the desalting column and fractionation of collected peak after elution from the desalting column. An additional wash step was performed, after the general wash step recommended by the manufacturer, with IMAC binding buffer containing 50-60 mM imidazole.

After elution, relevant fractions were pooled and the protein concentration was determined spectrophotometrically at A_{280}. The purity of protein preparations was analyzed using 12 % Bis-Tris NuPAGE® gels according to the manufacturer's recommendation. When appropriate, DTT (reducing agent) was added to a final concentration of 50 mM. The gels were subsequently stained with Coomassie.

Biosensor analysis

The purified fusion polypeptides' ability to bind $A\beta_{42}$ was analyzed using surface plasmon resonance on a Biacore® 2000 system. $A\beta_{42}$ was immobilized in different flow cells by amine coupling onto the carboxylated dextran layer on surfaces of a CM-5 chip, according to the manufacturer's recommendations. Immobilization of $A\beta_{42}$ resulted in 470 RU. One flow cell surface on each chip was activated and deactivated for use as reference cell during injections. Samples of His_6-$(Z_{A\beta 1,\ 3,\ 4,\ 12,\ 18\ and\ 19})_2$-myc were diluted in HBS-EP to a final concentration of 5 µM and injected in random order at a constant flow rate of 30 µl/min for 5 minutes. After each injection, the surfaces were regenerated with 2 injections of 50 mM NaOH. The samples' ability to interact with Aβ was confirmed, as illustrated by the sensorgrams in Figure 8.

Production of E. coli lysate

The *E. coli* strain HB101F' was cultivated in 50 ml TSB medium overnight at 37 °C. The cells were harvested by centrifugation at 6000 x *g* for 8 min and the pellets were stored in -20 °C until protein preparation. Cell pellets were resuspended in 40 ml PBS and proteins were released by sonication. The total protein concentration

from strain HB101F' lysate was determined using the Pierce BCA-protein assay as recommended by the manufacturer.

Affinity chromatography

The specificity of the polypeptides His_6-($Z_{A\beta 1,3,\ 4\ and\ 12}$)$_2$-myc was evaluated by applying *E. coli* lysate prepared as described above, human plasma and PBS, all spiked with $A\beta_{42}$, to columns having $Z_{A\beta}$ variants coupled thereto, followed by washing and elution. Spiked *E. coli* lysate was only applied to a column with His_6-($Z_{A\beta 1}$)$_2$-myc, whereas spiked plasma and spiked PBS were applied to all four columns. 1 mg of each His_6-($Z_{A\beta 1,\ 3,\ 4\ and\ 12}$)$_2$-myc variant were separately coupled to 1 ml HiTrap® affinity columns (with NHS-activated Sepharose® High Performance), according to the manufacturer's recommendations. The coupling efficiencies of the different $Z_{A\beta}$ variants to the columns were almost 100 %, determined by using a protocol from the manufacturer (data not shown). The columns were connected to an ÄKTA™explorer 10 system, and equilibrated with 5 column volumes of PBS buffer. 1 ml of *E. coli* lysate or plasma supplemented with 100 µg $A\beta_{42}$ was loaded onto the different $Z_{A\beta}$ columns at a flow rate of 0.2 ml/minute. PBS supplemented with 100 µg $A\beta_{42}$ was also separately loaded onto the columns. The $A\beta_{42}$ was eluted with 0.3 M HAc, pH 2.8. The selected fractions from the purification were lyophilized and dissolved in PBS or water. The purity of protein preparations was analyzed using 12 % Bis-Tris NuPAGE®. A NuPAGE® gel stained with Coomassie indicated that $A\beta_{42}$ was the only product detected in the eluates originating from the spiked *E. coli* lysate separation experiment (data not shown). The other gels were stained with silver. The results for spiked PBS and spiked plasma are shown in Figure 9. As shown in Figure 9, $A\beta_{42}$ was only found in the eluates and not detected in the flow through and wash fractions. Thus, from these experiments it is evident that $A\beta_{42}$ could be efficiently

captured from complex solutions using immobilised, $A\beta_{42}$-binding Z mutants according to the invention.

Example 5

Investigation of the possibility that selected Aβ-binding polypeptides bind also to Amyloid Precursor Protein (APP)

In this Example, the Aβ binding polypeptides His_6-$Z_{A\beta1}$-myc, His_6-$Z_{A\beta3}$-myc, His_6-$Z_{A\beta4}$-myc, and His_6-$Z_{A\beta12}$-myc were studied in order to determine whether they also bind to the Amyloid Precursor Protein (APP). In order to investigate this, immunofluorescence staining was performed on an APP^+ human neuroblastoma cell line, SH-SY5Y.

Cell preparation

SH-SY5Y cells (DSMZ, ACC209) were grown in a 25 cm^3 flask in DMEM (Dulbecco's Modified Eagles Medium, Gibco 41965-039) supplemented with 15 % FCS (Fetal Calf Serum, Gibco 10106-165). The cells were harvested from the flask by trypsination and pelleted by centrifugation. The pellet was resuspended in 3 ml cell culture medium and 1 drop was added per field/well of a Histolab glass slide with 8 fields. The density was checked by microscope examination and extra cells were added if the density was found to be too low. An extra drop of medium was added per field/well to avoid dehydration. The cells were left to grow overnight at 37 °C and 5 % CO_2. The cells were either stained directly or fixed with 2 % formaldehyde (Sigma F1635) in PBS for 10 minutes.

Immunofluorescence staining

The volumes used were approximately 20 ml/well for all incubations. Dilutions and washes were made with PBS or PBS + 0,1 % saponin (Sigma S4521) and washing was done for 3 x 5 min. All incubations were performed at room temperature inside a humidified, dark chamber. The last

wash was done in PBS for 5 min, followed by addition of 1 drop DAPI (4,6-diamidino-2-phenylindole, Molecular Probes, D1306) per well. DAPI was removed after 20 seconds by washing in PBS. The slides were left to dry and thereafter mounted with α-fading (Vector H-1000) before examination in an UV-microscope. Two pictures were taken for each photo, for ALEXA (green) and DAPI (blue) staining at 400x or 630x magnification using a CCD camera (Leica).

The first step staining with myc-tagged Aβ-specific polypeptides according to the invention (5 mg/ml) or goat-α-APP (Biosite A2275-69R) (1:100) was done on living or fixed cells. Incubation was carried out at 37 °C in 5 % CO_2 for 1 h. The slides were rinsed in PBS and living cells were then fixed with 2 % formaldehyde in PBS for 10 min. Slides were then blocked for 30 min with blocking solution and emptied. Then, the second step mouse-α-myc (Sigma F1635) was added, with or without saponin (separate slides), to the wells that had previously been incubated with Aβ-specific polypeptides. The second step was incubated for 1 h at RT. In addition, wells that received the positive control, goat-α-APP, were incubated for one additional hour with the same antibody. Slides were washed and anti-mouse/anti-goat Alexa 488 antibodies (Molecular Probes, D1306/A211467) were added. The slides were washed after 30 min of incubation and nuclei of cells were stained with DAPI before mounting and examination.

The resulting immunostaining images are presented as Figures 10A-D. The goat-α-APP antibody gave a bright intracellular staining (Figure 10A). Addition of saponin resulted in a considerably higher percentage of positive cells (Figure 10B). All cells that were fixed with formaldehyde after the first step staining with Aβ binding polypeptides according to the invention, and had no saponin added, were completely negative (Figure 10C). The His_6-$Z_{aβ4}$-myc and His_6-$Z_{aβ12}$-myc molecules gave a weak in-

tracellular, unspecific signal if saponin was added (Figure 10D), but the His_6-$Z_{A\beta1}$-myc and His_6-$Z_{A\beta3}$-myc molecules remained completely negative also in the presence of saponin (data not shown). In conclusion, the experiment suggests that the four tested Aβ binding polypeptides do not bind to APP.

Example 6

Expression of Aβ binding polypeptides His_6-$(Z_{A\beta1\_C28S})_2$-Cys and His_6-$(Z_{A\beta3\ C28S})_2$-Cys, and use of His_6-$(Z_{A\beta3\ C28S})_2$-Cys as capture ligand in affinity chromatography

In this Example, the two Aβ binding polypeptide dimers His_6-$(Z_{A\beta1\_C28S})_2$-Cys and His_6-$(Z_{A\beta3\ C28S})_2$-Cys were created and studied. The two variants $Z_{A\beta1}$ and $Z_{A\beta3}$ each contain an internal cysteine residue, giving rise to multimerization of the Aβ binding polypeptides. It is thought that some of the binding sites will, upon multimerization, be hidden from the Aβ target, thus resulting in a less efficient binding of Aβ peptides. Therefore, the Aβ binding polypeptides $Z_{A\beta1}$ and $Z_{A\beta3}$ were subjected to site-directed mutagenesis of the amino acid at position 28 from a cysteine residue to serine. These new variants still exhibited good binding to $A\beta_{42}$. The constructs His_6-$(Z_{A\beta1\ C28S})_2$-Cys and His_6-$(Z_{A\beta3\ C28S})_2$-Cys made it possible to direct immobilization of the Aβ binding polypeptides, using the C-terminal cysteine residue (disulphide bridges).

Mutagenesis, expression and purification

The sequences of Aβ binding variants $Z_{A\beta1}$ and $Z_{A\beta3}$ were subjected to site-directed mutagenesis using conventional methods. In this way, the cysteine residue at position 28 of both molecules was replaced with a serine residue. The resulting Aβ binding variants were denoted $Z_{A\beta1\ C28S}$ and $Z_{A\beta3\ C28S}$, and are represented in Figure 1 by SEQ

ID NO:45 and 46, respectively. By way of conventional methods of molecular biology, the mutated $Z_{A\beta}$ polypeptides were expressed using expression vectors encoding His_6-$(Z_{A\beta1\ C28S\ and\ 3\ C28S})_2$-Cys constructs His_6 represents a hexahistidyl tag, and $Z_{A\beta}$ represents either of the Aβ binding domains having the sequences $Z_{A\beta1\ C28S\ and\ 3\ C28S}$. A C-terminal cysteine residue was added.

Immobilization of biotinylated His_6-$(Z_{A\beta3\ C28S})_2$-Cys

The Aβ binding polypeptide His_6-$(Z_{A\beta3\ C28S})_2$-Cys was biotinylated so it could become immobilized on streptavidin columns (HiTrap streptavidin HP, Amersham Biosciences). The procedure for biotinylation was to reduce the sample with DTT in a molar excess of 200x for 45 min at 37 °C followed by buffer exchange on NAP™5 columns (Amersham Biosciences) according to the manufacturer's instructions. A 33x molar excess of biotin (NoWeight™ Maleimide PEO_2-Biotin, Pierce) was dissolved in PBS and added to the sample with incubation for 2 h at room temperature. A final buffer exchange was performed overnight to get the biotinylated Aβ binding polypeptides in "binding buffer" (20 mM sodium phosphate, 0.15 M NaCl, pH 7.5), using a Slide-A-Lyzer® Dialysis Cassette with a cut-off of 3500 Da (Pierce).

To equilibrate the streptavidin column, 10 ml of binding buffer was passed through the column with a flow rate of 1 ml/min. For immobilization, His_6-$(Z_{A\beta3\ C28S})_2$-Cys was added (1.25 mg of the biotinylated His_6-$(Z_{A\beta3\ C28S})_2$-Cys in a volume of 1 ml) at a flow rate of 0.1 ml/min. Washing with binding buffer was performed (10 ml, 1 ml/min), and the column was stored in binding buffer containing 20 % EtOH.

Affinity chromatography

The specificity of the Aβ binding polypeptide His_6-$(Z_{A\beta3\ C28S})_2$-Cys was evaluated by applying 1 ml of human serum (H4522, Sigma-Aldrich Sweden AB) or human plasma

(provided by Affibody AB, samples from 20 individuals), unspiked or spiked with 100 µg $A\beta_{1-42}$ (American Peptide Company) to the column with immobilized Aβ binding polypeptides, followed by washing and elution. The chromatographic method used is illustrated in Table 2.

Briefly, 100 µg Aβ was added to 1 ml serum and let through the column. 1 ml unspiked serum was used as a negative control. Washing with binding buffer was followed by a preliminary elution step (0.3 M HAc, pH 3.5) to release some of the "sticky" serum proteins. Finally, the Aβ was eluted with 0.3 M HAc, pH 2.8.

TABLE 2. Chromatography of samples with 100 µg/ml Aβ in serum ("spiked") and control samples ("unspiked")

Steps	No. of steps	Flow rate [ml/min]	Total volume [ml]
1. Wash (binding buffer/HAc, pH 2.8)	2	1	10*2
2. Equilibration (binding buffer)	1	1	10
3. Sample loading	1	0.1	~1
4. Wash (binding buffer)	1	1	30
5. Pre-elution (0.3 M HAc, pH 3.5)	1	1	10
6. Elution (0.3 M HAc, pH 2.8)	1	0.25	15
7. Regeneration wash (binding buffer)	1	1	30
8. Wash (HAc, pH 2.8 / binding buffer)	2	1	10*2
9. Re-equilibration (binding buffer)	1	1	30

During purification of Aβ peptide, fractions from each step were collected, concentrated (SpeedVac® System, Savant) and dissolved in reducing agent. Purity of protein preparations was analyzed with SDS-PAGE (Novex system, Invitrogen) in gels with 12 wells (NuPAGE™ 4-12 % Bis-Tris Gel, Invitrogen). A molecular weight marker

ranging from 3-185 kD was used (MultiMark® Multi-colored standard, Invitrogen). After the first addition of reducing agent, the samples were heated (96 °C, 5 min) giving completely reduced proteins. 10 µl of loading buffer (reducing agent, 20 % glycerol (87 % stock), 1 ml) was added to 3 µl of the protein samples. The gels were run in NuPAGE® MES SDS Running buffer (50 mM MES, 50 mM Tris Base, 0.1 % SDS, 1 mM EDTA, pH 7.3) at 125 V for 1.5 h, and stained by silver stain (SilverXpress® Silver Staining Kit, Invitrogen) according to the manufacturer's instructions.

Western Blot with antibodies against Aβ was used to find out what bands on the gels corresponded to Aβ. Transfer buffer (25 mM Bicine, 25 mM Bis-Tris, 1 mM EDTA, pH 7.2) was used for transferring of the proteins (Novex system, Invitrogen), 25 V for 2h. The membrane (Nitrocellulose Membrane Filter Paper Sandwich, Invitrogen) was blocked overnight in blocking solution (1 % milk powder in TST) before 3 x 5 min washing in TST (0.025 M Tris-HCl, 0.2 M NaCl, 1 mM EDTA, 0.05 % Tween 20, pH 8.0). A primary antibody (directed against amino acids 1-16 of Aβ, mouse monoclonal, Nordic BioSite) was added (1:2000), and samples incubated for 1.5 h. Another wash was performed before addition of a secondary antibody (rabbit-anti-mouse, conjugated with alkaline phosphatase, Dako) (1:1000), and incubation for 1.5 h. A final wash of the membrane was performed before developing of the membrane (alkaline phosphatase substrate tablet, insoluble, B-5655 Sigma) according to the supplier's recommendation.

The results of the experiment are displayed in Figure 11, showing the SDS-PAGE and Western Blot analysis of fractions from affinity chromatography of (A) 1 ml serum spiked with 100 µg $A\beta_{42}$ peptide and (B) 1 ml unspiked serum. Fifteen 1 ml fractions were eluted with 0.3 M HAc pH 2.8 and analyzed in lanes 6-8 of the gel. Lane 1: MultiMark® Multi-colored Standard (kD). Lane 2: Unspiked serum (1:100). Lane 3: Spiked serum (1:100). Lane 4: Flow

through (1:100). Lane 5: Pre-elution wash with 0.3 M HAc pH 3.5. Lane 6: Eluted fractions 1-2. Lane 7: Eluted fractions 3-10. Lane 8: Eluted fractions 11-15. Lane 9: Empty. Lane 10: Wash after equilibration of column, acidic pulses (HAc 0.3 M, pH 2.8). Lane 11: Aβ control (1 µg). Lane 12: HSA control (1 µg).

As can be seen in Figure 11A, the affinity column with biotinylated His_6-$(Z_{Aβ3\ C28S})_2$-Cys immobilized thereto was able to efficiently separate the content of Aβ peptide from the spiked serum samples.

Chromatography of physiological levels of Aβ peptides in serum

A patient suffering from Alzheimer's disease normally has a concentration of Aβ peptides in blood in the range of picograms to nanograms per ml. Therefore, the Aβ binding polypeptide His_6-$(Z_{Aβ3\ C28S})_2$-Cys was tested in conditions closer to physiological levels of the target Aβ peptide, i e a lower concentration of Aβ in serum was let through the columns than in the previous experiment.

Immobilization of biotinylated Aβ binding polypeptide to the column was performed as above, except that 372 µg of biotinylated His_6-$(Z_{Aβ3\ C28S})_2$-Cys in a volume of 300 µl was used.

100 ng Aβ peptide was added to 1 ml serum and run through the column using the chromatography protocol illustrated in Table 3.

TABLE 3. Chromatography of samples with physiological levels of Aβ in serum (100 ng/ml)

Steps	No. of steps	Flow rate [ml/min]	Total volume [ml]
1. Equilibration (binding buffer)	1	1	15
2. Sample loading	1	0.1	~1
3. Wash (binding buffer)	1	1	30
4. Wash (0.3 M HAc, pH 4.3)	1	1	15
5. Elution (0.3 M HAc, pH 2.8)	1	0.25	15
6. Regeneration wash (binding buffer, pH 7.5)	1	1	10
7. Regeneration wash (HAc, pH 4.3)	1	1	10
8. Regeneration wash (HAc, pH 2.8 / binding buffer)	2	1	10*2
9. Re-equilibration step (binding buffer)	1	1	10

The "physiological samples" were treated differently after concentration (SpeedVac® System, Savant) than the samples from the previous experiment, since everything in the eluted fractions (split in 15 x 1 ml eppendorf tubes) needed to be loaded in the gel, to make detection of these small amounts of Aβ possible. What was left in the eppendorf tubes was dissolved in 200 µl sterile H_2O. 800 µl of acetone was added for precipitation of the proteins (5 minutes, -20 °C). The samples were centrifuged (13000 rpm, 20 minutes, 4 °C) and the pellets were left to dry. 10 µl of reducing agent was added to the first eluted fraction and dissolved. This mixture was then transferred to the second fraction, followed by the third and so on. Thus, everything in the fifteen eluted fractions was finally collected in three test tubes, each with a volume of 10 µl reducing agent. The whole amount was loaded on the gel, together with 10 µl of loading buffer. Collected

fractions at the end of each wash were dissolved in 10 µl of reducing agent, where of 3 µl was added in the gel, mixed with 10 µl of loading buffer.

Western Blot was performed as described above.

The results of the experiment are displayed in Figure 12, showing the SDS-PAGE and Western Blot analysis of fractions from affinity chromatography of 1 ml serum spiked with 100 ng $A\beta_{42}$ peptide using the biotinylated $His_6-(Z_{A\beta3\ C28S})_2$-Cys immobilized to a streptavidin column. Fifteen 1 ml fractions were eluted with 0.3 M HAc pH 2.8, collected as described above, and analyzed in lanes 5-7 of the gel. Lane 1: MultiMark® Multi-colored Standard (kD). Lane 2: Unspiked serum (1:100). Lane 3: Spiked serum (1:100). Lane 4: Flow through (1:100). Lane 5: Eluted fractions 2-5. Lane 6: Eluted fractions 6-10. Lane 7: Eluted fractions 11-15. Lane 8: Empty. Lane 9: Wash before elution (0.3 M HAc pH 4.3). Lane 10: Wash after equilibrating the column, acidic pulses (HAc 0.3 M, pH 2.8). Lane 11: Aβ control (100 ng). Lane 12: Aβ control (1 µg).

