV AAPSVFIFPP SDEQLKSGTA SVVCLLNNFY PREAKVQWKV DNALQSGNSQ ESVTEQDSKD STYSLSSTLT		

	LSKADYEKHK VYACEVTHQG LSSPVTKSFN RGEC		
Heavy Chain	CDR1 (SEQ ID NO.: 38)	CDR2 (SEQ ID NO.: 39)	CDR3 (SEQ ID NO.: 40)
	DRTIH	YIYPGDGSTK YSQKFQG	RGAMDY
VH (SEQ ID NO.: 41)	QVQLVQSGAE VVKPGASVKI SCKASGYTFT DRTIHWVRQA PGQGLEWIGY IYPGDGSTKY SQKFQGRATL TADTSASTAY MELSSLRSED TAVYYCARRG AMDYWGQGTS VTVSSASTKG PSVFPLAPSS KSTSGGTAAL GCLVKDYFPE PVTVSWNSGA LTSGVHTFPA VLQSSGLYSL SSVVTVPSSS LGTQTYICNV NHKPSNTKVD KRVEPKSCDK THTCPPCPAP ELLGGPSVFL FPPKPKDTLM ISRTPEVTCV VVDVSHEDPE VKFNWYVDGV EVHNAKTKPR EEQYNSTYRV VSVLTVLHQD WLNGKEYKCK VSNKALPAPI EKTISKAKGQ PREPQVYTLP PSREEMTKNQ VSLTCLVKGF YPSDIAVEWE SNGQPENNYK TTPPVLDSDG SFFLYSKLTV DKSRWQQGNV FSCSVMHEAL HNHYTQKSLS LSPGK		
D55E			
Light Chain	CDR1	CDR2	CDR3
VL			
Heavy Chain	CDR1	CDR2 (SEQ ID NO.: 42)	CDR3
		YIYPGEGSTK YSQKFQGR	
VH (SEQ ID NO.: 43)	QVQLVQSGAE VVKPGASVKI SCKASGYTFT DRTIHWVRQA PGQGLEWIGY IYPGEGSTKY SQKFQGRATL TADTSASTAY MELSSLRSED TAVYYCARRG AMDYWGQGTS VTVSSASTKG PSVFPLAPSS KSTSGGTAAL GCLVKDYFPE PVTVSWNSGA LTSGVHTFPA VLQSSGLYSL SSVVTVPSSS LGTQTYICNV NHKPSNTKVD KRVEPKSCDK THTCPPCPAP ELLGGPSVFL		

	FPPKPKDTLM ISRTPEVTCV VVDVSHEDPE VKFNWYVDGV EVHNAKTKPR EEQYNSTYRV VSVLTVLHQD WLNGKEYKCK VSNKALPAPI EKTISKAKGQ PREPQVYTLP PSREEMTKNQ VSLTCLVKGF YPSDIAVEWE SNGQPENNYK TTPPVLDSDG SFFLYSKLTV DKSRWQQGNV FSCSVMHEAL HNHYTQKSLS LSPG		
D55Q			
Light Chain	CDR1	CDR2	CDR3
VL			
Heavy Chain	CDR1	CDR2 (SEQ ID NO.: 44)	CDR3
		YIYPGQGSTK YSQKFQGR	
VH (SEQ ID NO.: 45)	QVQLVQSGAE VVKPGASVKI SCKASGYTFT DRTIHWVRQA PGQGLEWIGY IYPGQGSTKY SQKFQGRATL TADTSASTAY MELSSLRSED TAVYYCARRG AMDYWGQGTS VTVSSASTKG PSVFPLAPSS KSTSGGTAAL GCLVKDYFPE PVTVSWNSGA LTSGVHTFPA VLQSSGLYSL SSVVTVPSSS LGTQTYICNV NHKPSNTKVD KRVEPKSCDK THTCPPCPAP ELLGGPSVFL FPPKPKDTLM ISRTPEVTCV VVDVSHEDPE VKFNWYVDGV EVHNAKTKPR EEQYNSTYRV VSVLTVLHQD WLNGKEYKCK VSNKALPAPI EKTISKAKGQ PREPQVYTLP PSREEMTKNQ VSLTCLVKGF YPSDIAVEWE SNGQPENNYK TTPPVLDSDG SFFLYSKLTV DKSRWQQGNV FSCSVMHEAL HNHYTQKSLS LSPG		
D55S			
Light Chain	CDR1	CDR2	CDR3
VL			
Heavy Chain	CDR1	CDR2	CDR3

		(SEQ ID NO.: 46)	
		YIYPGSGSTK YSQKFQGR	
VH (SEQ ID NO.: 47)	QVQLVQSGAE VVKPGASVKI SCKASGYTFT DRTIHWVRQA PGQGLEWIGY IYPGSGSTKY SQKFQGRATL TADTSASTAY MELSSLRSED TAVYYCARRG AMDYWGQGTS VTVSSASTKG PSVFPLAPSS KSTSGGTAAL GCLVKDYFPE PVTVSWNSGA LTSGVHTFPA VLQSSGLYSL SSVVTVPSSS LGTQTYICNV NHKPSNTKVD KRVEPKSCDK THTCPPCPAP ELLGGPSVFL FPPKPKDTLM ISRTPEVTCV VVDVSHEDPE VKFNWYVDGV EVHNAKTKPR EEQYNSTYRV VSVLTVLHQD WLNGKEYKCK VSNKALPAPI EKTISKAKGQ PREPQVYTLP PSREEMTKNQ VSLTCLVKGF YPSDIAVEWE SNGQPENNYK TTPPVLDSDG SFFLYSKLTV DKSRWQQGNV FSCSVMHEAL HNHYTQKSLS LSPG		
N32S			
Light Chain	CDR1 (SEQ ID NO.: 48)	CDR2	CDR3
	QASQDTSISL N		
VL (SEQ ID NO.: 49)	DVQMTQSPSS LSASVGDRVT MTCQASQDTS ISLNWFQQKP GKAPKLLIYG ASNLETGVPS RFSGSRSGTD FTLTISSLQP EDMATYYCLQ HTYLPFTFGS GTKLEIKRTV AAPSVFIFPP SDEQLKSGTA SVVCLLNNFY PREAKVQWKV DNALQSGNSQ ESVTEQDSKD STYSLSSTLT LSKADYEKHK VYACEVTHQG LSSPVTKSFN RGEC		
Heavy Chain	CDR1	CDR2	CDR3
VH			
N32Q			
Light Chain	CDR1 (SEQ ID NO.: 50)	CDR2	CDR3

	QASQDTSIQL Q		
VL (SEQ ID NO.: 51)	DVQMTQSPSS LSASVGDRVT MTCQASQDTS IQLNWFQQKP GKAPKLLIYG ASNLETGVPS RFSGSRSGTD FTLTISSLQP EDMATYYCLQ HTYLPFTFGS GTKLEIKRTV AAPSVFIFPP SDEQLKSGTA SVVCLLNNFY PREAKVQWKV DNALQSGNSQ ESVTEQDSKD STYSLSSTLT LSKADYEKHK VYACEVTHQG LSSPVTKSFN RGEC		
Heavy Chain	CDR1	CDR2	CDR3
VH			
N34S			
Light Chain	CDR1 (SEQ ID NO.: 52)	CDR2	CDR3
	QASQDTSINL S		
VL (SEQ ID NO.: 53)	DVQMTQSPSS LSASVGDRVT MTCQASQDTS INLSWFQQKP GKAPKLLIYG ASNLETGVPS RFSGSRSGTD FTLTISSLQP EDMATYYCLQ HTYLPFTFGS GTKLEIKRTV AAPSVFIFPP SDEQLKSGTA SVVCLLNNFY PREAKVQWKV DNALQSGNSQ ESVTEQDSKD STYSLSSTLT LSKADYEKHK VYACEVTHQG LSSPVTKSFN RGEC		
Heavy Chain	CDR1	CDR2	CDR3
VH			
N34Q			
Light Chain	CDR1 (SEQ ID NO.: 54)	CDR2	CDR3
	QASQDTSINL Q		
VL (SEQ ID NO.: 55)	DVQMTQSPSS LSASVGDRVT MTCQASQDTS INLQWFQQKP GKAPKLLIYG ASNLETGVPS		

	RFSGSRSGTD FTLTISSLQP EDMATYYCLQ HTYLPFTFGS GTKLEIKRTV AAPSVFIFPP SDEQLKSGTA SVVCLLNNFY PREAKVQWKV DNALQSGNSQ ESVTEQDSKD STYSLSSTLT LSKADYEKHK VYACEVTHQG LSSPVTKSFN RGEC		
Heavy Chain	CDR1	CDR2	CDR3
VH			
N32S,N34S			
Light Chain	CDR1 (SEQ ID NO.: 56)	CDR2	CDR3
	QASQDTSISL S		
VL (SEQ ID NO.: 57)	DVQMTQSPSS LSASVGDRVT MTCQASQDTS ISLSWFQQKP GKAPKLLIYG ASNLETGVPS RFSGSRSGTD FTLTISSLQP EDMATYYCLQ HTYLPFTFGS GTKLEIKRTV AAPSVFIFPP SDEQLKSGTA SVVCLLNNFY PREAKVQWKV DNALQSGNSQ ESVTEQDSKD STYSLSSTLT LSKADYEKHK VYACEVTHQG LSSPVTKSFN RGEC		
Heavy Chain	CDR1	CDR2	CDR3
VH			
N32Q, N34S			
Light Chain	CDR1 (SEQ ID NO.: 58)	CDR2	CDR3
	QASQDTSIQL S		
VL (SEQ ID NO.: 59)	DVQMTQSPSS LSASVGDRVT MTCQASQDTS IQLSWFQQKP GKAPKLLIYG ASNLETGVPS RFSGSRSGTD FTLTISSLQP EDMATYYCLQ HTYLPFTFGS GTKLEIKRTV AAPSVFIFPP SDEQLKSGTA SVVCLLNNFY PREAKVQWKV DNALQSGNSQ ESVTEQDSKD STYSLSSTLT LSKADYEKHK VYACEVTHQG LSSPVTKSFN RGEC		

Heavy Chain	CDR1	CDR2	CDR3
VH			
N32Q, N34Q			
Light Chain	CDR1 (SEQ ID NO.: 60)	CDR2	CDR3
	QASQDTSIQL Q		
VL (SEQ ID NO.: 61)	DVQMTQSPSS LSASVGDRVT MTCQASQDTS IQLQWFQQKP GKAPKLLIYG ASNLETGVPS RFSGSRSGTD FTLTISSLQP EDMATYYCLQ HTYLPFTFGS GTKLEIKRTV AAPSVFIFPP SDEQLKSGTA SVVCLLNNFY PREAKVQWKV DNALQSGNSQ ESVTEQDSKD STYSLSSTLT LSKADYEKHK VYACEVTHQG LSSPVTKSFN RGEC		
Heavy Chain	CDR1	CDR2	CDR3
VH			
N32S, N34Q			
Light Chain	CDR1 (SEQ ID NO.: 62)	CDR2	CDR3
	QASQDTSISL Q		
VL (SEQ ID NO.: 63)	DVQMTQSPSS LSASVGDRVT MTCQASQDTS ISLQWFQQKP GKAPKLLIYG ASNLETGVPS RFSGSRSGTD FTLTISSLQP EDMATYYCLQ HTYLPFTFGS GTKLEIKRTV AAPSVFIFPP SDEQLKSGTA SVVCLLNNFY PREAKVQWKV DNALQSGNSQ ESVTEQDSKD STYSLSSTLT LSKADYEKHK VYACEVTHQG LSSPVTKSFN RGEC		
Heavy Chain	CDR1	CDR2	CDR3
VH			