The results of the experiment demonstrate that a polypeptide according to the invention is useful for selective capture of amyloid beta peptide from plasma at physiological conditions.

Example 7

In vitro assay: Aβ sink

An *in vitro* assay was developed to identify the relative efficacy of Aβ binding polypeptides on sequestering soluble Aβ peptides. A set-up of two compartments, separated by a 10 kD cut off dialysis membrane (Mini Dialysis Units, Slide-A-Lyzer, Pierce) with volumes of 500 µl (top chamber) and 950 µl (bottom chamber) was used.

The size of monomeric Aβ peptides is 4 kD and the Aβ binding polypeptides have a size of 15 kD. Molecules lar-

ger than 10 kD cannot pass through the membrane. Since it is known that Aβ peptides tend to aggregate, forming multimeric complexes, the pore size of the membrane was chosen to be large enough to also let dimeric Aβ peptides through. It was presumed that the fraction of monomeric and dimeric Aβ peptides in the chamber containing Aβ peptides was large enough to show a possible "Aβ sink" effect by the Aβ binding polypeptides of the invention.

The Aβ binding polypeptide His_6-$(Z_{A\beta3\ C28S})_2$-Cys was placed in the bottom chamber at a concentration of 20 µg/ml (total volume 950 ml). The top chamber contained biotinylated $A\beta_{40}$ (US Biological) at a concentration of 10 ng/ml (total volume 500 ml). Aβ binding polypeptide and Aβ peptide were diluted in PBS. To compare the possible "pulling effect" of the Aβ binding polypeptide, a negative control (PBS in the bottom chamber) was used. As a positive control, a monoclonal antibody against Aβ (α-$A\beta_{17-26}$, US Biological) was used. The antibody was diluted in PBS at the same concentration as the Aβ binding polypeptide according to the invention (20 µg/ml). The three test tubes (test sample and the two controls) were placed upright in room temperature overnight. The set-up is shown schematically in Figure 13.

To detect the levels of Aβ in the different compartments, an ELISA was developed for detection of biotinylated Aβ peptides (see below). Samples from each compartment were taken after more than 14 h, and added in 2-step dilution series to double wells in the ELISA for detection. Standard samples were made with known concentrations of biotinylated Aβ in PBS (ranging from 0 to 25 ng/ml).

For the ELISA, 96-well plates (Costar) were coated with 100 µl/well of capture antibodies (mouse anti human-$A\beta_{1-16}$, Nordic BioSite) diluted in coating buffer (0.1 M sodium carbonate, pH 9.5) to a concentration of 0.1 µg/ml overnight. Washing (PBS with 0.05 % Tween 20) removed any free antibody. Blocking buffer (0.5 % casein in PBS) was

added for 2 h at room temperature and followed by another wash. 100 µl/well of sample or standard (Biotinylated $A\beta_{40}$, US Biological) was added in 2-step dilution series to duplicate wells and incubated for 1-2 h at room temperature. Washing was then performed to remove unbound sample. 100 µl/well of HRP conjugated streptavidin, 1:5000 (P0397 Dako) was added and incubated on a shaker for 1 h at room temperature. Washing was performed, and followed by addition of 100 µl/well of substrate (Immunopure TMB substrate kit, Pierce), which was incubated for 15-30 minutes. The color-changing reaction was stopped by adding 100 µl/well of stop solution (2 M H_2SO_4) according to the manufacturer's instructions. The plates were read at 450 nm (Magellan Sunrise).

With the help of a standard curve prepared using samples with known concentrations of Aβ, the ELISA result of the samples could be translated to a concentration.

Concentrations determined by ELISA after 14 h are shown in Table 4, and suggest that the Aβ binding polypeptides is able to sequester soluble Aβ through a membrane.

TABLE 4. Concentrations of Aβ in the *in vitro* assay

	His_6-$Z_{A\beta3\ 28S})_2$-Cys		α-$A\beta_{17-26}$		PBS	
	Top	Bottom	Top	Bottom	Top	Bottom
Aβ conc. at start [ng/ml], t = 0 h	10	0	10	0	10	0
Aβ conc. at stop [ng/ml], t = 14 h	3	0.7	3	0.1	3	0.2
Aβ in bottom chamber/ top chamber		23%		3%		7%

CLAIMS

1. Amyloid beta peptide binding polypeptide, which is related to a domain of staphylococcal protein A (SPA) in that the sequence of the polypeptide corresponds to the sequence of the SPA domain having 1 to about 20 substitution mutations.

2. Amyloid beta peptide binding polypeptide according to claim 1, which has a binding affinity for amyloid beta peptide such that the K_D value of the interaction is at most 5×10^{-6} M, as measured by surface plasmon resonance.

3. Amyloid beta peptide binding polypeptide according to claim 2, the K_D value of the interaction being at most 1×10^{-6} M, as measured by surface plasmon resonance.

4. Amyloid beta peptide binding polypeptide according to claim 3, the K_D value of the interaction being at most 9×10^{-7} M, as measured by surface plasmon resonance.

5. Amyloid beta peptide binding polypeptide according to any preceding claim, the sequence of which corresponds to the sequence of SPA protein Z, as set forth in SEQ ID NO:1, comprising 1 to about 20 substitution mutations.

6. Amyloid beta peptide binding polypeptide according to claim 5, comprising 4 to about 20 substitution mutations compared to the sequence of SPA protein Z in SEQ ID NO:1.

7. Amyloid beta peptide binding polypeptide according to claim 6, comprising 4 to about 13 substitution mutations compared to the sequence of SPA protein Z in SEQ ID NO:1.

8. Amyloid beta peptide binding polypeptide according to any of the claims 5-7, comprising substitution mutations at one or more of the positions 17, 18, 24, 27, 28 and 35 of the sequence of SPA protein Z in SEQ ID NO:1.

9. Amyloid beta peptide binding polypeptide according to claim 8, additionally comprising substitution mutations at one or more of the positions 10, 14, 25 and 32 of the sequence of SPA protein Z in SEQ ID NO:1.

10. Amyloid beta peptide binding polypeptide according to claim 8 or 9, additionally comprising substitution mutations at one or more of the positions 9, 11 and 13 of the sequence of SPA protein Z in SEQ ID NO:1.

11. Amyloid beta peptide binding polypeptide according to any one of claims 5-10, comprising a substitution mutation at a position corresponding to position 27 in SEQ ID NO:1 from arginine to leucine.

12. Amyloid beta peptide binding polypeptide according to claim 11, the amino acid sequence of which is as set out in any one of SEQ ID NO:2-38 and 45-46.

13. Amyloid beta peptide binding polypeptide according to any one of claims 5-12, comprising a substitution mutation at a position corresponding to position 17 in SEQ ID NO:1 from leucine to valine.

14. Amyloid beta peptide binding polypeptide according to claim 13, the amino acid sequence of which is as set out in any one of SEQ ID NO:2-6, 8-13, 15-19, 21, 23-24, 26-36, 38-40, 43, 45 and 46.

15. Amyloid beta peptide binding polypeptide according to any one of claims 5-14, comprising a substitution mutation at a position corresponding to position 24 in SEQ ID NO:1 from glutamic acid to alanine.

16. Amyloid beta peptide binding polypeptide according to claim 15, the amino acid sequence of which is as set out in any one of SEQ ID NO:2, 4-11, 13, 15, 17, 18, 22, 24-38 and 45.

17. Amyloid beta peptide binding polypeptide according to any one of claims 5-16, comprising a substitution mutation at a position corresponding to position 18 in SEQ ID NO:1 from histidine to an amino acid residue selected from tyrosine and phenylalanine.

18. Amyloid beta peptide binding polypeptide according to claim 17, the amino acid sequence of which is as set out in any one of SEQ ID NO:4-8, 11, 12, 14, 16-23, 25, 27, 29-34, 36, 39-41, 45 and 46.

19. Amyloid beta peptide binding polypeptide according to claim 17, comprising a substitution mutation at a position corresponding to position 18 in SEQ ID NO:1 from histidine to tyrosine.

20. Amyloid beta peptide binding polypeptide according to any one of claims 5-19, comprising a substitution mutation at a position corresponding to position 28 in SEQ ID NO:1 from asparagine to an amino acid residue selected from cysteine and serine.

21. Amyloid beta peptide binding polypeptide according to claim 20, the amino acid sequence of which is as set out in any one of SEQ ID NO:2-40 and 45-46.

22. Amyloid beta peptide binding polypeptide according to any one of claims 5-21, comprising a substitution mutation at a position corresponding to position 35 in SEQ ID NO:1 from lysine to an amino acid selected from glutamic acid and glutamine.

23. Amyloid beta peptide binding polypeptide according to claim 22, comprising a substitution mutation at a position corresponding to position 35 in SEQ ID NO:1 from lysine to glutamic acid.

24. Amyloid beta peptide binding polypeptide according to claim 22, the amino acid sequence of which is as set out in any one of SEQ ID NO:2-11, 13-17, 19-21, 23, 24, 26-38 and 45.

25. Amyloid beta peptide binding polypeptide according to any one of claims 5-24, comprising a substitution mutation at a position corresponding to position 10 in SEQ ID NO: 1 from glutamine to glycine.

26. Amyloid beta peptide binding polypeptide according to any one of claims 5-25, comprising a substitution mutation at a position corresponding to position 14 in SEQ ID NO: 1 from tyrosine to an amino acid residue selected from glycine and proline.

27. Amyloid beta peptide binding polypeptide according to any one of claims 5-26 comprising an acidic amino acid residue at a position corresponding to position 25 in SEQ ID NO:1.

28. Amyloid beta peptide binding polypeptide according to any one of claims 5-27, comprising a substitution mutation at a position corresponding to position 32 in SEQ ID NO:1 from glutamine to an amino acid residue selected from lysine, arginine and histidine.

29. Amyloid beta peptide binding polypeptide according to claim 28, comprising a substitution mutation at said position from glutamine to an amino acid residue selected from lysine and arginine.

30. Amyloid beta peptide binding polypeptide according to any one of claims 5-29, comprising a substitution mutation at one or more of the positions corresponding to positions 13-14 and 24-25 in SEQ ID NO:1 from the amino acid residue in the sequence according to SEQ ID NO:1 to proline.

31. Amyloid beta peptide binding polypeptide according to any one of claims 5-30, the amino acid sequence of which corresponds to that of SEQ ID NO:1 comprising at least the following mutations: L17V, H18Y, E24A and R27L.

32. Amyloid beta peptide binding polypeptide according to any one of claims 5-31, the amino acid sequence of which corresponds to that of SEQ ID NO:1 comprising at least the following mutations: P20K, H18V, L17F and I16F.

33. Amyloid beta peptide binding polypeptide according to any preceding claim, the amino acid sequence of which comprises substitution mutations in the sequence so as to contain the amino acid motif KLVFF.

34. Amyloid beta peptide binding polypeptide, whose amino acid sequence fulfils one definition selected from the following:

a) it is selected from SEQ ID NO:2-46;

b) it is an amino acid sequence having 85 % or greater identity to a sequence selected from SEQ ID NO:2-46.

35. Amyloid beta peptide binding polypeptide according to any preceding claim, in which at least one of the asparagine residues present in the domain of staphylococcal protein A (SPA) to which said polypeptide is related have been replaced with another amino acid residue.

36. Amyloid beta peptide binding polypeptide according to claim 35, the sequence of said domain of staphylococcal protein A (SPA) corresponding to the sequence of SPA protein Z as set forth in SEQ ID NO:1, and the polypeptide comprising substitution mutations at at least one position chosen from N3, N6, N11, N21, N23, N28, N43 and N52.

37. Amyloid beta peptide binding polypeptide according to claim 36, comprising at least one of the following mutations: N3A, N6A, N6D, N11S, N23T, N28A and N43E.

38. Amyloid beta peptide binding polypeptide, which constitutes a fragment of a polypeptide according to any preceding claim, which fragment retains binding affinity for an amyloid beta peptide.

39. Amyloid beta peptide binding polypeptide according to any preceding claim, which comprises additional amino acid residues at either terminal.

40. Amyloid beta peptide binding polypeptide according to claim 39, in which the additional amino acid residues comprise a cysteine residue at the N- or C-terminal of the polypeptide.

41. Amyloid beta peptide binding polypeptide according to any one of claims 39-40, in which the additional amino acid residues comprise a tag, preferably chosen from a hexahistidyl tag, a myc tag and a flag tag.

42. Amyloid beta peptide binding polypeptide according to claim 41, comprising an amino acid sequence selected from SEQ ID NO:66-75.

43. Amyloid beta peptide binding polypeptide according to any one of claims 40-42, in which the additional amino acid residues comprise at least one functional polypeptide domain, so that the polypeptide is a fusion polypeptide between a first moiety, consisting of the polypeptide according to any one of claims 1-38, and at least one second and optionally further moiety or moieties.

44. Amyloid beta peptide binding polypeptide according to claim 43, in which the second moiety consists of one or more polypeptide(s) according to any one of claims 1-38, making the polypeptide a multimer of amyloid beta peptide binding polypeptides according to any one of claims 1-38, the sequences of which may be the same or different.

45. Amyloid beta peptide binding polypeptide according to claim 44, comprising an amino acid sequence selected from SEQ ID NO:76-81.

46. Amyloid beta peptide binding polypeptide according to claim 43, in which the second moiety comprises at least one polypeptide domain capable of binding to a target molecule other than amyloid beta peptide.

47. Amyloid beta peptide binding polypeptide according to claim 46, in which the second moiety comprises at least one polypeptide domain capable of binding to human serum albumin.

48. Amyloid beta peptide binding polypeptide according to claim 47, in which the at least one polypeptide

domain capable of binding to human serum albumin is the albumin binding domain of streptococcal protein G.

49. Amyloid beta peptide binding polypeptide according to claim 48, comprising an amino acid sequence selected from SEQ ID NO:47-65.

50. Amyloid beta peptide binding polypeptide according to claim 46, in which the second moiety comprises a polypeptide which is related to a domain of staphylococcal protein A (SPA) in that the sequence of the polypeptide corresponds to the sequence of the SPA domain having 1 to about 20 substitution mutations.

51. Amyloid beta peptide binding polypeptide according claim 50, in which the sequence of the second moiety polypeptide corresponds to the sequence of SPA protein Z, as set forth in SEQ ID NO:1, having 1 to about 20 substitution mutations.

52. Amyloid beta peptide binding polypeptide according to claim 43, in which the second moiety is capable of enzymatic action.

53. Amyloid beta peptide binding polypeptide according to claim 43, in which the second moiety is capable of fluorescent action.

54. Amyloid beta peptide binding polypeptide according to claim 43, in which the second moiety is a phage coat protein or a fragment thereof.

55. Amyloid beta peptide binding polypeptide according to any preceding claim, which comprises a label group.

56. Amyloid beta peptide binding polypeptide according to claim 55, in which the label group is chosen from fluorescent labels, biotin and radioactive labels.

57. Nucleic acid molecule comprising a sequence encoding a polypeptide according to any one of claims 1-56.

58. Expression vector comprising the nucleic acid molecule according to claim 57.

59. Host cell comprising the expression vector according to claim 58.

60. Use of a polypeptide according to any one of claims 1-56 in a method of affinity separation.

61. Method of separation of amyloid beta peptide present in a sample from other constituents in the sample, comprising a step of affinity separation, in which step a polypeptide according to any one of claims 1-56 is used.

62. Method according to claim 61, comprising the steps:

a) applying the sample to an affinity matrix comprising a polypeptide according to any one of claims 1-56 under conditions that are conducive to binding of amyloid beta peptide to the affinity matrix;

b) washing the affinity matrix for removal of substances not bound thereto; and

c) eluting the bound amyloid beta peptide from the affinity matrix, thus obtaining an amyloid beta peptide fraction with an enriched amyloid beta peptide content; and

d) recovering said amyloid beta peptide fraction.

63. Method according to claim 61, comprising the steps:

a) applying the sample to an affinity matrix comprising a polypeptide according to any one of claims 1-56 under conditions that are conducive to binding of amyloid beta peptide to the affinity matrix;

b) washing the affinity matrix for recovery of substances not bound thereto, thus obtaining a depleted fraction with a substantially reduced amyloid beta peptide content; and

c) recovering said depleted fraction.

64. Method according to claim 61, comprising the steps:

a) applying the sample to an affinity matrix comprising a polypeptide according to any one of claims 1-56 under conditions that are conducive to binding of amyloid beta peptide to the affinity matrix; and

b) recovering the sample.

65. Method according to claim 61, comprising the steps:

a) applying of the sample to an affinity matrix comprising a polypeptide according to any one of claims 1-56 under conditions that are conducive to binding of amyloid beta peptide to the affinity matrix;

b) washing the affinity matrix for recovery of substances not bound thereto, thus obtaining a depleted fraction with a substantially reduced amyloid beta peptide content; and

c) eluting the bound amyloid beta peptide from the affinity matrix, thus obtaining an amyloid beta peptide fraction with an enriched amyloid beta peptide content; and

d) recovering said amyloid beta peptide fraction and said depleted fraction.

66. Affinity matrix comprising a polypeptide according to any one of claims 1-56.

67. Method according claim 61 for reducing the content of amyloid beta peptide in a portion of a body fluid of a human, comprising the steps to:

a) provide a portion of a body fluid from a human;

b) apply the portion of a body fluid to an affinity matrix comprising a polypeptide according to any one of claims 1-56 under conditions enabling binding of amyloid beta peptide to the affinity matrix, thereby causing a reduction of the content of amyloid beta peptide in the portion of body fluid; and

c) return at least a part of said portion of body fluid to said human.

68. Method according to claim 67, wherein said sample of a body fluid is obtained from a subject afflicted by Alzheimer's disease, and the symptoms of Alzheimer's disease are alleviated by performing said method.

69. Method according to any one of claims 67-68, wherein said body fluid is whole blood.

70. Method according to any one of claims 67-68, wherein said body fluid is plasma.

71. Method according to any one of claims 67-68, wherein said body fluid is serum.

72. Use of a polypeptide according to any one of claims 1-56 for detection of amyloid beta peptide.

73. Method for detection of amyloid beta peptide in a sample, in which method a polypeptide according to any one of claims 1-56 is used.

74. Method according to claim 73, comprising the steps: (i) providing a sample to be tested, (ii) applying a polypeptide according to any one of claims 1-56 to the sample under conditions such that binding of the polypeptide to any amyloid beta peptide present in the sample is enabled, (iii) removing non-bound polypeptide, and (iv) detecting bound polypeptide.

75. Method according to claim 73 or 74, in which the sample is a biological fluid sample, preferably a human blood plasma sample.

76. Method according to claim 73 or 74, in which the sample is a tissue sample.

77. Use of a polypeptide according to any one of claims 1-56 as a medicament.

78. Use of a polypeptide according to any one of claims 1-56 in the preparation of a medicament for the treatment of a disease characterized by an over-representation of Aβ.

79. Use according to claim 78, in which said disease characterized by an over-representation of Aβ is Alzheimer's disease.

80. Method for treatment of a disease characterized by an over-representation of Aβ, comprising administering to a subject in need of such treatment a therapeutically effective amount of a composition comprising a polypeptide according to any one of claims 1-56.

81. Method according to claim 80, in which said disease characterized by an over-representation of Aβ is Alzheimer's disease.