A101			
Light Chain	CDR1	CDR2	CDR3
VL			
Heavy Chain	CDR1	CDR2	CDR3 (SEQ ID NO.: 64)
			RGTMDY
VH (SEQ ID NO.: 65)	QVQLVQSGAE VVKPGASVKI SCKASGYTFT DRTIHWVRQA PGQGLEWIGY IYPGDGSTKY SQKFQGRATL TADTSASTAY MELSSLRSED TAVYYCARRG TMDYWGQGTS VTVSSASTKG PSVFPLAPSS KSTSGGTAAL GCLVKDYFPE PVTVSWNSGA LTSGVHTFPA VLQSSGLYSL SSVVTVPSSS LGTQTYICNV NHKPSNTKVD KRVEPKSCDK THTCPPCPAP ELLGGPSVFL FPPKPKDTLM ISRTPEVTCV VVDVSHEDPE VKFNWYVDGV EVHNAKTKPR EEQYNSTYRV VSVLTVLHQD WLNGKEYKCK VSNKALPAPI EKTISKAKGQ PREPQVYTLP PSREEMTKNQ VSLTCLVKGF YPSDIAVEWE SNGQPENNYK TTPPVLDSDG SFFLYSKLTV DKSRWQQGNV FSCSVMHEAL HNHYTQKSLS LSPG		
D55E, A101T			
Light Chain	CDR1	CDR2	CDR3
VL			
Heavy Chain	CDR1	CDR2 (SEQ ID NO.: 66)	CDR3 (SEQ ID NO.: 67)
		YIYPGQGSTK YSQKFQGR	RGTMDY
VH (SEQ ID NO.: 68)	QVQLVQSGAE VVKPGASVKI SCKASGYTFT DRTIHWVRQA PGQGLEWIGY IYPGEGSTKY SQKFQGRATL TADTSASTAY MELSSLRSED TAVYYCARRG TMDYWGQGTS VTVSSASTKG		

	PSVFPLAPSS KSTSGGTAAL GCLVKDYFPE PVTVSWNSGA LTSGVHTFPA VLQSSGLYSL SSVVTVPSSS LGTQTYICNV NHKPSNTKVD KRVEPKSCDK THTCPPCPAP ELLGGPSVFL FPPKPKDTLM ISRTPEVTCV VVDVSHEDPE VKFNWYVDGV EVHNAKTKPR EEQYNSTYRV VSVLTVLHQD WLNGKEYKCK VSNKALPAPI EKTISKAKGQ PREPQVYTLP PSREEMTKNQ VSLTCLVKGF YPSDIAVEWE SNGQPENNYK TTPPVLDSDG SFFLYSKLTV DKSRWQQGNV FSCSVMHEAL HNHYTQKSLS LSPG		
D55Q, A101T			
Light Chain	CDR1	CDR2	CDR3
VL			
Heavy Chain	CDR1	CDR2 (SEQ ID NO.: 69)	CDR3 (SEQ ID NO.: 70)
		YIYPGQGSTK YSQKFQGR	RGTMDY
VH (SEQ ID NO.: 71)	QVQLVQSGAE VVKPGASVKI SCKASGYTFT DRTIHWVRQA PGQGLEWIGY IYPGQGSTKY SQKFQGRATL TADTSASTAY MELSSLRSED TAVYYCARRG TMDYWGQGTS VTVSSASTKG PSVFPLAPSS KSTSGGTAAL GCLVKDYFPE PVTVSWNSGA LTSGVHTFPA VLQSSGLYSL SSVVTVPSSS LGTQTYICNV NHKPSNTKVD KRVEPKSCDK THTCPPCPAP ELLGGPSVFL FPPKPKDTLM ISRTPEVTCV VVDVSHEDPE VKFNWYVDGV EVHNAKTKPR EEQYNSTYRV VSVLTVLHQD WLNGKEYKCK VSNKALPAPI EKTISKAKGQ PREPQVYTLP PSREEMTKNQ VSLTCLVKGF YPSDIAVEWE SNGQPENNYK TTPPVLDSDG SFFLYSKLTV DKSRWQQGNV FSCSVMHEAL HNHYTQKSLS LSPG		
D55S, A101T			
Light Chain	CDR1	CDR2	CDR3

VL			
Heavy Chain	CDR1	CDR2 (SEQ ID NO.: 72)	CDR3 (SEQ ID NO.: 73)
		YIYPGSGSTK YSQKFQGR	RGTMDY
VH (SEQ ID NO.: 74)	QVQLVQSGAE VVKPGASVKI SCKASGYTFT DRTIHWVRQA PGQGLEWIGY IYPGSGSTKY SQKFQGRATL TADTSASTAY MELSSLRSED TAVYYCARRG TMDYWGQGTS VTVSSASTKG PSVFPLAPSS KSTSGGTAAL GCLVKDYFPE PVTVSWNSGA LTSGVHTFPA VLQSSGLYSL SSVVTVPSSS LGTQTYICNV NHKPSNTKVD KRVEPKSCDK THTCPPCPAP ELLGGPSVFL FPPKPKDTLM ISRTPEVTCV VVDVSHEDPE VKFNWYVDGV EVHNAKTKPR EEQYNSTYRV VSVLTVLHQD WLNGKEYKCK VSNKALPAPI EKTISKAKGQ PREPQVYTLP PSREEMTKNQ VSLTCLVKGF YPSDIAVEWE SNGQPENNYK TTPPVLDSDG SFFLYSKLTV DKSRWQQGNV FSCSVMHEAL HNHYTQKSLS LSPG		
N32S, A101T			
Light Chain	CDR1	CDR2	CDR3
	Same sequence as N32S CDR1		
VL	Same VL sequence as N32S		
Heavy Chain	CDR1	CDR2	CDR3
			Same sequence as A101 CDR3
VH	Same VH sequence as A101		
N32Q, A101T			
Light Chain	CDR1	CDR2	CDR3
	Same sequence as N32Q CDR1		
VL	Same VL sequence as N32Q		

Heavy Chain	CDR1	CDR2	CDR3
			Same sequence as A101 CDR3
VH	Same VH sequence as A101		
N34S, A101T			
Light Chain	CDR1	CDR2	CDR3
	Same sequence as N34s CDR1		
VL	Same VL sequence as N34S		
Heavy Chain	CDR1	CDR2	CDR3
			Same sequence as A101 CDR3
VH	Same VH sequence as A101T		
N34Q, A101T			
Light Chain	CDR1	CDR2	CDR3
	Same sequence as N34Q CDR1		
VL	Same VL sequence as N34Q		
Heavy Chain	CDR1	CDR2	CDR3
			Same sequence as A101 CDR3
VH	Same VH sequence as A101		

The detection/or detector (used interchangeably herein) antibodies used have been biotinylated as described in Example 2, and may bind the C-terminal, mid or N-terminal region of tau at a site different from the pS396 residue. The detector antibody may bind non-phosphorylated residues. In particular, epitopes of the C-terminus on tau are amino acids 1-20 (such as 6-18), the mid region of tau is amino acids 140-170 (such as 159-168) and N-terminus of tau is 400-441 (such as 404-421). In the Examples, the following antibodies are used:

Tau12 (#806502, BioLegend) is used and binds to the amino acids 6-18 of tau, detection antibody is HT7 (#MN1000, Invitrogen) binds mid region 159-168 amino acids, and Tau46 (#806601, BioLegend) binds the C-terminal region amino acids 404-441.