1/20

Polypeptide	Amino acid sequence	SEQ ID NO:
Z_{wt}	VDNKFNKEQQ NAFYEILHLP NLNEEQRNAF IQSLKDDPSQ SANLLAEAKK LNDAQAPK	1
	VDNKFNKEDA QATGEIVVLP NLNAEQLCAF IRSLEDDPSQ SANLLAEAKK LNDAQAPK	2
	VDNKFNKEEA RAGPEIVRLP NLNSTQLCAF IKSLEDDPSQ SANLLAEAKK LNDAQAPK	3
	VDNKFNKEFQ HANKEIVYLP NLNATQLCAF ISSLEDDPSQ SANLLAEAKK LNDAQAPK	4
	VDNKFNKEGG IATPEIVFLP NLNAAQLCAF IGSLEDDPSQ SANLLAEAKK LNDAQAPK	5
$Z_{Aβ1}$	VDNKFNKEGG LAPGEIVYLP NLNASQLCAF IHSLEDDPSQ SANLLAEAKK LNDAQAPK	6
$Z_{Aβ2}$	VDNKFNKEGK GAPGEIHYLP NLNADQLCAF IRSLEDDPSQ SANLLAEAKK LNDAQAPK	7
	VDNKFNKEGS KAHGEIVYLP NLNAEQLCAF IGSLEDDPSQ SANLLAEAKK LNDAQAPK	8
	VDNKFNKEGT LANPEIVKLP NLNANQLCAF IRSLEDDPSQ SANLLAEAKK LNDAQAPK	9
	VDNKFNKEGV SAAREIVPLP NLNADQLCAF IYSLQDDPSQ SANLLAEAKK LNDAQAPK	10
	VDNKFNKEIE VATGEIVYLP NLNADQLCAF INSLEDDPSQ SANLLAEAKK LNDAQAPK	11
$Z_{Aβ3}$	VDNKFNKEMA SAGGEIVYLP NLNPDQLCAF IHSLHDDPSQ SANLLAEAKK LNDAQAPK	12
$Z_{Aβ12}$	VDNKFNKENM KATGEIVWLP NLNASQLCAF IQSLEDDPSQ SANLLAEAKK LNDAQAPK	13
	VDNKFNKENR YAPGEITYLP NLNSDQLCAF IRSLEDDPSQ SANLLAEAKK LNDAQAPK	14
$Z_{Aβ4}$	VDNKFNKENS NAGPEIVLLP NLNAAQLCAF IQSLEDDPSQ SANLLAEAKK LNDAQAPK	15
	VDNKFNKEPM MAGGEIVYLP NLNSHQLCAF IKSLEDDPSQ SANLLAEAKK LNDAQAPK	16
	VDNKFNKEQM LADREIVYLP NLNANQLCAF IMSLEDDPSQ SANLLAEAKK LNDAQAPK	17
	VDNKFNKEQS EAPIEIVYLP NLNAEQLCAF IQSLYDDPSQ SANLLAEAKK LNDAQAPK	18
$Z_{Aβ5}$	VDNKFNKEQT MATGEIVYLP NLNPEQLCAF IHSLEDDPSQ SANLLAEAKK LNDAQAPK	19
	VDNKFNKEQG TAQPEITYLP NLNSDQLCAF IKSLEDDPSQ SANLLAEAKK LNDAQAPK	20
	VDNKFNKEQP KAGKEIVYLP NLNGTQLCAF IKSLEDDPSQ SANLLAEAKK LNDAQAPK	21
	VDNKFNKEPS KAKREITYLP NLNASQLCAF IKSLYDDPSQ SANLLAEAKK LNDAQAPK	22
	VDNKFNKERE GAAGEIVFLP NLNGEQLCAF IKSLEDDPSQ SANLLAEAKK LNDAQAPK	23
$Z_{Aβ13}$	VDNKFNKERG AAPPEIVRLP NLNAEQLCAF IQSLEDDPSQ SANLLAEAKK LNDAQAPK	24
$Z_{Aβ15}$	VDNKFNKERG QAQGEIHYLP NLNATQLCAF ITSLYDDPSQ SANLLAEAKK LNDAQAPK	25
$Z_{Aβ14}$	VDNKFNKERG QADREIVMLP NLNASQLCAF IKSLQDDPSQ SANLLAEAKK LNDAQAPK	26
$Z_{Aβ6}$	VDNKFNKERG RAEGEIVYLP NLNADQLCAF IFSLQDDPSQ SANLLAEAKK LNDAQAPK	27
$Z_{Aβ7}$	VDNKFNKERG VASPEIVRLP NLNAQQLCAF IRSLEDDPSQ SANLLAEAKK LNDAQAPK	28
	VDNKFNKERP EASREIVYLP NLNAAQLCAF IESLEDDPSQ SANLLAEAKK LNDAQAPK	29
	VDNKFNKERP EAAREIVYLP NLNADQLCAF IESLQDDPSQ SANLLAEAKK LNDAQAPK	30
$Z_{Aβ8}$	VDNKFNKERV RASGEIVYLP NLNADQLCAF IYSLQDDPSQ SANLLAEAKK LNDAQAPK	31
	VDNKFNKESE KAPVEIVYLP NLNAEQLCAF IYSLQDDPSQ SANLLAEAKK LNDAQAPK	32
	VDNKFNKESK GAKKEIVFLP NLNAIQLCAF IQSLEDDPSQ SANLLAEAKK LNDAQAPK	33
	VDNKFNKEST PATPEIVYLP NLNAAQLCAF IKSLEDDPSQ SANLLAEAKK LNDAQAPK	34
	VDNKFNKETR RAGREIVRLP NLNADQLCAF IRSLEDDPSQ SANLLAEAKK LNDAQAPK	35
	VDNKFNKETA GATREIVYLP NLNAEQLCAF IKSLEDDPSQ SANLLAEAKK LNDAQAPK	36
	VDNKFNKEVG SAAKEIIPLP NLNAEQLCAF IRSLEDDPSQ SANLLAEAKK LNDAQAPK	37
$Z_{Aβ16}$	VDNKFNKEVQ GAQPEIVRLP NLNASQLCAF IQSLQDDPSQ SANLLAEAKK LNDAQAPK	38
$Z_{Aβ9}$	VDNKFNKEGG PAQGEIVYLP NLNPAQICAF IQSLADDPSQ SANLLAEAKK LNDAQAPK	39
$Z_{Aβ10}$	VDNKFNKERS GANREIVYLP NLNGPQVCAF ILSLYDDPSQ SANLLAEAKK LNDAQAPK	40
$Z_{Aβ17}$	VDNKFNKEAQ CALVEITYLP NLNRKQCRAF IRSLDDDPSQ SANLLAEAKK LNDAQAPK	41
$Z_{Aβ18}$	VDNKFNKEHR CAHYEITVLP NLNPRQCRAF ISSLRDDPSQ SANLLAEAKK LNDAQAPK	42
$Z_{Aβ19}$	VDNKFNKEPK QATWEIVRLP NLNRQTEAAF IRSLLDDPSQ SANLLAEAKK LNDAQAPK	43
$Z_{Aβ20}$	VDNKFNKERE KASWEIWELP NLNEDQTSAF IQSLKDDPSQ SANLLAEAKK LNDAQAPK	44
$Z_{Aβ1\ C28S}$	VDNKFNKEGG LAPGEIVYLP NLNASQLSAF IHSLEDDPSQ SANLLAEAKK LNDAQAPK	45
$Z_{Aβ3\ C28S}$	VDNKFNKEMA SAGGEIVYLP NLNPDQLSAF IHSLHDDPSQ SANLLAEAKK LNDAQAPK	46

FIGURE 1

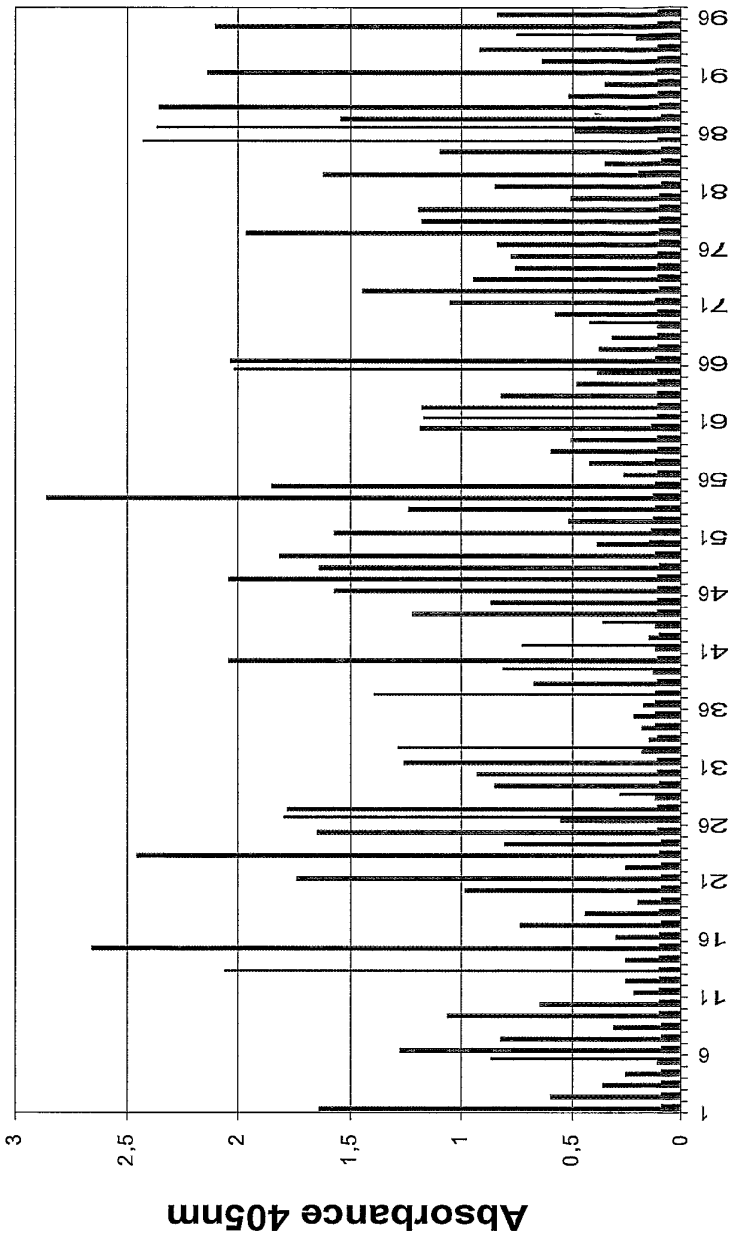

Absorbance 405nm
3
2,5
2
1,5
1
0,5
0
Well number
1
6
11
16
21
26
31
36
41
46
51
56
61
66
71
76
81
86
91
96

FIGURE 2A

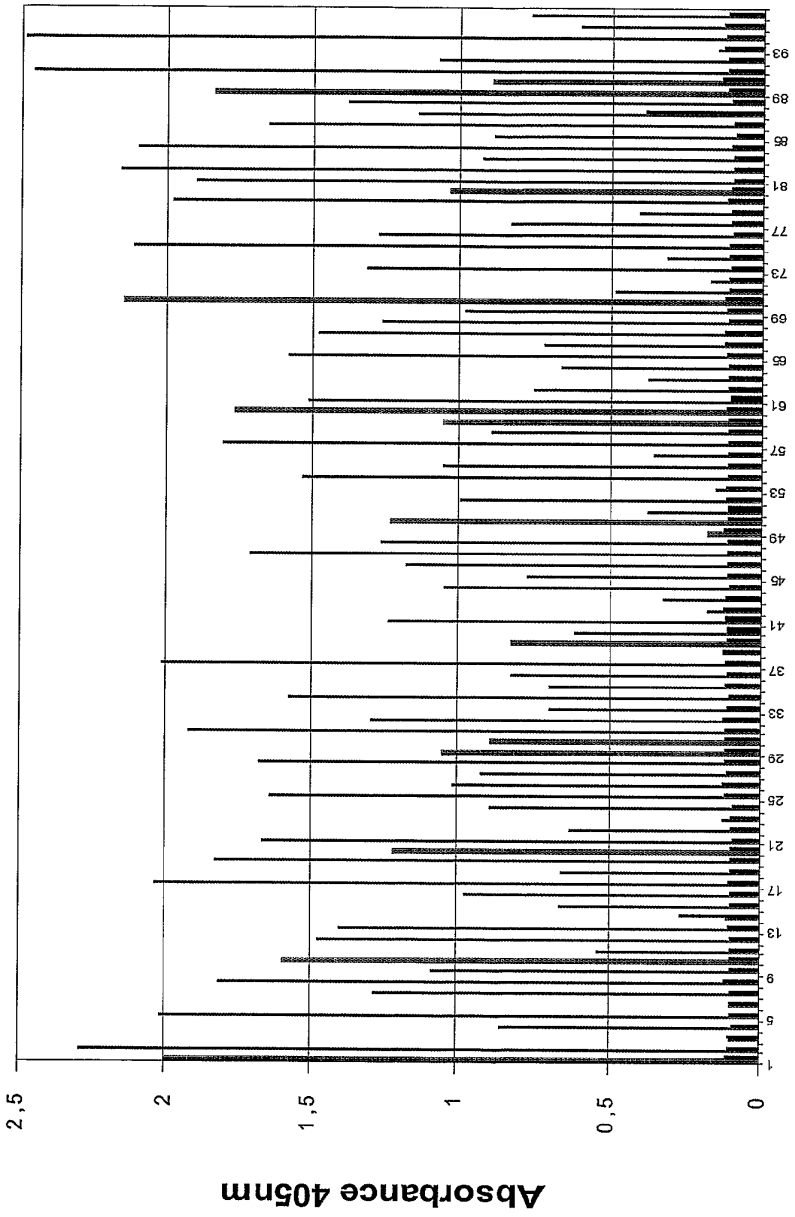

Absorbance 405nm
2,5
2
1,5
1
0,5
0
Well number
1
5
9
13
17
21
25
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45
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53
57
61
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69
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77
81
85
89
93

FIGURE 2B

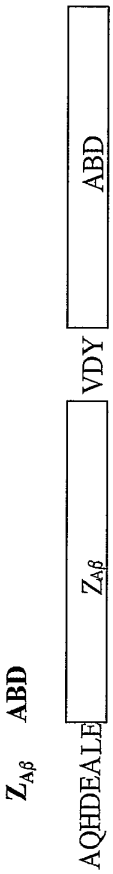


FIGURE 3

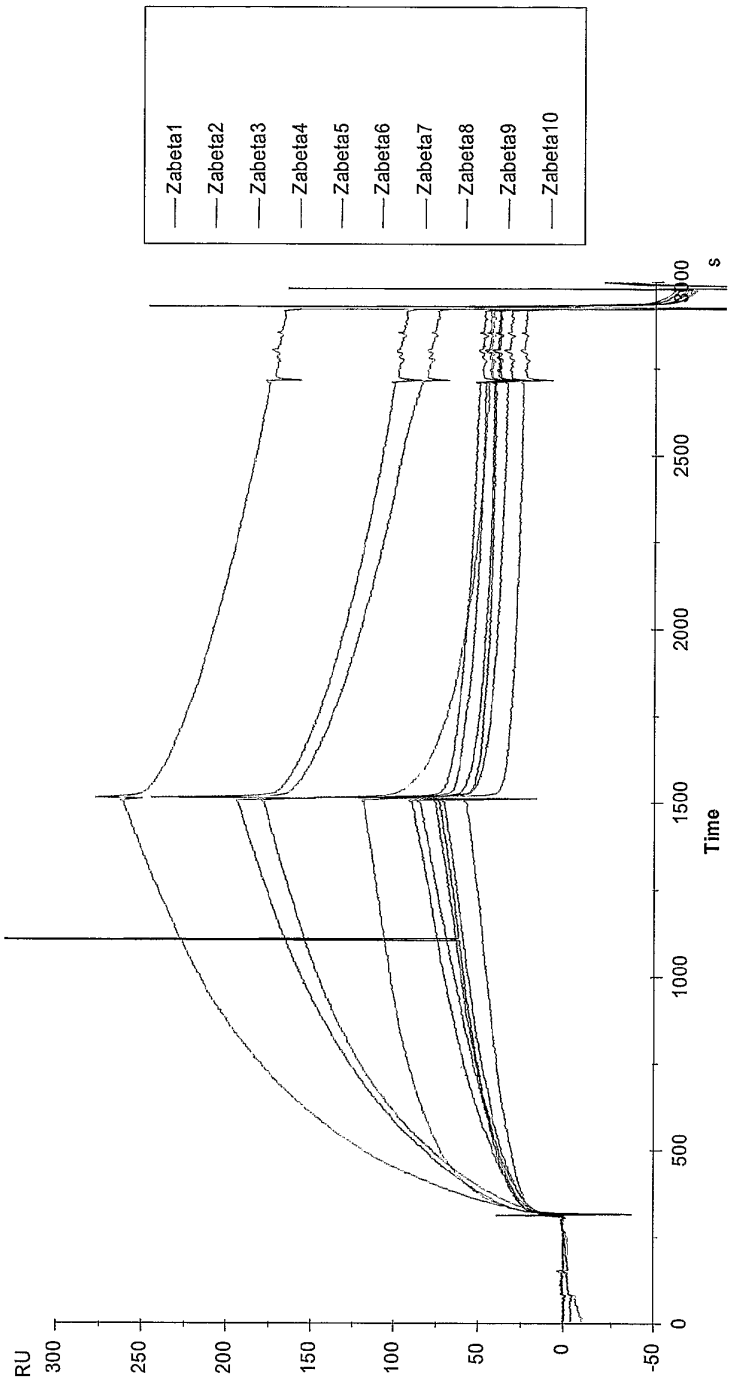

Zabeta1
Zabeta2
Zabeta3
Zabeta4
Zabeta5
Zabeta6
Zabeta7
Zabeta8
Zabeta9
Zabeta10
RU
300
250
200
150
100
50
0
-50
0
500
1000
1500
2000
2500
3000
s
Time

FIGURE 4A

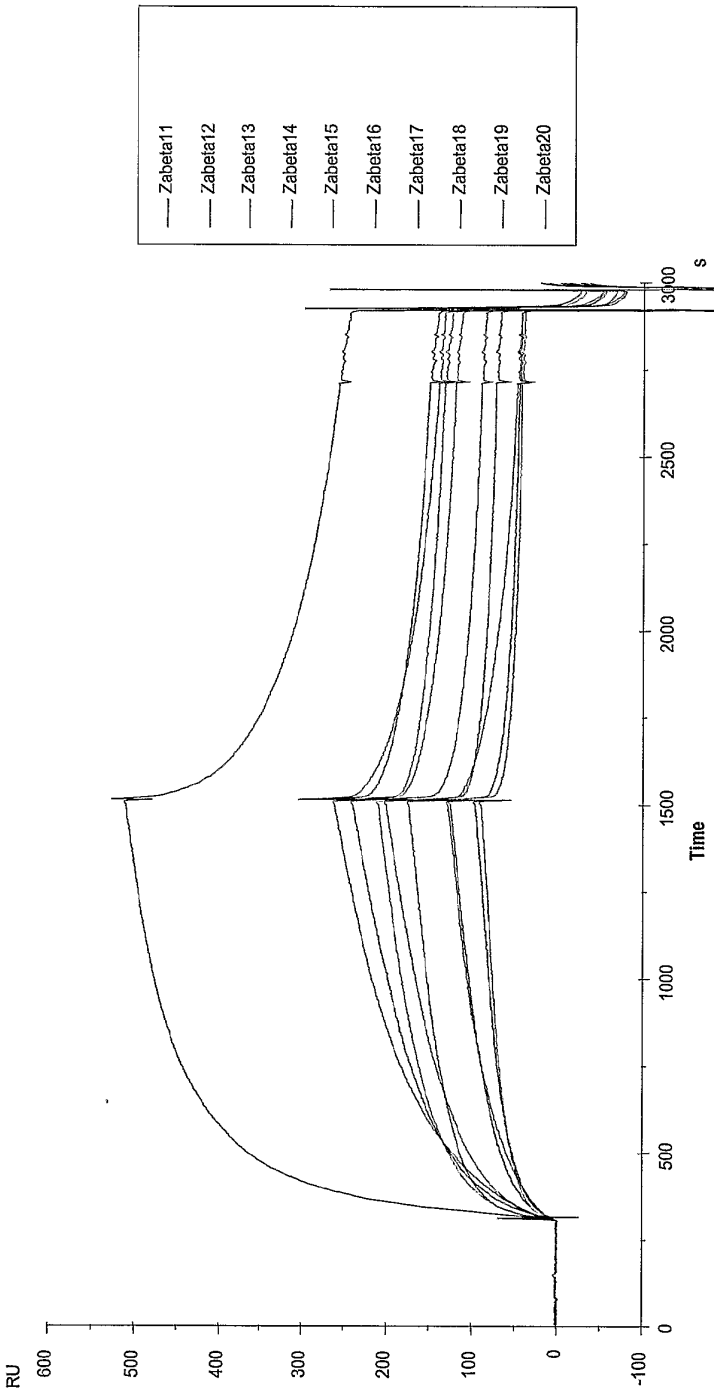

Zabeta11
Zabeta12
Zabeta13
Zabeta14
Zabeta15
Zabeta16
Zabeta17
Zabeta18
Zabeta19
Zabeta20
RU
600
500
400
300
200
100
0
-100
0
500
1000
1500
2000
2500
3000
s
Time

FIGURE 4B

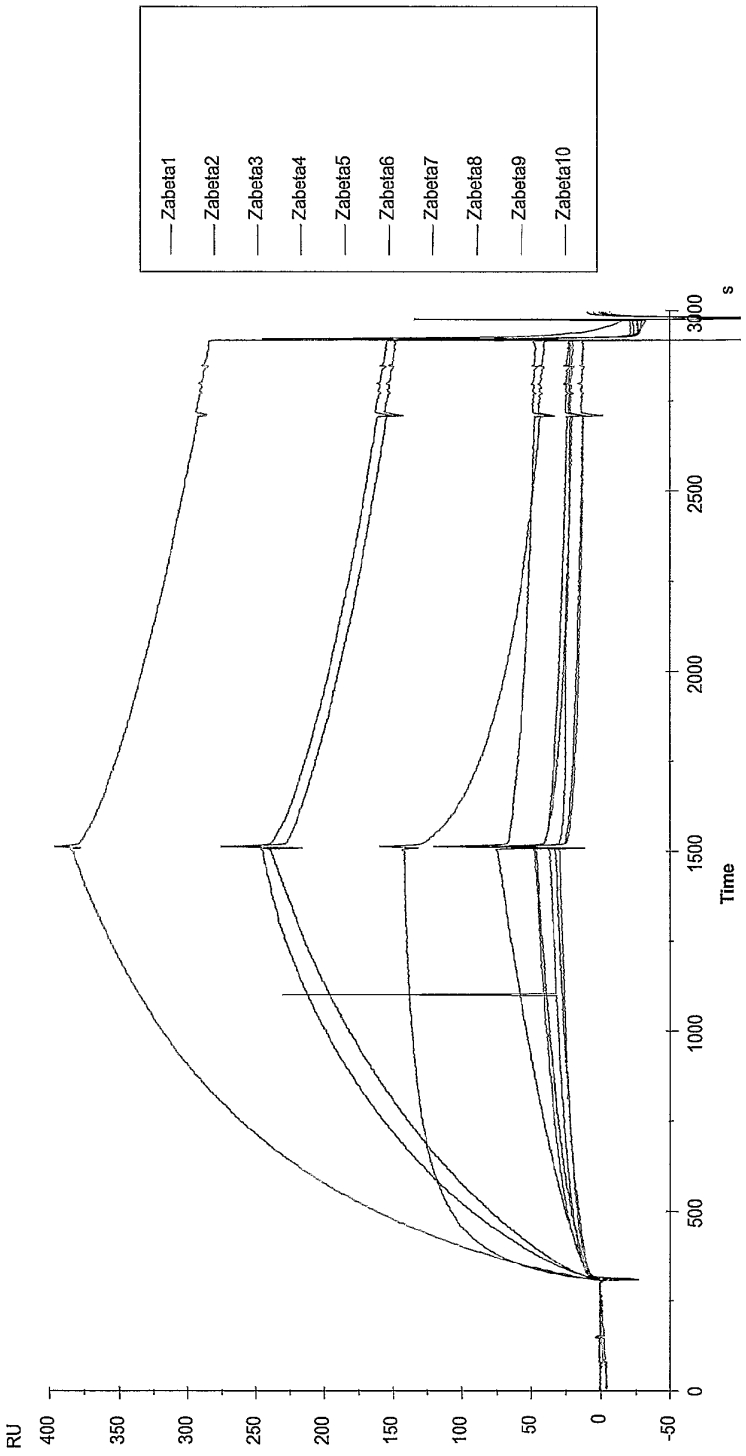

Zabeta1
Zabeta2
Zabeta3
Zabeta4
Zabeta5
Zabeta6
Zabeta7
Zabeta8
Zabeta9
Zabeta10
RU
400
350
300
250
200
150
100
50
0
-50
Time
0
500
1000
1500
2000
2500
3000
s

FIGURE 4C

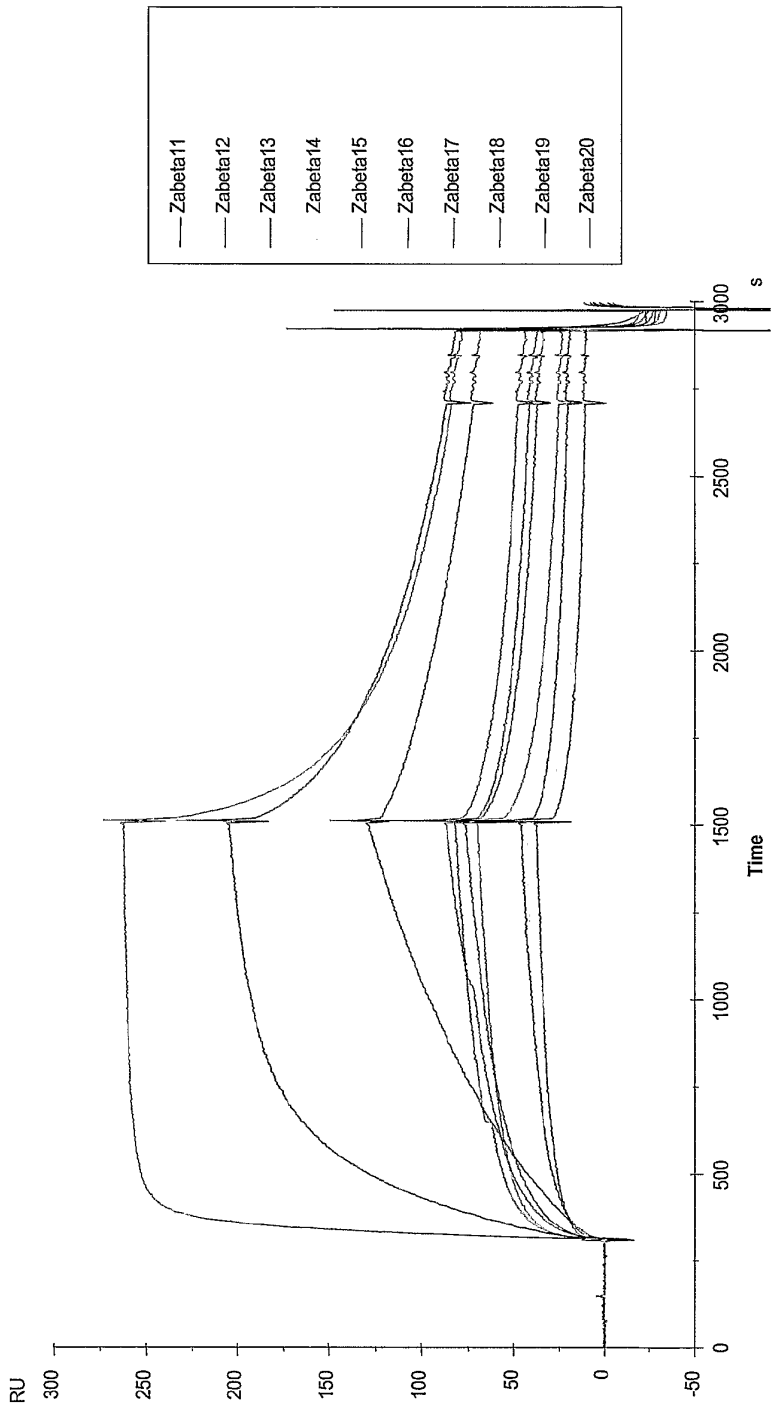

Zabeta11
Zabeta12
Zabeta13
Zabeta14
Zabeta15
Zabeta16
Zabeta17
Zabeta18
Zabeta19
Zabeta20
RU
300
250
200
150
100
50
0
-50
0
500
1000
1500
2000
2500
3000
s
Time

FIGURE 4D

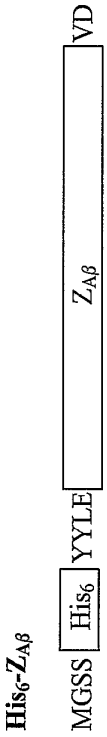


FIGURE 5

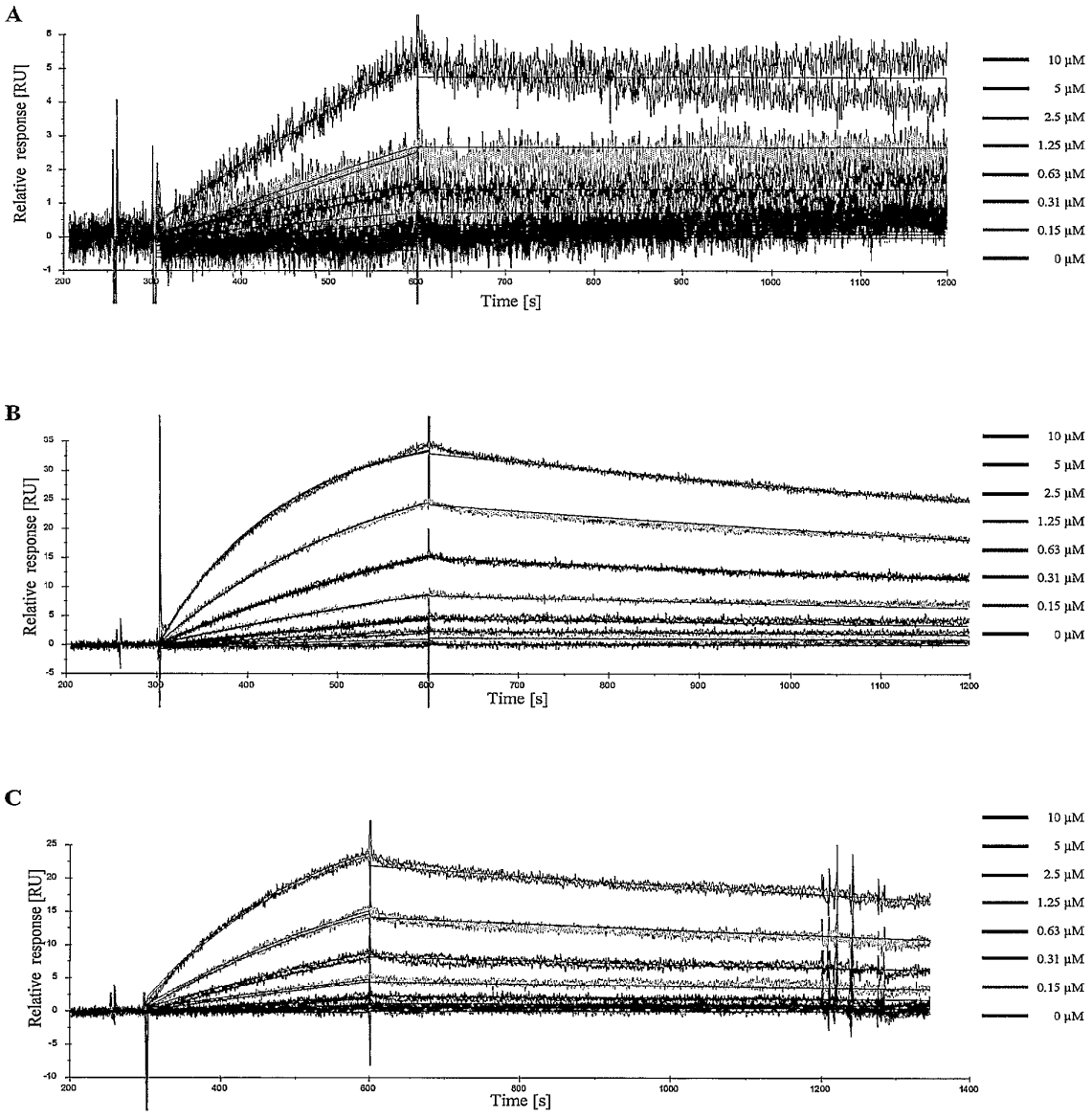

A
Relative response [RU]
Time [s]
10 µM
5 µM
2.5 µM
1.25 µM
0.63 µM
0.31 µM
0.15 µM
0 µM
B
Relative response [RU]
Time [s]
10 µM
5 µM
2.5 µM
1.25 µM
0.63 µM
0.31 µM
0.15 µM
0 µM
C
Relative response [RU]
Time [s]
10 µM
5 µM
2.5 µM
1.25 µM
0.63 µM
0.31 µM
0.15 µM
0 µM

FIGURE 6A-C

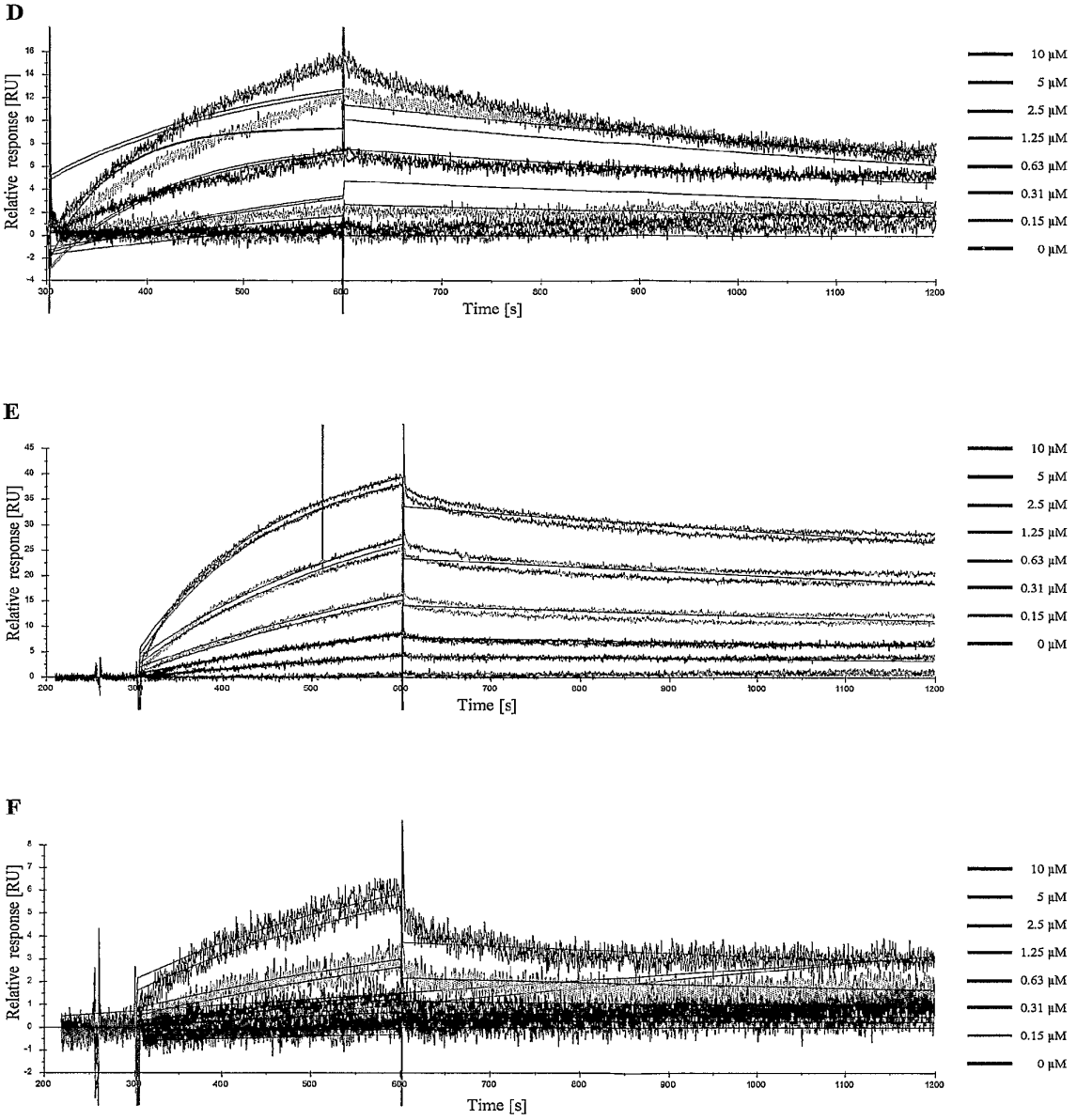

D
Relative response [RU]
Time [s]
10 µM
5 µM
2.5 µM
1.25 µM
0.63 µM
0.31 µM
0.15 µM
0 µM
E
Relative response [RU]
Time [s]
10 µM
5 µM
2.5 µM
1.25 µM
0.63 µM
0.31 µM
0.15 µM
0 µM
F
Relative response [RU]
Time [s]
10 µM
5 µM
2.5 µM
1.25 µM
0.63 µM
0.31 µM
0.15 µM
0 µM