Three assays measuring pS396 on different tau species have been developed (**Fig 1**); each uses the anti-pS396 capture antibody and share all other experimental conditions except the detection antibodies, which have been described below.

1. **Full length pS396** (FL assay): this assay measures pS396 phosphorylated tau species stretching from the N-terminus region. The detection antibody, monoclonal Tau12 (#806502, BioLegend), binds to the amino acids 6-18 of tau.
2. **Mid region pS396** (MR assay): this assay measures tau forms simultaneously carrying two epitopes: pS396 phosphorylation and the mid region 159-168 amino acids. The detection antibody is HT7 (#MN1000, Invitrogen).
3. **C-terminus pS396** (CT assay): the assay is specific for pS396 phosphorylated tau that contains the extreme carboxyl terminus region (amino acids 404-441). The detection antibody is Tau46 (#806601, BioLegend).

The method, as described in Example 4, comprises the steps of

a. Mixing capture antibodies specific for pS396 attached to paramagnetic beads with a biotinylated detection antibody and a sample,
b. Incubating the mixture at a sufficient time to allow the antibodies to bind to tau in the sample (e.g. 1, 2, 3 or 5 minutes or more),
c. Optionally, washing the mixture in step b) after incubation,
d. Adding streptavidin-conjugated beta-galactosidase and allowing said streptavidin-conjugate and the biotinylated detector antibody to react (e.g. 1, 2, 3 or 5 minutes or more),
e. Optionally washing the obtained mixture after step d),
f. Adding resorufin beta-D-galactopyranoside to the mixture in step d) or e) and allowing the hydrolysis of resorufin beta-D-galactopyranoside (e.g. 1, 2, 3 or 5 minutes or more),

g. Reading the fluorescence signal and comparing the signal with a standard

The amount of pS396 conjugated antibody beads used are usually at least 1000 beads such as at least 10,000, 100,000 beads or more. The sample may be a
5 CSF, plasma or bio-fluid sample from a mammal, for example a human CSF sample from a human suffering from a tauopathy such as Alzheimer's disease, Pick's disease, corticobasal degeneration, progressive supranuclear palsy or globular glial tauopathy. It may be advantageous to concentrate the sample with respect to tau for example by use of spin filtration columns and size exclusion
10 chromatography as shown in **Example 3**.

The results of the assays used individually or in combination can be used to diagnose or differentiate tauopathies, such as Alzheimer's disease, Pick's disease, corticobasal degeneration, progressive supranuclear palsy and globular
15 glial tauopathy, as shown in **Example 4**. For example, the CT assay can be used to diagnose Alzheimer's disease and Pick's disease. By comparing the MR/FL ratio it can be used to differentiate Alzheimer's disease over the other tauopathies and the control, and further the Pick's disease was significant different from corticobasal degeneration, progressive supranuclear palsy,
20 globular glial tauopathy and the control. By suing the CT/FL ratio, Alzheimer's disease can be used to differentiate over the other tauopathies and the control and Pick's disease was different compared to corticobasal degeneration, progressive supranuclear palsy and globular glial tauopathy and control.

25 **EXPERIMENTAL DETAILS**

Example 1: Conjugation of capture antibody (pS396) to paramagnetic beads

The pS396 antibody was buffer exchanged into bead conjugation buffer (BCB; 50mM MES pH 6.2) using Ultracel 50K spin filtration columns (#UFC505096, Amicon). The filter was first rinsed with 450 ul BCB by centrifuging at 14000 xg at
30 room temperature (RT) for 5 min, and discarding the flow-through. Thereafter, 1.6 g/L antibody was buffer exchanged into BCB by centrifuging at 14000 xg, RT, for 5 min. The flow-through was discarded and the filter returned to the collection tube. BCB was added to the retentate to bring the volume to 450 ul, and re-centrifuged under the same conditions. This step was repeated once after discarding the flow-

through. Subsequently, the filter was rinsed with 40 ul BCB, inverted into a new collection tube and the antibody recovered by centrifuging for 2 min at 1000 xg, RT. The concentration of the antibody was estimated with Nanodrop Lite (ThermoFisher Scientific) and stored at 4 º C until use.

5 Paramagnetic carboxylated singleplex beads (#103207, Quanterix) were washed thrice with bead wash buffer (BWB; 1x PBS + 1 % Tween 20) and then twice with BCB using a magnetic separator. The beads at a concentration of 1.4×10^6 beads/µL were activated by adding 0.3 g/L 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (#A35391, Thermo Scientific)

10 and incubating at 4 º C for 30 min. Thereafter, the activated beads were washed once with ice-cold BCB and the supernatant discarded. The antibody (0.2 g/L) was added to the beads and the mixture incubated for 2 h at RT with shaking to allow the antibodies bind to the beads. Shaking was always performed with a HulaMixer Sample Mixer (#15920D, ThermoFisher Scientific) under the following conditions

15 with each step lasting 5 sec: orbital = 5 rpm, reciprocal = 90 º, vibro/pause = 5º. Afterwards, the supernatant was removed and the antibody-conjugated beads washed twice with BWB. The reaction was blocked with bead blocking buffer (1 % BSA in 1xPBS) for 1 h at RT with shaking. Finally, the beads were washed twice with BWB and then once with bead diluent (BD; 50 mM Tris pH 7.8, 50 mM NaCl,

20 10 mM EDTA, 1 % BSA, 0.1 % Tween20). After removing the supernatant with a magnetic separator, the beads were resuspended in BD and stored at 4 º C until use.

Example 2: Biotin conjugation to detection antibody

25 The detection antibodies were buffer exchanged into biotinylation reaction buffer (BRB; 100 mM PBS pH 7.4) in Ultracel 50K spin filtration columns (#UFC505096, Amicon). After cleaning the column by centrifuging 450 ul BRB at 14000 xg, RT, for 5 min, the flow-through was discarded and the antibody transferred to the filter. BRB was added to bring the volume to 450 ul and centrifuged at 14000 xg at RT

30 for 5 min. The buffer exchange was repeated two more times, at each stage by bringing the antibody volume to 450 ul with BRB and centrifuging for 5 min at 14000 xg, RT. The filter was rinsed with 40ul BRB, inverted into a new collection tube and the antibody recovered by centrifuging for 2 min at 1000 xg, RT. The concentration of the antibody was estimated using Nanodrop Lite. Forty times excess of EZ-Link

NHS-PEG4-Biotin (#21329, Thermo Scientific) was added to the antibody and incubated for 30 min at RT. Free biotin was removed by repeating the buffer exchange process performed prior to the biotin labelling. The biotin-conjugated antibodies were stored at 4 º C until use.

5 **Example 3: Pre-analytical processing of samples and calibrators**

Appropriate concentrations of the assay calibrator (recombinant tau 441 phosphorylated *in vitro* by Glycogen Synthase Kinase 3β (#TO8-50FN, SignalChem)) were prepared by diluting stock concentrations with the assay diluent (Tau 2.0 diluent, #101556, Quanterix) before analysis. Quality control
10 samples include TBS-soluble human Alzheimer's disease brain extract diluted 500 and 5000 times with the assay diluent.

Tris buffered saline (TBS)-soluble human brain extracts, rTg4510 transgenic mice CSF and plasma samples were diluted with Tau 2.0 diluent to the desired concentrations indicated in Figures 2 and 3 and their legends.

15 The level of pS396 tau in human CSF was enriched by concentrating samples in spin filtration columns and fractionating the retentate by size exclusion chromatography (SEC) on a Superdex S200 10/300 GL column (#17-5175-01, GE Healthcare) running on an Ethan LC system (GE Healthcare). The running buffer was 50 mM Tris pH 7.5 + 10% glycerol. Collected fractions were analysed directly
20 using the Simoa pS396 assays. This method has been verified using spin filtration columns of different capacities and properties (**Table 1**).

Table 1. Details of centrifugal filter devices and conditions used to concentrate human CSF prior to size-based fractionation to enrich pS396 signal. All devices
25 were purchased from Merck Millipore.

Filter name	**Catalogue number**	**Centrifugation conditions**	**CSF volume**
Ultracel®-YM3	4203	6500 xg, 4 h, 4 º C	12 x 2 ml*
Ultracel®-3K	UFC900308	4000 xg, 40 min, 25 º C	15 ml
Centriprep	4303	3000 xg, 95 min, 4 º C	15 ml
Microcon® -10	MRCPRT010	14000 xg, 40 min, RT	8 x 0.5 ml*
Amicon® Ultra-2ml	UFC200324	7500 xg, 40 min, RT	2 ml

**Retentate fractions from the indicated number of columns were pooled for further analyses.*

Example 4: Single molecule array (Simoa) assays

5 Each pS396 assay uses a two-step protocol on the Simoa HD-1 instrument (Quanterix, Lexington, MA, USA). In this assay configuration, 100ul of the bead mixture, consisting of 1000 beads/ul each of pS396 antibody-coated beads and Helper Beads (#103208, Quanterix), is aspirated into a reaction cuvette. Thereafter, 20ul biotinylated detection antibody (2ug/ml) and 100ul of the analyte 10 of interest were added and the reaction mixture incubated for 47 cadences (1 cadence = 45 sec) to allow the analyte to react with the capture and detection antibodies. The beads were subsequently washed and 100ul of 450 pM streptavidin-conjugated β-galactosidase (SBG; #100439, Quanterix). Following another incubation for 7 cadences and a subsequent wash, 25 µl resorufin β−D-15 galactopyranoside (RGP; #103159, Quanterix) was added. Hydrolysis of RGP was catalysed by SBG, yielding the fluorescent product resorufin. The beads were transferred onto a disc of 200,000 wells, each only large enough to accommodate one bead. Extra beads were removed and the disc surface sealed before imaging. The fluorescent signals were converted to average enzyme per bead (AEB) and 20 the sample concentrations extrapolated from a four-parametric logistic calibration curve generated with known protein concentrations.

Assay setups

Three assays measuring pS396 on different tau species have been developed (Fig 25 1); each uses the anti-pS396 capture antibody and share all other experimental conditions except the detection antibodies, which have been described below.

1. Full length pS396 (FL assay): this assay measures pS396 phosphorylated tau species stretching from the N-terminus region. The detection antibody, monoclonal Tau12 (#806502, BioLegend), binds to the amino acids 6-18 of 30 tau.
2. Mid region pS396 (MR assay): this assay measures tau forms simultaneously carrying two epitopes: pS396 phosphorylation and the mid region 159-168 amino acids. The detection antibody is HT7 (#MN1000, Invitrogen).

3. C-terminus pS396 (CT assay): the assay is specific for pS396 phosphorylated tau that contains the extreme carboxyl terminus region (amino acids 404-441). The detection antibody is Tau46 (#806601, BioLegend).