FIGURE 6D-F

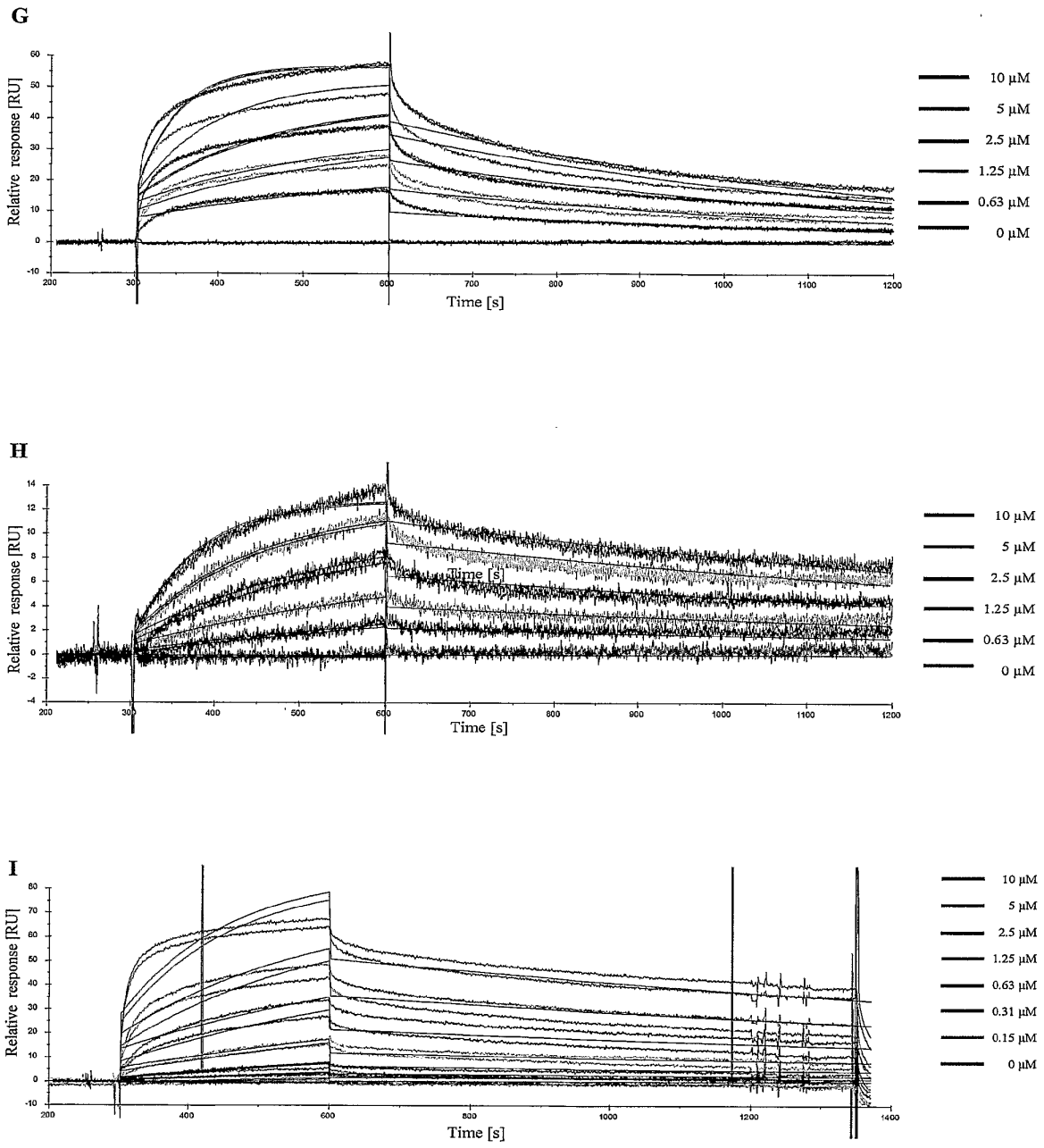

G
Relative response [RU]
Time [s]
10 µM
5 µM
2.5 µM
1.25 µM
0.63 µM
0 µM
H
Relative response [RU]
Time [s]
10 µM
5 µM
2.5 µM
1.25 µM
0.63 µM
0 µM
I
Relative response [RU]
Time [s]
10 µM
5 µM
2.5 µM
1.25 µM
0.63 µM
0.31 µM
0.15 µM
0 µM

FIGURE 6G-I

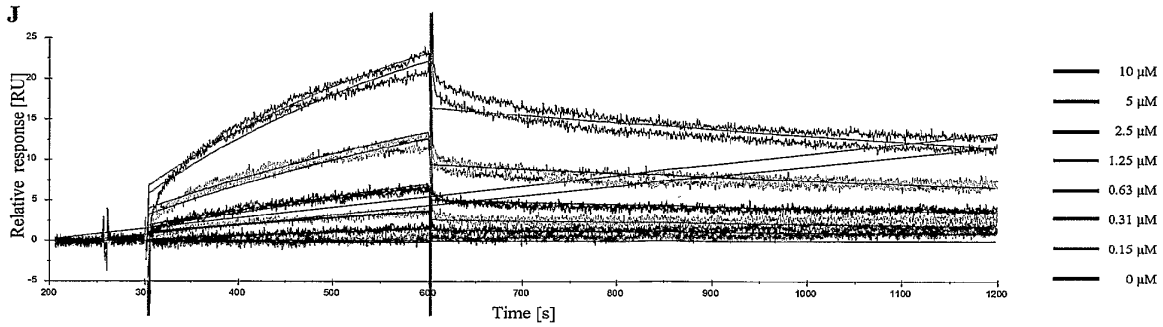

J
Relative response [RU]
Time [s]
10 µM
5 µM
2.5 µM
1.25 µM
0.63 µM
0.31 µM
0.15 µM
0 µM

FIGURE 6J



FIGURE 7

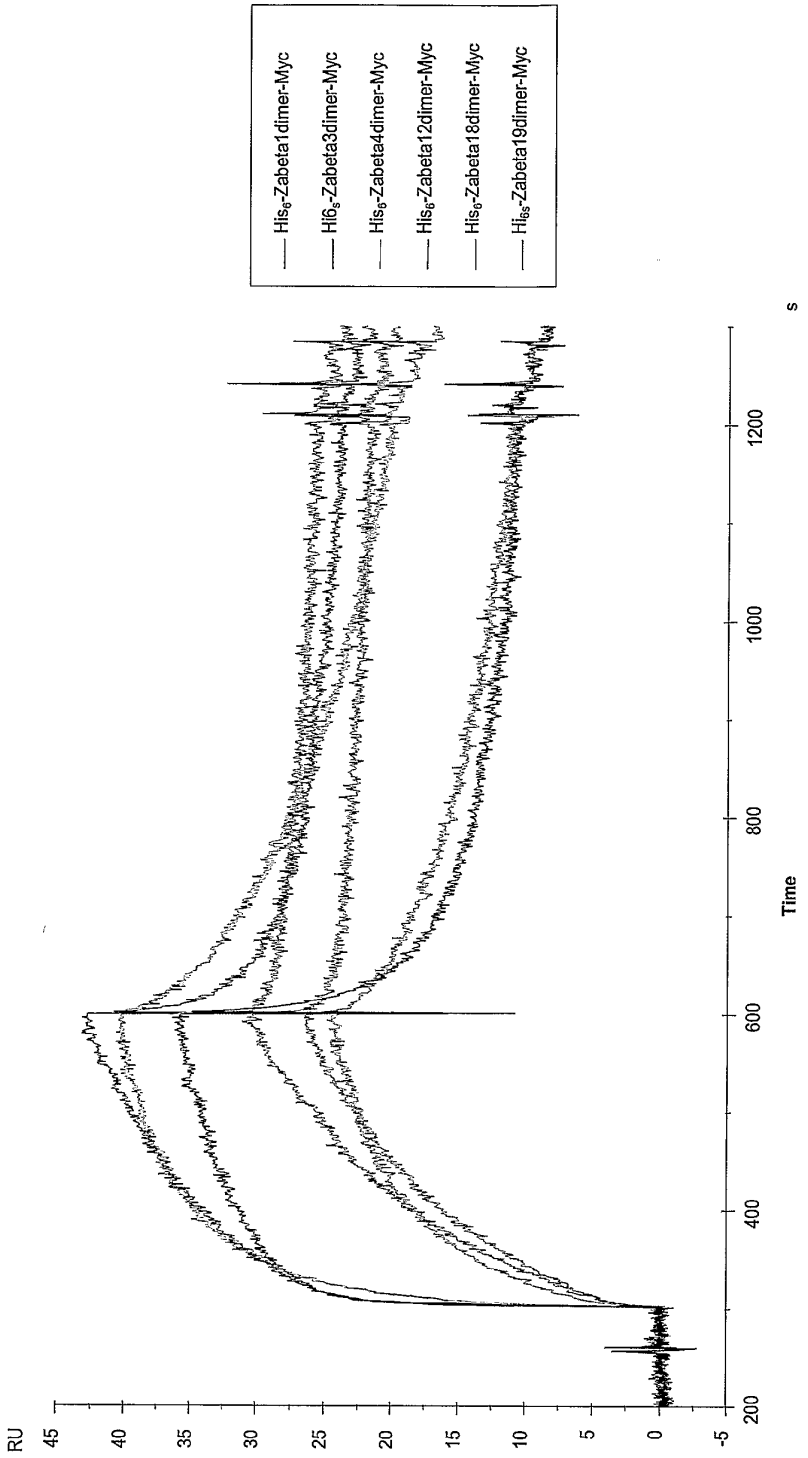

His6-Zabeta1dimer-Myc
His6-Zabeta3dimer-Myc
His6-Zabeta4dimer-Myc
His6-Zabeta12dimer-Myc
His6-Zabeta18dimer-Myc
His6-Zabeta19dimer-Myc
RU
45
40
35
30
25
20
15
10
5
0
-5
200
400
600
800
1000
1200
Time
s

FIGURE 8

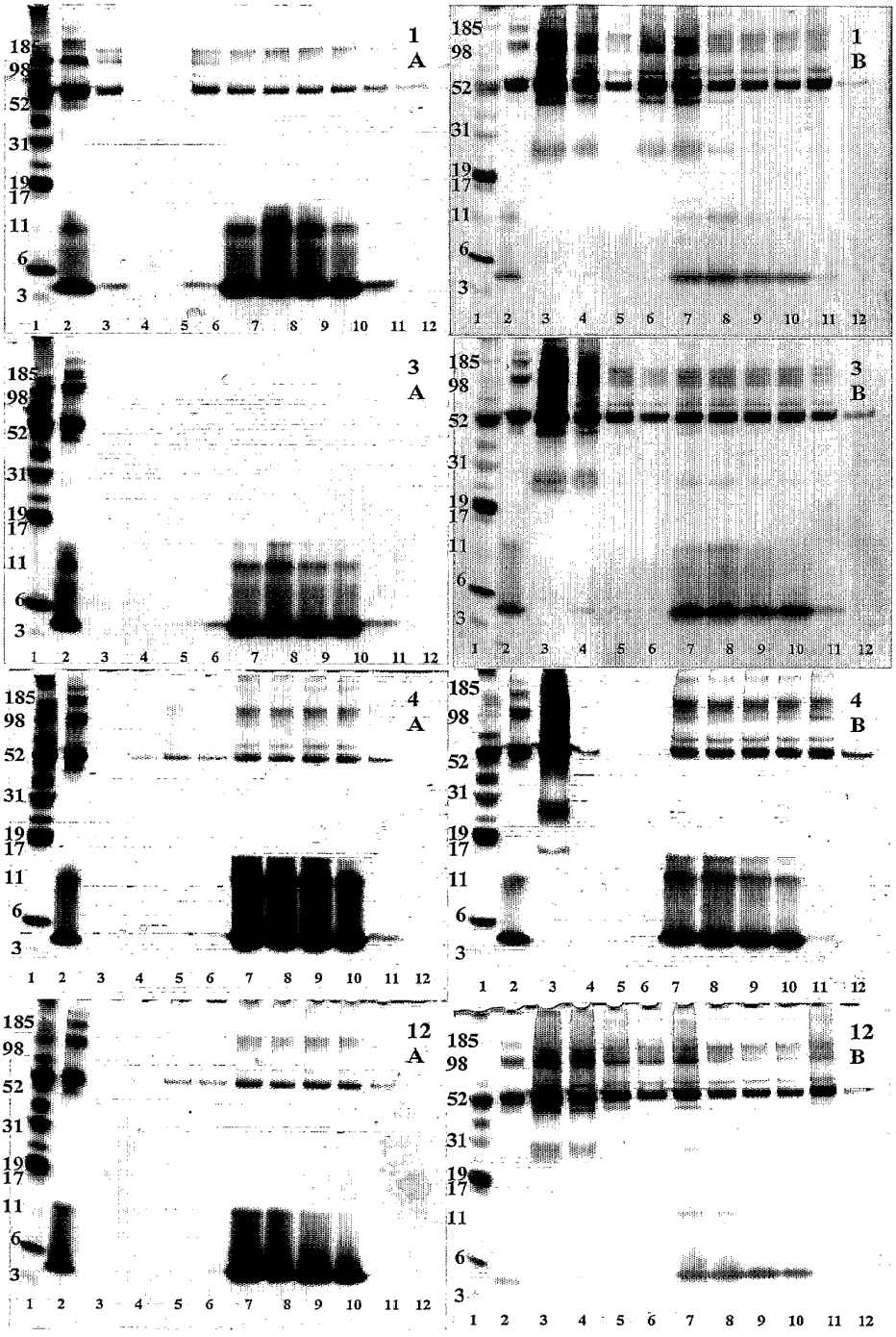

1 A
1 B
3 A
3 B
4 A
4 B
12 A
12 B
185
98
52
31
19
17
11
6
3
1 2 3 4 5 6 7 8 9 10 11 12

FIGURE 9

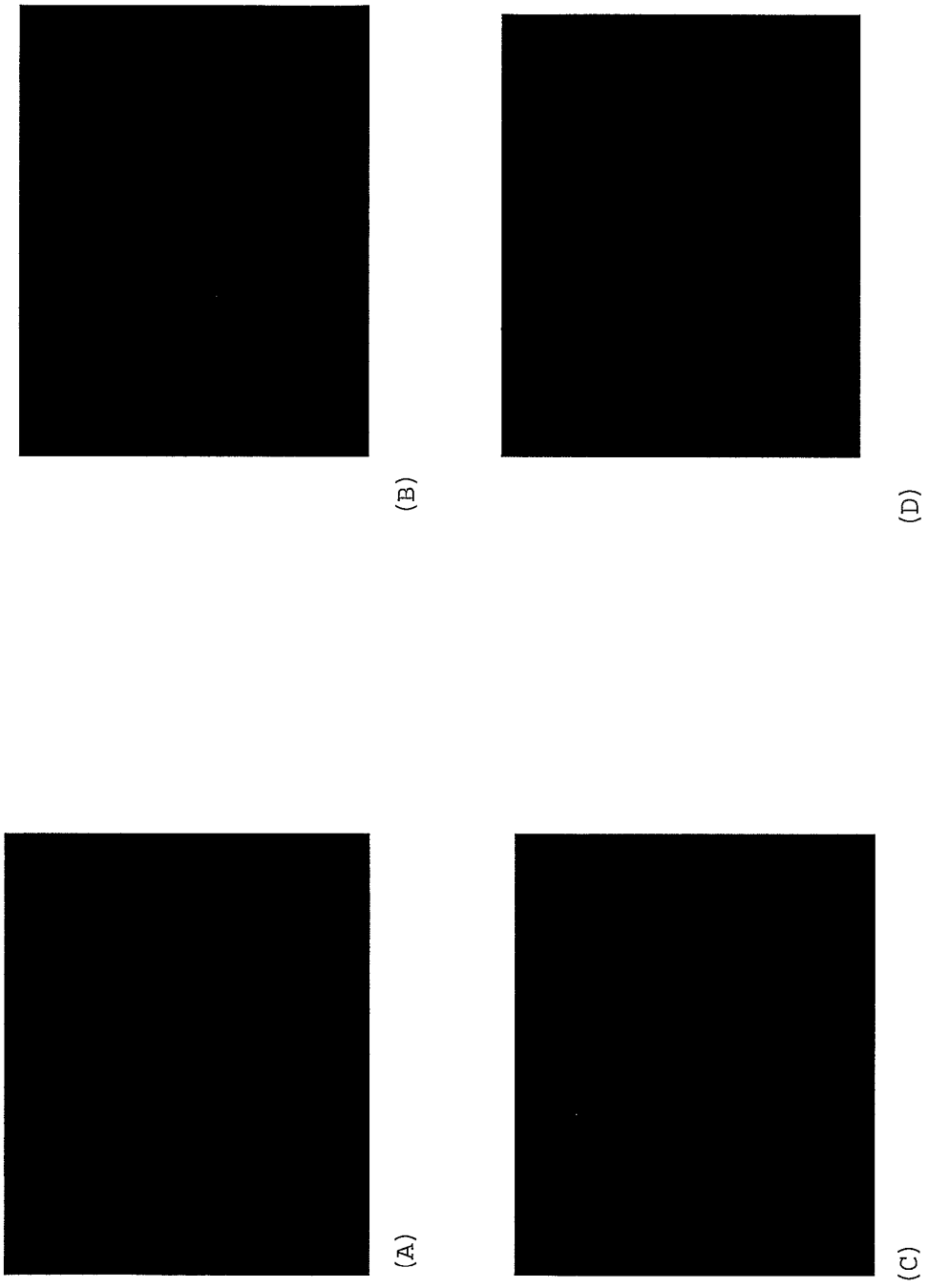


FIGURE 10

Fujirebio Diagnostics, Inc. v. Quanterix Corp.
EX-1045
7642

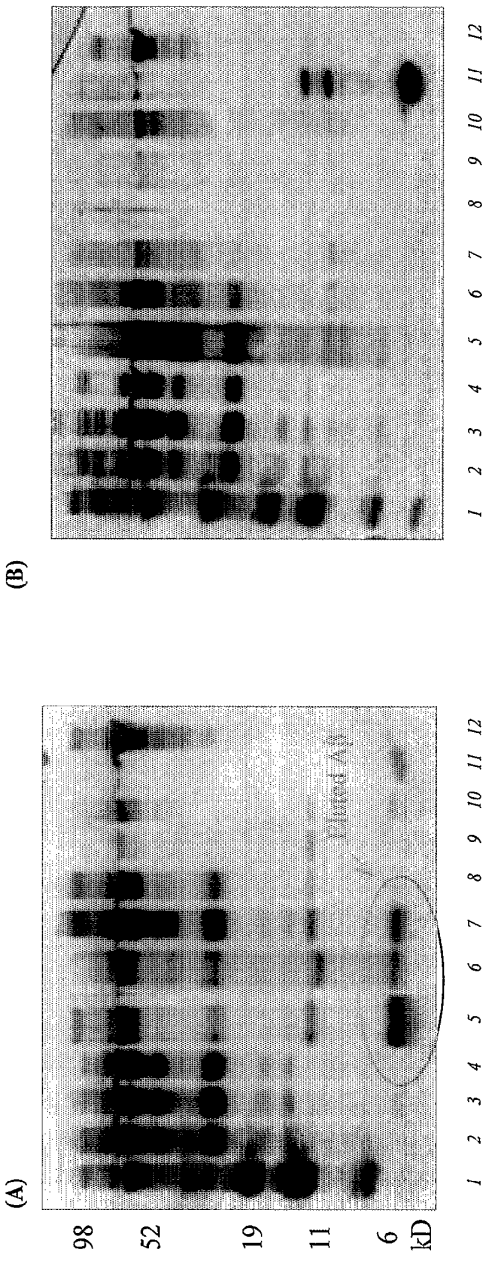


FIGURE 11

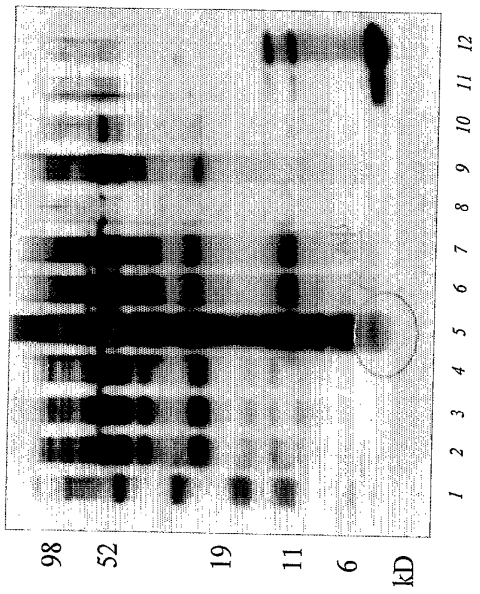


FIGURE 12

Fujirebio Diagnostics, Inc. v. Quanterix Corp.
EX-1045
7644

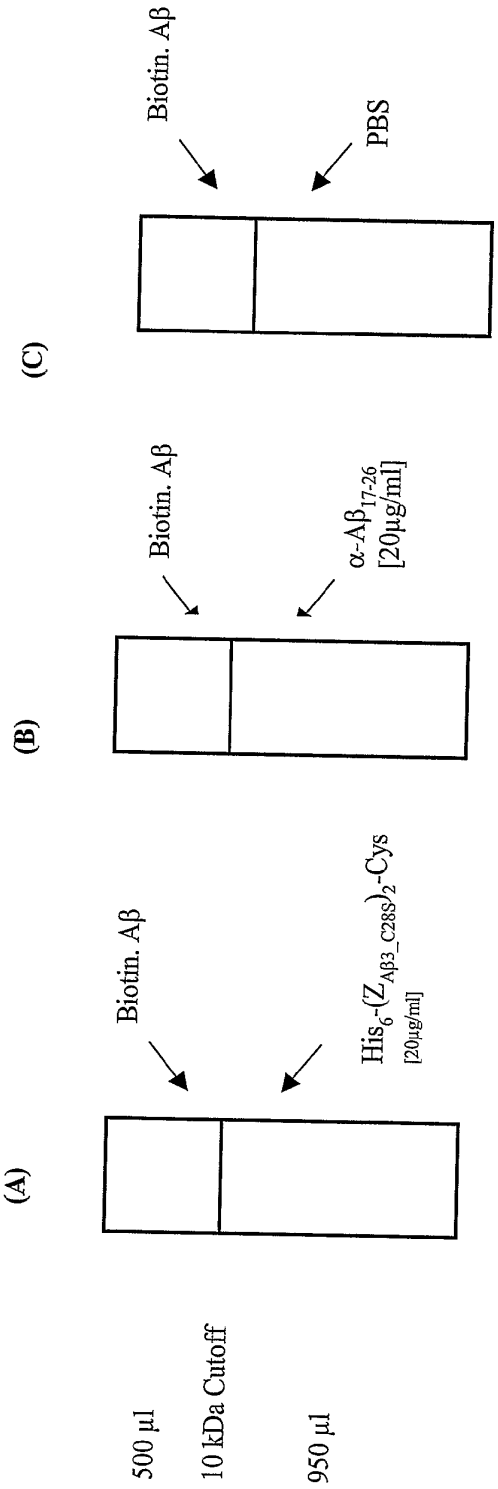

(A)
Biotin. Aβ
His_6-($Z_{Aβ3\_C28S}$)$_2$-Cys
[20μg/ml]
500 μl
10 kDa Cutoff
950 μl
(B)
Biotin. Aβ
α-$Aβ_{17-26}$
[20μg/ml]
(C)
Biotin. Aβ
PBS