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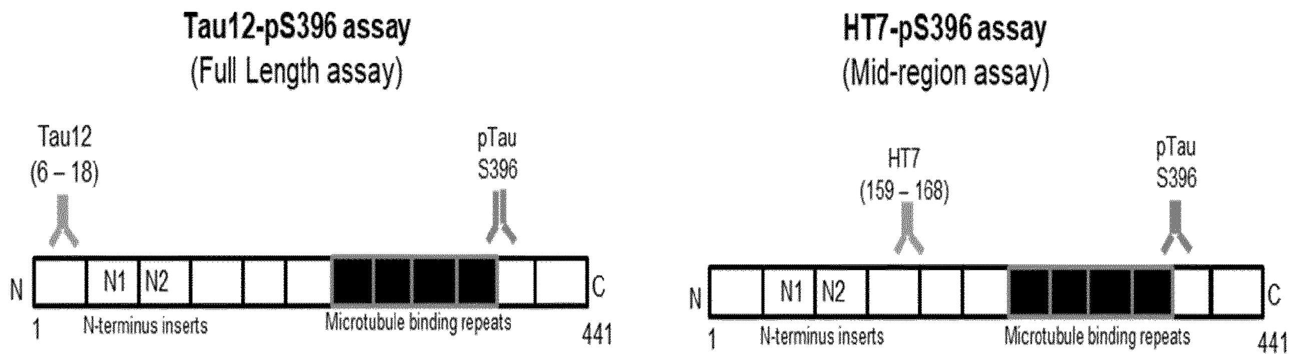
CLAIMS

1. An *in vitro* assay for measuring phosphorylated tau in a sample, said assay comprises the use of 2 antibodies i) a capture antibody specific for pS396 on tau and ii) a detection antibody binding tau on a different epitope than the capture antibody.
2. The assay according to claim 1, wherein said detection antibody is binding to an epitope within amino acids 1-20 on tau (such as 6-18), amino acids 140-170 (such as 159-168) on tau, or 400-441 (such as 404-421) on tau.
3. The assay according to claims 1-2, wherein the detection antibody is biotinylated.
4. The assay according to any one of the previous claims, wherein the capture antibody is attached to the surface of paramagnetic beads.
5. The assay according to any one of the previous claims, wherein beta-galactosidase conjugated streptavidin is used to generate a signal readout.
6. The assay according to any one of the previous claims, wherein the sample is a CSF, plasma or other bio-fluid sample from a mammal.
7. The assay according to any one of the previous claims, wherein the sample is a CSF or plasma sample from a human suffering from a tauopathy such as Alzheimer's disease, Pick's disease, corticobasal degeneration, progressive supranuclear palsy or globular glial tauopathy.
8. The assay according to any one of the previous claims, wherein the CSF sample has been concentrated with respect to tau for example by use of spin filtration columns and size exclusion chromatography.
9. Use of an assay according to any one of the previous claims for diagnosing a tauopathy such as Alzheimer's disease, Pick's disease, corticobasal degeneration, progressive supranuclear palsy or globular glial tauopathy.
10. A method for measuring phosphorylated tau in a sample, which method comprises the steps of
    a. Mixing capture antibodies specific for pS396 attached to paramagnetic beads with biotinylated detection antibodies and a sample,
    b. Incubating the mixture at a sufficient time to allow the antibodies to bind to tau in the sample (e.g. 1, 2, 3 or 5 minutes or more),
    c. Optionally, washing the mixture in step b) after incubation,

d. Adding streptavidin-conjugated beta-galactosidase and allowing said streptavidin-conjugate and the biotinylated detector antibody to react (e.g. 1, 2, 3 or 5 minutes or more),
e. Optionally washing the obtained mixture after step d),
5 f. Adding resorufin beta-D-galactopyranoside to the mixture in step d) or e) and allowing the hydrolysis of resorufin beta-D-galactopyranoside (e.g. 1, 2, 3 or 5 minutes or more),
g. Reading the fluorescence signal and comparing the signal with a standard

10 11. The method according to claim 10, wherein said capture antibodies are attached to paramagnetic beads and at least 1000 of said paramagnetic beads are added to the sample, such as at least 10000, 100,000 beads or more,
12. The method according to claim 10, wherein said detection antibody is binding
15 to an epitope within amino acids 1-20 on tau (such as 6-18), amino acids 140-170 (such as 159-168) on tau, or 400-441 (such as 404-421) on tau
13. The method according to claims 10-12, wherein the sample is a CSF, plasma or bio-fluid sample from a mammal.
14. The method according to claims 10-13, wherein the sample is a CSF or
20 plasma sample from a human suffering from a tauopathy such as Alzheimer’s disease, Pick’s disease, corticobasal degeneration, progressive supranuclear palsy or globular glial tauopathy.
15. The method according to claims 10-14, wherein said sample has been concentrated with respect to tau for example by use of spin filtration columns
25 and size exclusion chromatography.
16. Use of a method according to claims 10-15 for diagnosing a tauopathy such as Alzheimer’s disease, Pick’s disease, corticobasal degeneration, progressive supranuclear palsy or globular glial tauopathy.
17. Use of a method according to claims 10-17, wherein said detector antibody is
30 binding an epitope within amino acids 400-441 (such as 404-421) on tau to diagnose Alzheimer’s disease or Pick’s disease

A



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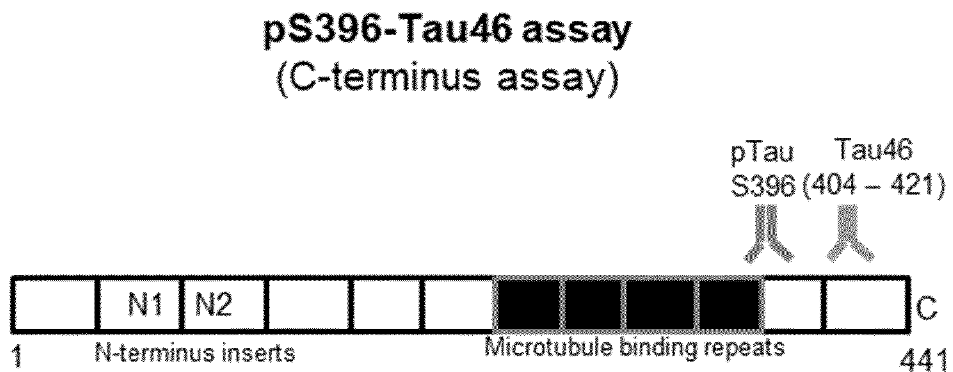
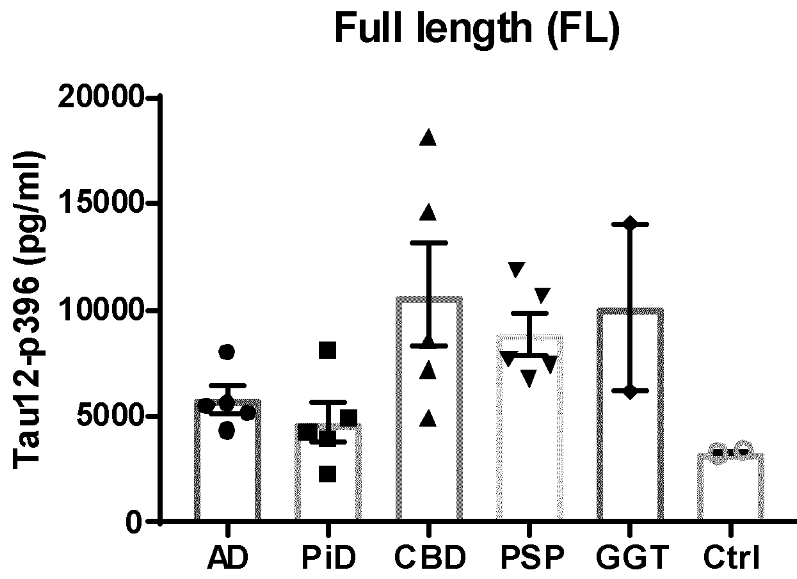