FIGURE 13

SEQUENCE LISTING

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<120> New Polypeptide

<130> 21017756

<150> SE0400274-4

<151> 2004-02-09

<160> 81

<170> PatentIn version 3.1

<210> 1

<211> 58

<212> PRT

<213> Artificial

<400> 1

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Leu His Leu Pro Asn Leu Asn Glu Glu Gln Arg Asn Ala Phe Ile Gln
20 25 30

Ser Leu Lys Asp Asp Pro Ser Gln Ser Ala Asn Leu Leu Ala Glu Ala
35 40 45

Lys Lys Leu Asn Asp Ala Gln Ala Pro Lys
50 55

<210> 2

<211> 58

<212> PRT

<213> Artificial

<400> 2

Val Asp Asn Lys Phe Asn Lys Glu Asp Ala Gln Ala Thr Gly Glu Ile
1 5 10 15

Val Val Leu Pro Asn Leu Asn Ala Glu Gln Leu Cys Ala Phe Ile Arg
20 25 30

Ser Leu Glu Asp Asp Pro Ser Gln Ser Ala Asn Leu Leu Ala Glu Ala
35 40 45

Lys Lys Leu Asn Asp Ala Gln Ala Pro Lys
50 55

<210> 3

<211> 58

<212> PRT

<213> Artificial

<400> 3

Val Asp Asn Lys Phe Asn Lys Glu Glu Ala Arg Ala Gly Pro Glu Ile
1 5 10 15

Val Arg Leu Pro Asn Leu Asn Ser Thr Gln Leu Cys Ala Phe Ile Lys
20 25 30

Ser Leu Glu Asp Asp Pro Ser Gln Ser Ala Asn Leu Leu Ala Glu Ala
35 40 45

Lys Lys Leu Asn Asp Ala Gln Ala Pro Lys
50 55

<210> 4

<211> 58

<212> PRT

<213> Artificial

<400> 4

Val Asp Asn Lys Phe Asn Lys Glu Phe Gln His Ala Asn Lys Glu Ile
1 5 10 15

Val Tyr Leu Pro Asn Leu Asn Ala Thr Gln Leu Cys Ala Phe Ile Ser
20 25 30

Ser Leu Glu Asp Asp Pro Ser Gln Ser Ala Asn Leu Leu Ala Glu Ala
35 40 45

Lys Lys Leu Asn Asp Ala Gln Ala Pro Lys
50 55

<210> 5

<211> 58

<212> PRT

<213> Artificial

<400> 5

Val Asp Asn Lys Phe Asn Lys Glu Gly Gly Ile Ala Thr Pro Glu Ile
1 5 10 15

Val Phe Leu Pro Asn Leu Asn Ala Ala Gln Leu Cys Ala Phe Ile Gly
20 25 30

Ser Leu Glu Asp Asp Pro Ser Gln Ser Ala Asn Leu Leu Ala Glu Ala
35 40 45

Lys Lys Leu Asn Asp Ala Gln Ala Pro Lys
50 55

<210> 6

<211> 58

<212> PRT

<213> Artificial

<400> 6

Val Asp Asn Lys Phe Asn Lys Glu Gly Gly Leu Ala Pro Gly Glu Ile
1 5 10 15

Val Tyr Leu Pro Asn Leu Asn Ala Ser Gln Leu Cys Ala Phe Ile His
20 25 30

Ser Leu Glu Asp Asp Pro Ser Gln Ser Ala Asn Leu Leu Ala Glu Ala
35 40 45

Lys Lys Leu Asn Asp Ala Gln Ala Pro Lys
50 55

<210> 7

<211> 58

<212> PRT

<213> Artificial

<400> 7

Val Asp Asn Lys Phe Asn Lys Glu Gly Lys Gly Ala Pro Gly Glu Ile
1 5 10 15

His Tyr Leu Pro Asn Leu Asn Ala Asp Gln Leu Cys Ala Phe Ile Arg
20 25 30

Ser Leu Glu Asp Asp Pro Ser Gln Ser Ala Asn Leu Leu Ala Glu Ala
35 40 45

Lys Lys Leu Asn Asp Ala Gln Ala Pro Lys
50 55

<210> 8

<211> 58

<212> PRT

<213> Artificial

<400> 8

Val Asp Asn Lys Phe Asn Lys Glu Gly Ser Lys Ala His Gly Glu Ile
1 5 10 15

Val Tyr Leu Pro Asn Leu Asn Ala Glu Gln Leu Cys Ala Phe Ile Gly
20 25 30

Ser Leu Glu Asp Asp Pro Ser Gln Ser Ala Asn Leu Leu Ala Glu Ala
35 40 45

Lys Lys Leu Asn Asp Ala Gln Ala Pro Lys
50 55

<210> 9

<211> 58

<212> PRT

<213> Artificial

<400> 9

Val Asp Asn Lys Phe Asn Lys Glu Gly Thr Leu Ala Asn Pro Glu Ile
1 5 10 15

Val Lys Leu Pro Asn Leu Asn Ala Asn Gln Leu Cys Ala Phe Ile Arg
20 25 30

Ser Leu Glu Asp Asp Pro Ser Gln Ser Ala Asn Leu Leu Ala Glu Ala
35 40 45

Lys Lys Leu Asn Asp Ala Gln Ala Pro Lys
50 55

<210> 10

<211> 58

<212> PRT

<213> Artificial

<400> 10

Val Asp Asn Lys Phe Asn Lys Glu Gly Val Ser Ala Ala Arg Glu Ile
1 5 10 15

Val Pro Leu Pro Asn Leu Asn Ala Asp Gln Leu Cys Ala Phe Ile Tyr
20 25 30

Ser Leu Gln Asp Asp Pro Ser Gln Ser Ala Asn Leu Leu Ala Glu Ala
35 40 45

Lys Lys Leu Asn Asp Ala Gln Ala Pro Lys
50 55

<210> 11

<211> 58

<212> PRT

<213> Artificial

<400> 11

Val Asp Asn Lys Phe Asn Lys Glu Ile Glu Val Ala Thr Gly Glu Ile
1 5 10 15

Val Tyr Leu Pro Asn Leu Asn Ala Asp Gln Leu Cys Ala Phe Ile Asn
20 25 30

Ser Leu Glu Asp Asp Pro Ser Gln Ser Ala Asn Leu Leu Ala Glu Ala
35 40 45

Lys Lys Leu Asn Asp Ala Gln Ala Pro Lys
50 55

<210> 12

<211> 58

<212> PRT

<213> Artificial

<400> 12

Val Asp Asn Lys Phe Asn Lys Glu Met Ala Ser Ala Gly Gly Glu Ile
1 5 10 15

Val Tyr Leu Pro Asn Leu Asn Pro Asp Gln Leu Cys Ala Phe Ile His
20 25 30

Ser Leu His Asp Asp Pro Ser Gln Ser Ala Asn Leu Leu Ala Glu Ala
35 40 45

Lys Lys Leu Asn Asp Ala Gln Ala Pro Lys
50 55

<210> 13

<211> 58

<212> PRT

<213> Artificial

<400> 13

Val Asp Asn Lys Phe Asn Lys Glu Asn Met Lys Ala Thr Gly Glu Ile
1 5 10 15

Val Trp Leu Pro Asn Leu Asn Ala Ser Gln Leu Cys Ala Phe Ile Gln
20 25 30

Ser Leu Glu Asp Asp Pro Ser Gln Ser Ala Asn Leu Leu Ala Glu Ala
35 40 45

Lys Lys Leu Asn Asp Ala Gln Ala Pro Lys
50 55

<210> 14

<211> 58

<212> PRT

<213> Artificial

<400> 14

```
Val Asp Asn Lys Phe Asn Lys Glu Asn Arg Tyr Ala Pro Gly Glu Ile
1               5                   10                  15

Thr Tyr Leu Pro Asn Leu Asn Ser Asp Gln Leu Cys Ala Phe Ile Arg
            20                  25                  30

Ser Leu Glu Asp Asp Pro Ser Gln Ser Ala Asn Leu Leu Ala Glu Ala
        35                  40                  45

Lys Lys Leu Asn Asp Ala Gln Ala Pro Lys
    50                  55
```

<210> 15

<211> 58

<212> PRT

<213> Artificial

<400> 15

```
Val Asp Asn Lys Phe Asn Lys Glu Asn Ser Asn Ala Gly Pro Glu Ile
1               5                   10                  15

Val Leu Leu Pro Asn Leu Asn Ala Ala Gln Leu Cys Ala Phe Ile Gln
            20                  25                  30

Ser Leu Glu Asp Asp Pro Ser Gln Ser Ala Asn Leu Leu Ala Glu Ala
        35                  40                  45

Lys Lys Leu Asn Asp Ala Gln Ala Pro Lys
    50                  55
```