Fig 1

A



B

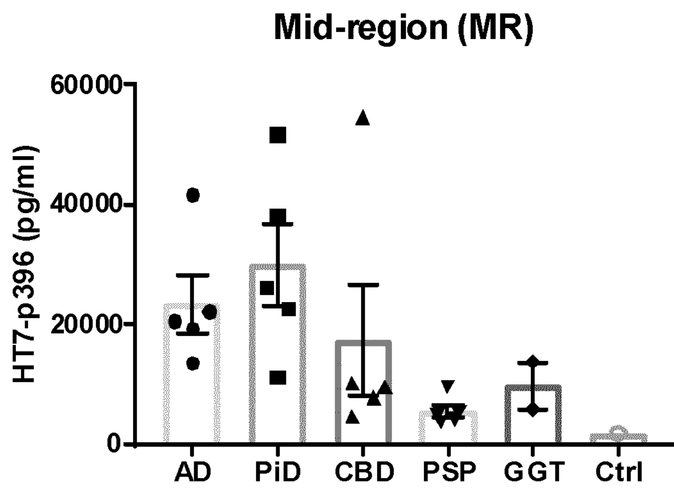
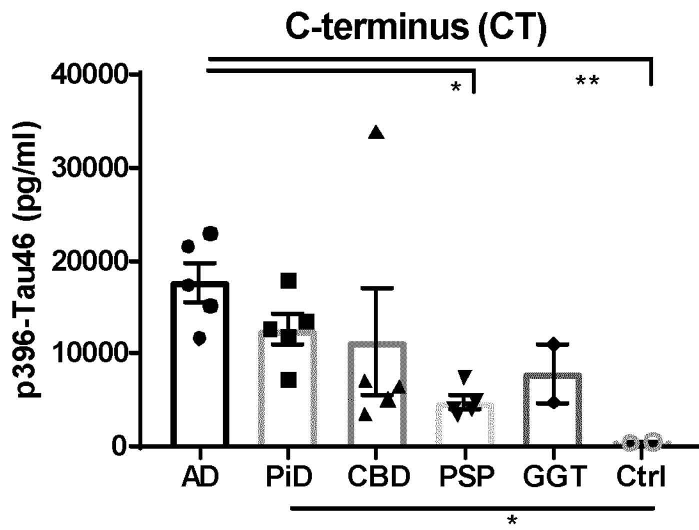


Fig 2A and Fig 2B

C



D

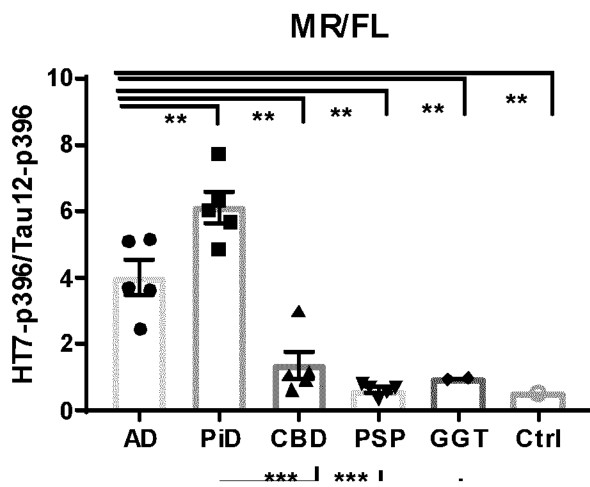


Fig 2C and Fig 2D

E

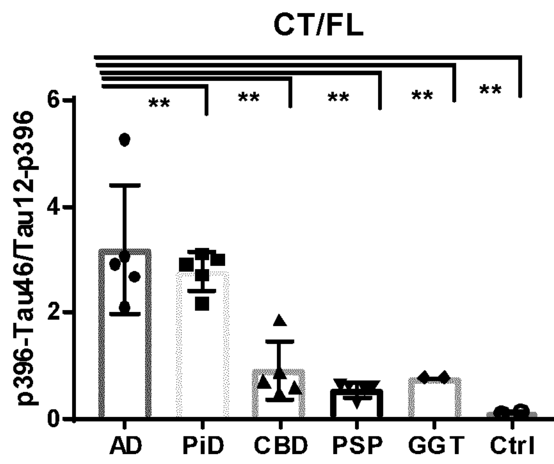
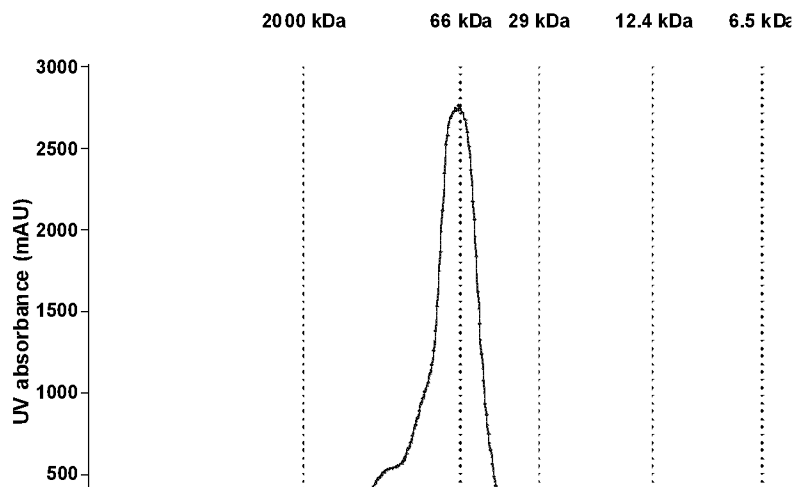