<210> 16

<211> 58

<212> PRT

<213> Artificial

<400> 16

Val Asp Asn Lys Phe Asn Lys Glu Pro Met Met Ala Gly Gly Glu Ile
1 5 10 15

Val Tyr Leu Pro Asn Leu Asn Ser His Gln Leu Cys Ala Phe Ile Lys
20 25 30

Ser Leu Glu Asp Asp Pro Ser Gln Ser Ala Asn Leu Leu Ala Glu Ala
35 40 45

Lys Lys Leu Asn Asp Ala Gln Ala Pro Lys
50 55

<210> 17

<211> 58

<212> PRT

<213> Artificial

<400> 17

Val Asp Asn Lys Phe Asn Lys Glu Gln Met Leu Ala Asp Arg Glu Ile
1 5 10 15

Val Tyr Leu Pro Asn Leu Asn Ala Asn Gln Leu Cys Ala Phe Ile Met
20 25 30

Ser Leu Glu Asp Asp Pro Ser Gln Ser Ala Asn Leu Leu Ala Glu Ala
35 40 45

Lys Lys Leu Asn Asp Ala Gln Ala Pro Lys
50 55

<210> 18

<211> 58

<212> PRT

<213> Artificial

<400> 18

Val Asp Asn Lys Phe Asn Lys Glu Gln Ser Glu Ala Pro Ile Glu Ile
1 5 10 15

Val Tyr Leu Pro Asn Leu Asn Ala Glu Gln Leu Cys Ala Phe Ile Gln
20 25 30

Ser Leu Tyr Asp Asp Pro Ser Gln Ser Ala Asn Leu Leu Ala Glu Ala
35 40 45

Lys Lys Leu Asn Asp Ala Gln Ala Pro Lys
50 55

<210> 19

<211> 58

<212> PRT

<213> Artificial

<400> 19

Val Asp Asn Lys Phe Asn Lys Glu Gln Thr Met Ala Thr Gly Glu Ile
1 5 10 15

Val Tyr Leu Pro Asn Leu Asn Pro Glu Gln Leu Cys Ala Phe Ile His
20 25 30

Ser Leu Glu Asp Asp Pro Ser Gln Ser Ala Asn Leu Leu Ala Glu Ala
35 40 45

Lys Lys Leu Asn Asp Ala Gln Ala Pro Lys
50 55

<210> 20

<211> 58

<212> PRT

<213> Artificial

<400> 20

Val Asp Asn Lys Phe Asn Lys Glu Gln Gly Thr Ala Gln Pro Glu Ile
1 5 10 15

Thr Tyr Leu Pro Asn Leu Asn Ser Asp Gln Leu Cys Ala Phe Ile Lys
20 25 30

Ser Leu Glu Asp Asp Pro Ser Gln Ser Ala Asn Leu Leu Ala Glu Ala
35 40 45

Lys Lys Leu Asn Asp Ala Gln Ala Pro Lys
50 55

<210> 21

<211> 58

<212> PRT

<213> Artificial

<400> 21

Val Asp Asn Lys Phe Asn Lys Glu Gln Pro Lys Ala Gly Lys Glu Ile
1 5 10 15

Val Tyr Leu Pro Asn Leu Asn Gly Thr Gln Leu Cys Ala Phe Ile Lys
20 25 30

Ser Leu Glu Asp Asp Pro Ser Gln Ser Ala Asn Leu Leu Ala Glu Ala
35 40 45

Lys Lys Leu Asn Asp Ala Gln Ala Pro Lys
50 55

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<210> 22

<211> 58

<212> PRT

<213> Artificial

<400> 22

Val Asp Asn Lys Phe Asn Lys Glu Pro Ser Lys Ala Lys Arg Glu Ile
1 5 10 15

Thr Tyr Leu Pro Asn Leu Asn Ala Ser Gln Leu Cys Ala Phe Ile Lys
20 25 30

Ser Leu Tyr Asp Asp Pro Ser Gln Ser Ala Asn Leu Leu Ala Glu Ala
35 40 45

Lys Lys Leu Asn Asp Ala Gln Ala Pro Lys
50 55

<210> 23

<211> 58

<212> PRT

<213> Artificial

<400> 23

Val Asp Asn Lys Phe Asn Lys Glu Arg Glu Gly Ala Ala Gly Glu Ile
1 5 10 15

Val Phe Leu Pro Asn Leu Asn Gly Glu Gln Leu Cys Ala Phe Ile Lys
20 25 30

Ser Leu Glu Asp Asp Pro Ser Gln Ser Ala Asn Leu Leu Ala Glu Ala
35 40 45

Lys Lys Leu Asn Asp Ala Gln Ala Pro Lys
50 55

<210> 24

<211> 58

<212> PRT

<213> Artificial

<400> 24

Val Asp Asn Lys Phe Asn Lys Glu Arg Gly Ala Ala Pro Pro Glu Ile
1 5 10 15

Val Arg Leu Pro Asn Leu Asn Ala Glu Gln Leu Cys Ala Phe Ile Gln
20 25 30

Ser Leu Glu Asp Asp Pro Ser Gln Ser Ala Asn Leu Leu Ala Glu Ala
35 40 45

Lys Lys Leu Asn Asp Ala Gln Ala Pro Lys
50 55

<210> 25

<211> 58

<212> PRT

<213> Artificial

<400> 25

Val Asp Asn Lys Phe Asn Lys Glu Arg Gly Gln Ala Gln Gly Glu Ile
1 5 10 15

His Tyr Leu Pro Asn Leu Asn Ala Thr Gln Leu Cys Ala Phe Ile Thr
20 25 30

Ser Leu Tyr Asp Asp Pro Ser Gln Ser Ala Asn Leu Leu Ala Glu Ala
35 40 45

Lys Lys Leu Asn Asp Ala Gln Ala Pro Lys
50 55

<210> 26

<211> 58

<212> PRT

<213> Artificial

<400> 26

Val Asp Asn Lys Phe Asn Lys Glu Arg Gly Gln Ala Asp Arg Glu Ile
1 5 10 15

Val Met Leu Pro Asn Leu Asn Ala Ser Gln Leu Cys Ala Phe Ile Lys
20 25 30

Ser Leu Gln Asp Asp Pro Ser Gln Ser Ala Asn Leu Leu Ala Glu Ala
35 40 45

Lys Lys Leu Asn Asp Ala Gln Ala Pro Lys
50 55

<210> 27

<211> 58

<212> PRT

<213> Artificial

<400> 27

Val Asp Asn Lys Phe Asn Lys Glu Arg Gly Arg Ala Glu Gly Glu Ile
1 5 10 15

Val Tyr Leu Pro Asn Leu Asn Ala Asp Gln Leu Cys Ala Phe Ile Phe
20 25 30

Ser Leu Gln Asp Asp Pro Ser Gln Ser Ala Asn Leu Leu Ala Glu Ala
35 40 45

Lys Lys Leu Asn Asp Ala Gln Ala Pro Lys
50 55

<210> 28

<211> 58

<212> PRT

<213> Artificial

<400> 28

Val Asp Asn Lys Phe Asn Lys Glu Arg Gly Val Ala Ser Pro Glu Ile
1 5 10 15

Val Arg Leu Pro Asn Leu Asn Ala Gln Gln Leu Cys Ala Phe Ile Arg
20 25 30

Ser Leu Glu Asp Asp Pro Ser Gln Ser Ala Asn Leu Leu Ala Glu Ala
35 40 45

Lys Lys Leu Asn Asp Ala Gln Ala Pro Lys
50 55

<210> 29

<211> 58

<212> PRT

<213> Artificial

<400> 29

Val Asp Asn Lys Phe Asn Lys Glu Arg Pro Glu Ala Ser Arg Glu Ile
1 5 10 15

Val Tyr Leu Pro Asn Leu Asn Ala Ala Gln Leu Cys Ala Phe Ile Glu
20 25 30

Ser Leu Glu Asp Asp Pro Ser Gln Ser Ala Asn Leu Leu Ala Glu Ala
35 40 45

Lys Lys Leu Asn Asp Ala Gln Ala Pro Lys
50 55

<210> 30

<211> 58

<212> PRT

<213> Artificial

<400> 30

Val Asp Asn Lys Phe Asn Lys Glu Arg Pro Glu Ala Ala Arg Glu Ile
1 5 10 15

Val Tyr Leu Pro Asn Leu Asn Ala Asp Gln Leu Cys Ala Phe Ile Glu
20 25 30

Ser Leu Gln Asp Asp Pro Ser Gln Ser Ala Asn Leu Leu Ala Glu Ala
35 40 45

Lys Lys Leu Asn Asp Ala Gln Ala Pro Lys
50 55

<210> 31

<211> 58

<212> PRT

<213> Artificial

<400> 31

Val Asp Asn Lys Phe Asn Lys Glu Arg Val Arg Ala Ser Gly Glu Ile
1 5 10 15

Val Tyr Leu Pro Asn Leu Asn Ala Asp Gln Leu Cys Ala Phe Ile Tyr
20 25 30

Ser Leu Gln Asp Asp Pro Ser Gln Ser Ala Asn Leu Leu Ala Glu Ala
35 40 45

Lys Lys Leu Asn Asp Ala Gln Ala Pro Lys
50 55

<210> 32

<211> 58

<212> PRT

<213> Artificial

<400> 32

Val Asp Asn Lys Phe Asn Lys Glu Ser Glu Lys Ala Pro Val Glu Ile
1 5 10 15

Val Tyr Leu Pro Asn Leu Asn Ala Glu Gln Leu Cys Ala Phe Ile Tyr
20 25 30

Ser Leu Gln Asp Asp Pro Ser Gln Ser Ala Asn Leu Leu Ala Glu Ala
35 40 45

Lys Lys Leu Asn Asp Ala Gln Ala Pro Lys
50 55

<210> 33

<211> 58

<212> PRT

<213> Artificial

<400> 33

Val Asp Asn Lys Phe Asn Lys Glu Ser Lys Gly Ala Lys Lys Glu Ile
1 5 10 15

Val Phe Leu Pro Asn Leu Asn Ala Ile Gln Leu Cys Ala Phe Ile Gln
20 25 30

Ser Leu Glu Asp Asp Pro Ser Gln Ser Ala Asn Leu Leu Ala Glu Ala
35 40 45

Lys Lys Leu Asn Asp Ala Gln Ala Pro Lys
50 55

<210> 34

<211> 58

<212> PRT

<213> Artificial

<400> 34

Val Asp Asn Lys Phe Asn Lys Glu Ser Thr Pro Ala Thr Pro Glu Ile
1 5 10 15

Val Tyr Leu Pro Asn Leu Asn Ala Ala Gln Leu Cys Ala Phe Ile Lys
20 25 30

Ser Leu Glu Asp Asp Pro Ser Gln Ser Ala Asn Leu Leu Ala Glu Ala
35 40 45

Lys Lys Leu Asn Asp Ala Gln Ala Pro Lys
50 55

<210> 35

<211> 58

<212> PRT

<213> Artificial

<400> 35

Val Asp Asn Lys Phe Asn Lys Glu Thr Arg Arg Ala Gly Arg Glu Ile
1 5 10 15

Val Arg Leu Pro Asn Leu Asn Ala Asp Gln Leu Cys Ala Phe Ile Arg
20 25 30

Ser Leu Glu Asp Asp Pro Ser Gln Ser Ala Asn Leu Leu Ala Glu Ala
35 40 45

Lys Lys Leu Asn Asp Ala Gln Ala Pro Lys
50 55

<210> 36

<211> 58

<212> PRT

<213> Artificial

<400> 36

Val Asp Asn Lys Phe Asn Lys Glu Thr Ala Gly Ala Thr Arg Glu Ile
1 5 10 15

Val Tyr Leu Pro Asn Leu Asn Ala Glu Gln Leu Cys Ala Phe Ile Lys
20 25 30

Ser Leu Glu Asp Asp Pro Ser Gln Ser Ala Asn Leu Leu Ala Glu Ala
35 40 45

Lys Lys Leu Asn Asp Ala Gln Ala Pro Lys
50 55

<210> 37

<211> 58

<212> PRT

<213> Artificial

<400> 37

Val Asp Asn Lys Phe Asn Lys Glu Val Gly Ser Ala Ala Lys Glu Ile
1 5 10 15

Ile Pro Leu Pro Asn Leu Asn Ala Glu Gln Leu Cys Ala Phe Ile Arg
20 25 30

Ser Leu Glu Asp Asp Pro Ser Gln Ser Ala Asn Leu Leu Ala Glu Ala
35 40 45

Lys Lys Leu Asn Asp Ala Gln Ala Pro Lys
50 55

<210> 38

<211> 58

<212> PRT

<213> Artificial

<400> 38

Val Asp Asn Lys Phe Asn Lys Glu Val Gln Gly Ala Gln Pro Glu Ile
1 5 10 15

Val Arg Leu Pro Asn Leu Asn Ala Ser Gln Leu Cys Ala Phe Ile Gln
20 25 30

Ser Leu Gln Asp Asp Pro Ser Gln Ser Ala Asn Leu Leu Ala Glu Ala
35 40 45

Lys Lys Leu Asn Asp Ala Gln Ala Pro Lys
50 55

<210> 39

<211> 58

<212> PRT

<213> Artificial

<400> 39

Val Asp Asn Lys Phe Asn Lys Glu Gly Gly Pro Ala Gln Gly Glu Ile
1 5 10 15

Val Tyr Leu Pro Asn Leu Asn Pro Ala Gln Ile Cys Ala Phe Ile Gln
20 25 30

Ser Leu Ala Asp Asp Pro Ser Gln Ser Ala Asn Leu Leu Ala Glu Ala
35 40 45

Lys Lys Leu Asn Asp Ala Gln Ala Pro Lys
50 55

<210> 40

<211> 58

<212> PRT

<213> Artificial

<400> 40

Val Asp Asn Lys Phe Asn Lys Glu Arg Ser Gly Ala Asn Arg Glu Ile
1 5 10 15

Val Tyr Leu Pro Asn Leu Asn Gly Pro Gln Val Cys Ala Phe Ile Leu
20 25 30

Ser Leu Tyr Asp Asp Pro Ser Gln Ser Ala Asn Leu Leu Ala Glu Ala
35 40 45

Lys Lys Leu Asn Asp Ala Gln Ala Pro Lys
50 55

<210> 41

<211> 58

<212> PRT

<213> Artificial

<400> 41

Val Asp Asn Lys Phe Asn Lys Glu Ala Gln Cys Ala Leu Val Glu Ile
1 5 10 15

Thr Tyr Leu Pro Asn Leu Asn Arg Lys Gln Cys Arg Ala Phe Ile Arg
20 25 30

Ser Leu Asp Asp Asp Pro Ser Gln Ser Ala Asn Leu Leu Ala Glu Ala
35 40 45

Lys Lys Leu Asn Asp Ala Gln Ala Pro Lys
50 55

<210> 42

<211> 58

<212> PRT

<213> Artificial

<400> 42

Val Asp Asn Lys Phe Asn Lys Glu His Arg Cys Ala His Tyr Glu Ile
1 5 10 15

Thr Val Leu Pro Asn Leu Asn Pro Arg Gln Cys Arg Ala Phe Ile Ser
20 25 30

Ser Leu Arg Asp Asp Pro Ser Gln Ser Ala Asn Leu Leu Ala Glu Ala
35 40 45

Lys Lys Leu Asn Asp Ala Gln Ala Pro Lys
50 55

<210> 43

<211> 58

<212> PRT

<213> Artificial

<400> 43

Val Asp Asn Lys Phe Asn Lys Glu Pro Lys Gln Ala Thr Trp Glu Ile
1 5 10 15

Val Arg Leu Pro Asn Leu Asn Arg Gln Thr Glu Ala Ala Phe Ile Arg
20 25 30

Ser Leu Leu Asp Asp Pro Ser Gln Ser Ala Asn Leu Leu Ala Glu Ala
35 40 45

Lys Lys Leu Asn Asp Ala Gln Ala Pro Lys
50 55

<210> 44

<211> 58

<212> PRT

<213> Artificial

<400> 44

Val Asp Asn Lys Phe Asn Lys Glu Arg Glu Lys Ala Ser Trp Glu Ile
1 5 10 15

Trp Glu Leu Pro Asn Leu Asn Glu Asp Gln Thr Ser Ala Phe Ile Gln
20 25 30

Ser Leu Lys Asp Asp Pro Ser Gln Ser Ala Asn Leu Leu Ala Glu Ala
35 40 45

Lys Lys Leu Asn Asp Ala Gln Ala Pro Lys
50 55

<210> 45

<211> 58

<212> PRT

<213> Artificial

<400> 45

Val Asp Asn Lys Phe Asn Lys Glu Gly Gly Leu Ala Pro Gly Glu Ile
1 5 10 15

Val Tyr Leu Pro Asn Leu Asn Ala Ser Gln Leu Ser Ala Phe Ile His
20 25 30

Ser Leu Glu Asp Asp Pro Ser Gln Ser Ala Asn Leu Leu Ala Glu Ala
35 40 45

Lys Lys Leu Asn Asp Ala Gln Ala Pro Lys
50 55

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<210> 46

<211> 58

<212> PRT

<213> Artificial

<400> 46

Val Asp Asn Lys Phe Asn Lys Glu Met Ala Ser Ala Gly Gly Glu Ile
1 5 10 15

Val Tyr Leu Pro Asn Leu Asn Pro Asp Gln Leu Ser Ala Phe Ile His
20 25 30

Ser Leu His Asp Asp Pro Ser Gln Ser Ala Asn Leu Leu Ala Glu Ala
35 40 45

Lys Lys Leu Asn Asp Ala Gln Ala Pro Lys
50 55

<210> 47

<211> 115

<212> PRT

<213> Artificial

<400> 47

Ala Gln His Asp Glu Ala Leu Glu Val Asp Asn Lys Phe Asn Lys Glu
1 5 10 15

Gly Gly Leu Ala Pro Gly Glu Ile Val Tyr Leu Pro Asn Leu Asn Ala
20 25 30

Ser Gln Leu Cys Ala Phe Ile His Ser Leu Glu Asp Asp Pro Ser Gln
35 40 45

Ser Ala Asn Leu Leu Ala Glu Ala Lys Lys Leu Asn Asp Ala Gln Ala
50 55 60

Pro Lys Val Asp Tyr Leu Ala Glu Ala Lys Val Leu Ala Asn Arg Glu

65 70 75 80

Leu Asp Lys Tyr Gly Val Ser Asp Tyr Tyr Lys Asn Leu Ile Asn Asn
85 90 95

Ala Lys Thr Val Glu Gly Val Lys Ala Leu Ile Asp Glu Ile Leu Ala
100 105 110

Ala Leu Pro
115

<210> 48

<211> 115

<212> PRT

<213> Artificial

<400> 48

Ala Gln His Asp Glu Ala Leu Glu Val Asp Asn Lys Phe Asn Lys Glu
1 5 10 15

Gly Lys Gly Ala Pro Gly Glu Ile His Tyr Leu Pro Asn Leu Asn Ala
20 25 30

Asp Gln Leu Cys Ala Phe Ile Arg Ser Leu Glu Asp Asp Pro Ser Gln
35 40 45

Ser Ala Asn Leu Leu Ala Glu Ala Lys Lys Leu Asn Asp Ala Gln Ala
50 55 60

Pro Lys Val Asp Tyr Leu Ala Glu Ala Lys Val Leu Ala Asn Arg Glu
65 70 75 80

Leu Asp Lys Tyr Gly Val Ser Asp Tyr Tyr Lys Asn Leu Ile Asn Asn
85 90 95

Ala Lys Thr Val Glu Gly Val Lys Ala Leu Ile Asp Glu Ile Leu Ala
100 105 110

Ala Leu Pro
115

<210> 49

<211> 115

<212> PRT

<213> Artificial

<400> 49

Ala Gln His Asp Glu Ala Leu Glu Val Asp Asn Lys Phe Asn Lys Glu
1 5 10 15

Met Ala Ser Ala Gly Gly Glu Ile Val Tyr Leu Pro Asn Leu Asn Pro
20 25 30

Asp Gln Leu Cys Ala Phe Ile His Ser Leu His Asp Asp Pro Ser Gln
35 40 45

Ser Ala Asn Leu Leu Ala Glu Ala Lys Lys Leu Asn Asp Ala Gln Ala
50 55 60

Pro Lys Val Asp Tyr Leu Ala Glu Ala Lys Val Leu Ala Asn Arg Glu
65 70 75 80

Leu Asp Lys Tyr Gly Val Ser Asp Tyr Tyr Lys Asn Leu Ile Asn Asn
85 90 95

Ala Lys Thr Val Glu Gly Val Lys Ala Leu Ile Asp Glu Ile Leu Ala
100 105 110

Ala Leu Pro
115

<210> 50

<211> 115

<212> PRT

<213> Artificial

<400> 50

```
Ala Gln His Asp Glu Ala Leu Glu Val Asp Asn Lys Phe Asn Lys Glu
1               5                  10                  15

Asn Met Lys Ala Thr Gly Glu Ile Val Trp Leu Pro Asn Leu Asn Ala
            20                  25                  30

Ser Gln Leu Cys Ala Phe Ile Gln Ser Leu Glu Asp Asp Pro Ser Gln
        35                  40                  45

Ser Ala Asn Leu Leu Ala Glu Ala Lys Lys Leu Asn Asp Ala Gln Ala
    50                  55                  60

Pro Lys Val Asp Tyr Leu Ala Glu Ala Lys Val Leu Ala Asn Arg Glu
65                  70                  75                  80

Leu Asp Lys Tyr Gly Val Ser Asp Tyr Tyr Lys Asn Leu Ile Asn Asn
                85                  90                  95

Ala Lys Thr Val Glu Gly Val Lys Ala Leu Ile Asp Glu Ile Leu Ala
            100                 105                 110

Ala Leu Pro
        115
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<210> 51

<211> 115

<212> PRT

<213> Artificial

<400> 51

```
Ala Gln His Asp Glu Ala Leu Glu Val Asp Asn Lys Phe Asn Lys Glu
1               5                  10                  15

Asn Ser Asn Ala Gly Pro Glu Ile Val Leu Leu Pro Asn Leu Asn Ala
            20                  25                  30

Ala Gln Leu Cys Ala Phe Ile Gln Ser Leu Glu Asp Asp Pro Ser Gln
        35                  40                  45
```

```
Ser Ala Asn Leu Leu Ala Glu Ala Lys Lys Leu Asn Asp Ala Gln Ala
    50                  55                  60

Pro Lys Val Asp Tyr Leu Ala Glu Ala Lys Val Leu Ala Asn Arg Glu
65                  70                  75                  80

Leu Asp Lys Tyr Gly Val Ser Asp Tyr Tyr Lys Asn Leu Ile Asn Asn
                85                  90                  95

Ala Lys Thr Val Glu Gly Val Lys Ala Leu Ile Asp Glu Ile Leu Ala
            100                 105                 110

Ala Leu Pro
        115
```

<210> 52

<211> 115

<212> PRT

<213> Artificial

<400> 52

```
Ala Gln His Asp Glu Ala Leu Glu Val Asp Asn Lys Phe Asn Lys Glu
1               5                   10                  15

Gln Thr Met Ala Thr Gly Glu Ile Val Tyr Leu Pro Asn Leu Asn Pro
            20                  25                  30

Glu Gln Leu Cys Ala Phe Ile His Ser Leu Glu Asp Asp Pro Ser Gln
        35                  40                  45

Ser Ala Asn Leu Leu Ala Glu Ala Lys Lys Leu Asn Asp Ala Gln Ala
    50                  55                  60

Pro Lys Val Asp Tyr Leu Ala Glu Ala Lys Val Leu Ala Asn Arg Glu
65                  70                  75                  80

Leu Asp Lys Tyr Gly Val Ser Asp Tyr Tyr Lys Asn Leu Ile Asn Asn
                85                  90                  95

Ala Lys Thr Val Glu Gly Val Lys Ala Leu Ile Asp Glu Ile Leu Ala
```

100 105 110

Ala Leu Pro
115

<210> 53

<211> 115

<212> PRT

<213> Artificial

<400> 53

Ala Gln His Asp Glu Ala Leu Glu Val Asp Asn Lys Phe Asn Lys Glu
1 5 10 15

Arg Gly Ala Ala Pro Pro Glu Ile Val Arg Leu Pro Asn Leu Asn Ala
20 25 30

Glu Gln Leu Cys Ala Phe Ile Gln Ser Leu Glu Asp Asp Pro Ser Gln
35 40 45

Ser Ala Asn Leu Leu Ala Glu Ala Lys Lys Leu Asn Asp Ala Gln Ala
50 55 60

Pro Lys Val Asp Tyr Leu Ala Glu Ala Lys Val Leu Ala Asn Arg Glu
65 70 75 80

Leu Asp Lys Tyr Gly Val Ser Asp Tyr Tyr Lys Asn Leu Ile Asn Asn
85 90 95

Ala Lys Thr Val Glu Gly Val Lys Ala Leu Ile Asp Glu Ile Leu Ala
100 105 110

Ala Leu Pro
115

<210> 54

<211> 115

<212> PRT

<213> Artificial

<400> 54

Ala Gln His Asp Glu Ala Leu Glu Val Asp Asn Lys Phe Asn Lys Glu
1 5 10 15

Arg Gly Gln Ala Gln Gly Glu Ile His Tyr Leu Pro Asn Leu Asn Ala
20 25 30

Thr Gln Leu Cys Ala Phe Ile Thr Ser Leu Tyr Asp Asp Pro Ser Gln
35 40 45

Ser Ala Asn Leu Leu Ala Glu Ala Lys Lys Leu Asn Asp Ala Gln Ala
50 55 60

Pro Lys Val Asp Tyr Leu Ala Glu Ala Lys Val Leu Ala Asn Arg Glu
65 70 75 80

Leu Asp Lys Tyr Gly Val Ser Asp Tyr Tyr Lys Asn Leu Ile Asn Asn
85 90 95

Ala Lys Thr Val Glu Gly Val Lys Ala Leu Ile Asp Glu Ile Leu Ala
100 105 110

Ala Leu Pro
115

<210> 55

<211> 115

<212> PRT

<213> Artificial

<400> 55

Ala Gln His Asp Glu Ala Leu Glu Val Asp Asn Lys Phe Asn Lys Glu
1 5 10 15

Arg Gly Gln Ala Asp Arg Glu Ile Val Met Leu Pro Asn Leu Asn Ala
20 25 30

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Ser Gln Leu Cys Ala Phe Ile Lys Ser Leu Gln Asp Asp Pro Ser Gln
        35                  40                  45

Ser Ala Asn Leu Leu Ala Glu Ala Lys Lys Leu Asn Asp Ala Gln Ala
    50                  55                  60

Pro Lys Val Asp Tyr Leu Ala Glu Ala Lys Val Leu Ala Asn Arg Glu
65                  70                  75                  80

Leu Asp Lys Tyr Gly Val Ser Asp Tyr Tyr Lys Asn Leu Ile Asn Asn
                85                  90                  95

Ala Lys Thr Val Glu Gly Val Lys Ala Leu Ile Asp Glu Ile Leu Ala
            100                 105                 110

Ala Leu Pro
        115
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<210> 56

<211> 115

<212> PRT

<213> Artificial

<400> 56

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Ala Gln His Asp Glu Ala Leu Glu Val Asp Asn Lys Phe Asn Lys Glu
1               5                   10                  15

Arg Gly Arg Ala Glu Gly Glu Ile Val Tyr Leu Pro Asn Leu Asn Ala
            20                  25                  30

Asp Gln Leu Cys Ala Phe Ile Phe Ser Leu Gln Asp Asp Pro Ser Gln
        35                  40                  45

Ser Ala Asn Leu Leu Ala Glu Ala Lys Lys Leu Asn Asp Ala Gln Ala
    50                  55                  60

Pro Lys Val Asp Tyr Leu Ala Glu Ala Lys Val Leu Ala Asn Arg Glu
65                  70                  75                  80
```