Fig 2E

Sample ID	Dilution	Measured (pg/ml)	Dilution corrected (pg/ml)
	CSF		
82-7	1/50	3.52	176.17
83-18	1/50	2.15	107.53
83-16	1/75	6.51	488.20
	Plasma		
82-7	1/10	17.43	174.29
82-8	1/10	12.93	129.34
83-18	1/10	15.06	150.55
83-17	1/10	15.95	159.54
83-16	1/20	3.27	65.38
82-9	1/20	17.32	346.31

Fig 3

A



B

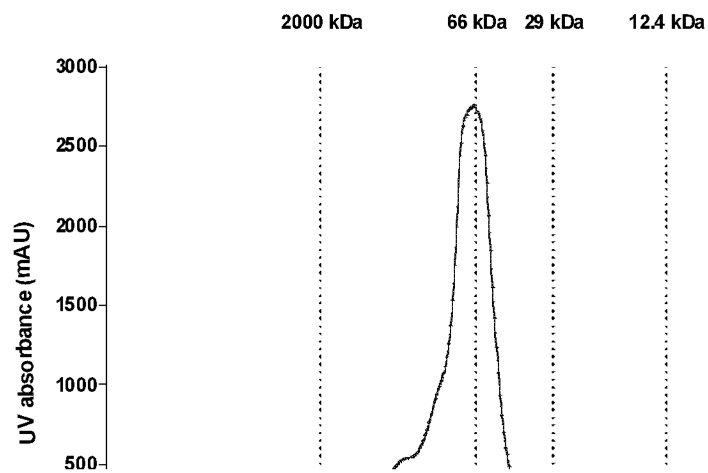


Fig 4A and Fig 4B

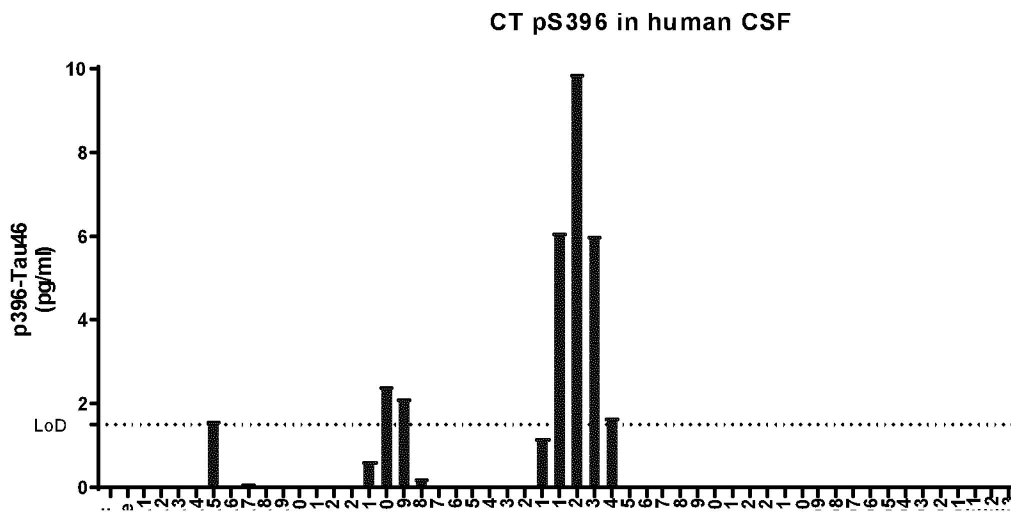


Fig 4C

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2020/058062

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, WPI Data, BIOSIS, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 01/55725 A2 (INNOGENETICS NV [BE]; VANMECHELEN EUGEEN [BE] ET AL.) 2 August 2001 (2001-08-02) paragraphs [01.2], [01.3]; claims 1-19; examples 1, 2 -----	1-17
Y	WO 2013/050567 A1 (AC IMMUNE SA [CH]; UNIV LEUVEN KATH [BE] ET AL.) 11 April 2013 (2013-04-11) claims 67,68; examples 7, 11 -----	1-17
	-/--	

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

[X] 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

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
7 July 2020	13/07/2020

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	Moreno de Vega, C

2

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	LIXIN SONG ET AL: "Analysis of tau post-translational modifications in rTg4510 mice, a model of tau pathology", MOLECULAR NEURODEGENERATION, BIOMED CENTRAL LTD, LO, vol. 10, no. 1, 26 March 2015 (2015-03-26), page 14, XP021215332, ISSN: 1750-1326, DOI: 10.1186/S13024-015-0011-1 abstract; figures 2, 7, S1; table 1 -----	1-17
X	CLARA THEUNIS ET AL: "Novel Phospho-Tau Monoclonal Antibody Generated Using a Liposomal Vaccine, with Enhanced Recognition of a Conformational Tauopathy Epitope", JOURNAL OF ALZHEIMER'S DISEASE, vol. 56, no. 2, 24 January 2017 (2017-01-24), pages 585-599, XP055531434, NL ISSN: 1387-2877, DOI: 10.3233/JAD-160695	1-3
Y	page 589, left-hand column, paragraph 2 - page 590, right-hand column, paragraph 2; figures 1, 5 page 597, left-hand column, paragraph 2 - paragraph 3 -----	1-17
Y	US 2017/254817 A1 (GRAFMAN JORDAN [US] ET AL) 7 September 2017 (2017-09-07) paragraph [0071] -----	1-17
X	NINA ROSENQVIST ET AL: "Highly specific and selective anti-pS396-tau antibody C10.2 targets seeding-competent tau", ALZHEIMER'S & DEMENTIA: TRANSLATIONAL RESEARCH & CLINICAL INTERVENTIONS, vol. 4, no. 1, 4 January 2018 (2018-01-04), pages 521-534, XP055702432, ISSN: 2352-8737, DOI: 10.1016/j.trci.2018.09.005	1,6
Y	abstract paragraph [02.6]; table 1 -----	1-17

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2020/058062

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