Leu Asp Lys Tyr Gly Val Ser Asp Tyr Tyr Lys Asn Leu Ile Asn Asn
85 90 95

Ala Lys Thr Val Glu Gly Val Lys Ala Leu Ile Asp Glu Ile Leu Ala
100 105 110

Ala Leu Pro
115

<210> 57

<211> 115

<212> PRT

<213> Artificial

<400> 57

Ala Gln His Asp Glu Ala Leu Glu Val Asp Asn Lys Phe Asn Lys Glu
1 5 10 15

Arg Gly Val Ala Ser Pro Glu Ile Val Arg Leu Pro Asn Leu Asn Ala
20 25 30

Gln Gln Leu Cys Ala Phe Ile Arg Ser Leu Glu Asp Asp Pro Ser Gln
35 40 45

Ser Ala Asn Leu Leu Ala Glu Ala Lys Lys Leu Asn Asp Ala Gln Ala
50 55 60

Pro Lys Val Asp Tyr Leu Ala Glu Ala Lys Val Leu Ala Asn Arg Glu
65 70 75 80

Leu Asp Lys Tyr Gly Val Ser Asp Tyr Tyr Lys Asn Leu Ile Asn Asn
85 90 95

Ala Lys Thr Val Glu Gly Val Lys Ala Leu Ile Asp Glu Ile Leu Ala
100 105 110

Ala Leu Pro
115

<210> 58

<211> 115

<212> PRT

<213> Artificial

<400> 58

Ala Gln His Asp Glu Ala Leu Glu Val Asp Asn Lys Phe Asn Lys Glu
1 5 10 15

Arg Val Arg Ala Ser Gly Glu Ile Val Tyr Leu Pro Asn Leu Asn Ala
20 25 30

Asp Gln Leu Cys Ala Phe Ile Tyr Ser Leu Gln Asp Asp Pro Ser Gln
35 40 45

Ser Ala Asn Leu Leu Ala Glu Ala Lys Lys Leu Asn Asp Ala Gln Ala
50 55 60

Pro Lys Val Asp Tyr Leu Ala Glu Ala Lys Val Leu Ala Asn Arg Glu
65 70 75 80

Leu Asp Lys Tyr Gly Val Ser Asp Tyr Tyr Lys Asn Leu Ile Asn Asn
85 90 95

Ala Lys Thr Val Glu Gly Val Lys Ala Leu Ile Asp Glu Ile Leu Ala
100 105 110

Ala Leu Pro
115

<210> 59

<211> 115

<212> PRT

<213> Artificial

<400> 59

Ala Gln His Asp Glu Ala Leu Glu Val Asp Asn Lys Phe Asn Lys Glu

1 5 10 15

Val Gln Gly Ala Gln Pro Glu Ile Val Arg Leu Pro Asn Leu Asn Ala
20 25 30

Ser Gln Leu Cys Ala Phe Ile Gln Ser Leu Gln Asp Asp Pro Ser Gln
35 40 45

Ser Ala Asn Leu Leu Ala Glu Ala Lys Lys Leu Asn Asp Ala Gln Ala
50 55 60

Pro Lys Val Asp Tyr Leu Ala Glu Ala Lys Val Leu Ala Asn Arg Glu
65 70 75 80

Leu Asp Lys Tyr Gly Val Ser Asp Tyr Tyr Lys Asn Leu Ile Asn Asn
85 90 95

Ala Lys Thr Val Glu Gly Val Lys Ala Leu Ile Asp Glu Ile Leu Ala
100 105 110

Ala Leu Pro
115

<210> 60

<211> 115

<212> PRT

<213> Artificial

<400> 60

Ala Gln His Asp Glu Ala Leu Glu Val Asp Asn Lys Phe Asn Lys Glu
1 5 10 15

Gly Gly Pro Ala Gln Gly Glu Ile Val Tyr Leu Pro Asn Leu Asn Pro
20 25 30

Ala Gln Ile Cys Ala Phe Ile Gln Ser Leu Ala Asp Asp Pro Ser Gln
35 40 45

Ser Ala Asn Leu Leu Ala Glu Ala Lys Lys Leu Asn Asp Ala Gln Ala
50 55 60

```
Pro Lys Val Asp Tyr Leu Ala Glu Ala Lys Val Leu Ala Asn Arg Glu
65                  70                  75                  80

Leu Asp Lys Tyr Gly Val Ser Asp Tyr Tyr Lys Asn Leu Ile Asn Asn
                85                  90                  95

Ala Lys Thr Val Glu Gly Val Lys Ala Leu Ile Asp Glu Ile Leu Ala
            100                 105                 110

Ala Leu Pro
        115
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<210> 61

<211> 115

<212> PRT

<213> Artificial

<400> 61

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Ala Gln His Asp Glu Ala Leu Glu Val Asp Asn Lys Phe Asn Lys Glu
1               5                   10                  15

Arg Ser Gly Ala Asn Arg Glu Ile Val Tyr Leu Pro Asn Leu Asn Gly
            20                  25                  30

Pro Gln Val Cys Ala Phe Ile Leu Ser Leu Tyr Asp Asp Pro Ser Gln
        35                  40                  45

Ser Ala Asn Leu Leu Ala Glu Ala Lys Lys Leu Asn Asp Ala Gln Ala
    50                  55                  60

Pro Lys Val Asp Tyr Leu Ala Glu Ala Lys Val Leu Ala Asn Arg Glu
65                  70                  75                  80

Leu Asp Lys Tyr Gly Val Ser Asp Tyr Tyr Lys Asn Leu Ile Asn Asn
                85                  90                  95

Ala Lys Thr Val Glu Gly Val Lys Ala Leu Ile Asp Glu Ile Leu Ala
            100                 105                 110
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Ala Leu Pro
115

<210> 62

<211> 115

<212> PRT

<213> Artificial

<400> 62

Ala Gln His Asp Glu Ala Leu Glu Val Asp Asn Lys Phe Asn Lys Glu
1 5 10 15

Ala Gln Cys Ala Leu Val Glu Ile Thr Tyr Leu Pro Asn Leu Asn Arg
20 25 30

Lys Gln Cys Arg Ala Phe Ile Arg Ser Leu Asp Asp Asp Pro Ser Gln
35 40 45

Ser Ala Asn Leu Leu Ala Glu Ala Lys Lys Leu Asn Asp Ala Gln Ala
50 55 60

Pro Lys Val Asp Tyr Leu Ala Glu Ala Lys Val Leu Ala Asn Arg Glu
65 70 75 80

Leu Asp Lys Tyr Gly Val Ser Asp Tyr Tyr Lys Asn Leu Ile Asn Asn
85 90 95

Ala Lys Thr Val Glu Gly Val Lys Ala Leu Ile Asp Glu Ile Leu Ala
100 105 110

Ala Leu Pro
115

<210> 63

<211> 115

<212> PRT

<213> Artificial

<400> 63

Ala Gln His Asp Glu Ala Leu Glu Val Asp Asn Lys Phe Asn Lys Glu
1 5 10 15

His Arg Cys Ala His Tyr Glu Ile Thr Val Leu Pro Asn Leu Asn Pro
20 25 30

Arg Gln Cys Arg Ala Phe Ile Ser Ser Leu Arg Asp Asp Pro Ser Gln
35 40 45

Ser Ala Asn Leu Leu Ala Glu Ala Lys Lys Leu Asn Asp Ala Gln Ala
50 55 60

Pro Lys Val Asp Tyr Leu Ala Glu Ala Lys Val Leu Ala Asn Arg Glu
65 70 75 80

Leu Asp Lys Tyr Gly Val Ser Asp Tyr Tyr Lys Asn Leu Ile Asn Asn
85 90 95

Ala Lys Thr Val Glu Gly Val Lys Ala Leu Ile Asp Glu Ile Leu Ala
100 105 110

Ala Leu Pro
115

<210> 64

<211> 115

<212> PRT

<213> Artificial

<400> 64

Ala Gln His Asp Glu Ala Leu Glu Val Asp Asn Lys Phe Asn Lys Glu
1 5 10 15

Pro Lys Gln Ala Thr Trp Glu Ile Val Arg Leu Pro Asn Leu Asn Arg
20 25 30

Gln Thr Glu Ala Ala Phe Ile Arg Ser Leu Leu Asp Asp Pro Ser Gln

35 40 45

Ser Ala Asn Leu Leu Ala Glu Ala Lys Lys Leu Asn Asp Ala Gln Ala
50 55 60

Pro Lys Val Asp Tyr Leu Ala Glu Ala Lys Val Leu Ala Asn Arg Glu
65 70 75 80

Leu Asp Lys Tyr Gly Val Ser Asp Tyr Tyr Lys Asn Leu Ile Asn Asn
85 90 95

Ala Lys Thr Val Glu Gly Val Lys Ala Leu Ile Asp Glu Ile Leu Ala
100 105 110

Ala Leu Pro
115

<210> 65

<211> 115

<212> PRT

<213> Artificial

<400> 65

Ala Gln His Asp Glu Ala Leu Glu Val Asp Asn Lys Phe Asn Lys Glu
1 5 10 15

Arg Glu Lys Ala Ser Trp Glu Ile Trp Glu Leu Pro Asn Leu Asn Glu
20 25 30

Asp Gln Thr Ser Ala Phe Ile Gln Ser Leu Lys Asp Asp Pro Ser Gln
35 40 45

Ser Ala Asn Leu Leu Ala Glu Ala Lys Lys Leu Asn Asp Ala Gln Ala
50 55 60

Pro Lys Val Asp Tyr Leu Ala Glu Ala Lys Val Leu Ala Asn Arg Glu
65 70 75 80

Leu Asp Lys Tyr Gly Val Ser Asp Tyr Tyr Lys Asn Leu Ile Asn Asn
85 90 95

Ala Lys Thr Val Glu Gly Val Lys Ala Leu Ile Asp Glu Ile Leu Ala
100 105 110

Ala Leu Pro
115

<210> 66

<211> 74

<212> PRT

<213> Artificial

<400> 66

Met Gly Ser Ser His His His His His His Tyr Tyr Leu Glu Val Asp
1 5 10 15

Asn Lys Phe Asn Lys Glu Gly Gly Leu Ala Pro Gly Glu Ile Val Tyr
20 25 30

Leu Pro Asn Leu Asn Ala Ser Gln Leu Cys Ala Phe Ile His Ser Leu
35 40 45

Glu Asp Asp Pro Ser Gln Ser Ala Asn Leu Leu Ala Glu Ala Lys Lys
50 55 60

Leu Asn Asp Ala Gln Ala Pro Lys Val Asp
65 70

<210> 67

<211> 74

<212> PRT

<213> Artificial

<400> 67

Met Gly Ser Ser His His His His His His Tyr Tyr Leu Glu Val Asp
1 5 10 15

Asn Lys Phe Asn Lys Glu Met Ala Ser Ala Gly Gly Glu Ile Val Tyr
20 25 30

Leu Pro Asn Leu Asn Pro Asp Gln Leu Cys Ala Phe Ile His Ser Leu
35 40 45

His Asp Asp Pro Ser Gln Ser Ala Asn Leu Leu Ala Glu Ala Lys Lys
50 55 60

Leu Asn Asp Ala Gln Ala Pro Lys Val Asp
65 70

<210> 68

<211> 74

<212> PRT

<213> Artificial

<400> 68

Met Gly Ser Ser His His His His His His Tyr Tyr Leu Glu Val Asp
1 5 10 15

Asn Lys Phe Asn Lys Glu Asn Met Lys Ala Thr Gly Glu Ile Val Trp
20 25 30

Leu Pro Asn Leu Asn Ala Ser Gln Leu Cys Ala Phe Ile Gln Ser Leu
35 40 45

Glu Asp Asp Pro Ser Gln Ser Ala Asn Leu Leu Ala Glu Ala Lys Lys
50 55 60

Leu Asn Asp Ala Gln Ala Pro Lys Val Asp
65 70

<210> 69

<211> 74

<212> PRT

<213> Artificial

<400> 69

Met Gly Ser Ser His His His His His His Tyr Tyr Leu Glu Val Asp
1 5 10 15

Asn Lys Phe Asn Lys Glu Asn Ser Asn Ala Gly Pro Glu Ile Val Leu
20 25 30

Leu Pro Asn Leu Asn Ala Ala Gln Leu Cys Ala Phe Ile Gln Ser Leu
35 40 45

Glu Asp Asp Pro Ser Gln Ser Ala Asn Leu Leu Ala Glu Ala Lys Lys
50 55 60

Leu Asn Asp Ala Gln Ala Pro Lys Val Asp
65 70

<210> 70

<211> 74

<212> PRT

<213> Artificial

<400> 70

Met Gly Ser Ser His His His His His His Tyr Tyr Leu Glu Val Asp
1 5 10 15

Asn Lys Phe Asn Lys Glu Gln Thr Met Ala Thr Gly Glu Ile Val Tyr
20 25 30

Leu Pro Asn Leu Asn Pro Glu Gln Leu Cys Ala Phe Ile His Ser Leu
35 40 45

Glu Asp Asp Pro Ser Gln Ser Ala Asn Leu Leu Ala Glu Ala Lys Lys
50 55 60

Leu Asn Asp Ala Gln Ala Pro Lys Val Asp
65 70

<210> 71

<211> 74

<212> PRT

<213> Artificial

<400> 71

Met Gly Ser Ser His His His His His His Tyr Tyr Leu Glu Val Asp
1 5 10 15

Asn Lys Phe Asn Lys Glu Val Gln Gly Ala Gln Pro Glu Ile Val Arg
20 25 30

Leu Pro Asn Leu Asn Ala Ser Gln Leu Cys Ala Phe Ile Gln Ser Leu
35 40 45

Gln Asp Asp Pro Ser Gln Ser Ala Asn Leu Leu Ala Glu Ala Lys Lys
50 55 60

Leu Asn Asp Ala Gln Ala Pro Lys Val Asp
65 70

<210> 72

<211> 74

<212> PRT

<213> Artificial

<400> 72

Met Gly Ser Ser His His His His His His Tyr Tyr Leu Glu Val Asp
1 5 10 15

Asn Lys Phe Asn Lys Glu Ala Gln Cys Ala Leu Val Glu Ile Thr Tyr
20 25 30

Leu Pro Asn Leu Asn Arg Lys Gln Cys Arg Ala Phe Ile Arg Ser Leu
35 40 45

Asp Asp Asp Pro Ser Gln Ser Ala Asn Leu Leu Ala Glu Ala Lys Lys

50 55 60

Leu Asn Asp Ala Gln Ala Pro Lys Val Asp
65 70

<210> 73

<211> 74

<212> PRT

<213> Artificial

<400> 73

Met Gly Ser Ser His His His His His His Tyr Tyr Leu Glu Val Asp
1 5 10 15

Asn Lys Phe Asn Lys Glu His Arg Cys Ala His Tyr Glu Ile Thr Val
20 25 30

Leu Pro Asn Leu Asn Pro Arg Gln Cys Arg Ala Phe Ile Ser Ser Leu
35 40 45

Arg Asp Asp Pro Ser Gln Ser Ala Asn Leu Leu Ala Glu Ala Lys Lys
50 55 60

Leu Asn Asp Ala Gln Ala Pro Lys Val Asp
65 70

<210> 74

<211> 74

<212> PRT

<213> Artificial

<400> 74

Met Gly Ser Ser His His His His His His Tyr Tyr Leu Glu Val Asp
1 5 10 15

Asn Lys Phe Asn Lys Glu Pro Lys Gln Ala Thr Trp Glu Ile Val Arg
20 25 30

Leu Pro Asn Leu Asn Arg Gln Thr Glu Ala Ala Phe Ile Arg Ser Leu
35 40 45

Leu Asp Asp Pro Ser Gln Ser Ala Asn Leu Leu Ala Glu Ala Lys Lys
50 55 60

Leu Asn Asp Ala Gln Ala Pro Lys Val Asp
65 70

<210> 75

<211> 74

<212> PRT

<213> Artificial

<400> 75

Met Gly Ser Ser His His His His His His Tyr Tyr Leu Glu Val Asp
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Asn Lys Phe Asn Lys Glu Arg Glu Lys Ala Ser Trp Glu Ile Trp Glu
20 25 30

Leu Pro Asn Leu Asn Glu Asp Gln Thr Ser Ala Phe Ile Gln Ser Leu
35 40 45

Lys Asp Asp Pro Ser Gln Ser Ala Asn Leu Leu Ala Glu Ala Lys Lys
50 55 60

Leu Asn Asp Ala Gln Ala Pro Lys Val Asp
65 70

<210> 76

<211> 142

<212> PRT

<213> Artificial

<400> 76

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Met Gly Ser Ser His His His His His His Tyr Tyr Leu Glu Val Asp
1               5                   10                  15

Asn Lys Phe Asn Lys Glu Gly Gly Leu Ala Pro Gly Glu Ile Val Tyr
            20                  25                  30

Leu Pro Asn Leu Asn Ala Ser Gln Leu Cys Ala Phe Ile His Ser Leu
        35                  40                  45

Glu Asp Asp Pro Ser Gln Ser Ala Asn Leu Leu Ala Glu Ala Lys Lys
    50                  55                  60

Leu Asn Asp Ala Gln Ala Pro Lys Val Asp Val Asp Asn Lys Phe Asn
65                  70                  75                  80

Lys Glu Gly Gly Leu Ala Pro Gly Glu Ile Val Tyr Leu Pro Asn Leu
                85                  90                  95

Asn Ala Ser Gln Leu Cys Ala Phe Ile His Ser Leu Glu Asp Asp Pro
            100                 105                 110

Ser Gln Ser Ala Asn Leu Leu Ala Glu Ala Lys Lys Leu Asn Asp Ala
        115                 120                 125

Gln Ala Pro Lys Glu Gln Lys Leu Ile Ser Glu Glu Asp Leu
    130                 135                 140
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<210> 77

<211> 142

<212> PRT

<213> Artificial

<400> 77

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Met Gly Ser Ser His His His His His His Tyr Tyr Leu Glu Val Asp
1               5                   10                  15

Asn Lys Phe Asn Lys Glu Met Ala Ser Ala Gly Gly Glu Ile Val Tyr
            20                  25                  30
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Leu Pro Asn Leu Asn Pro Asp Gln Leu Cys Ala Phe Ile His Ser Leu
        35                  40                  45

His Asp Asp Pro Ser Gln Ser Ala Asn Leu Leu Ala Glu Ala Lys Lys
    50                  55                  60

Leu Asn Asp Ala Gln Ala Pro Lys Val Asp Val Asp Asn Lys Phe Asn
65                  70                  75                  80

Lys Glu Met Ala Ser Ala Gly Gly Glu Ile Val Tyr Leu Pro Asn Leu
                85                  90                  95

Asn Pro Asp Gln Leu Cys Ala Phe Ile His Ser Leu His Asp Asp Pro
            100                 105                 110

Ser Gln Ser Ala Asn Leu Leu Ala Glu Ala Lys Lys Leu Asn Asp Ala
        115                 120                 125

Gln Ala Pro Lys Glu Gln Lys Leu Ile Ser Glu Glu Asp Leu
    130                 135                 140
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<210> 78

<211> 142

<212> PRT

<213> Artificial

<400> 78

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Met Gly Ser Ser His His His His His His Tyr Tyr Leu Glu Val Asp
1               5                   10                  15

Asn Lys Phe Asn Lys Glu Asn Met Lys Ala Thr Gly Glu Ile Val Trp
            20                  25                  30

Leu Pro Asn Leu Asn Ala Ser Gln Leu Cys Ala Phe Ile Gln Ser Leu
        35                  40                  45

Glu Asp Asp Pro Ser Gln Ser Ala Asn Leu Leu Ala Glu Ala Lys Lys
    50                  55                  60

Leu Asn Asp Ala Gln Ala Pro Lys Val Asp Val Asp Asn Lys Phe Asn
```

65 70 75 80

Lys Glu Asn Met Lys Ala Thr Gly Glu Ile Val Trp Leu Pro Asn Leu
85 90 95

Asn Ala Ser Gln Leu Cys Ala Phe Ile Gln Ser Leu Glu Asp Asp Pro
100 105 110

Ser Gln Ser Ala Asn Leu Leu Ala Glu Ala Lys Lys Leu Asn Asp Ala
115 120 125

Gln Ala Pro Lys Glu Gln Lys Leu Ile Ser Glu Glu Asp Leu
130 135 140

<210> 79

<211> 142

<212> PRT

<213> Artificial

<400> 79

Met Gly Ser Ser His His His His His His Tyr Tyr Leu Glu Val Asp
1 5 10 15

Asn Lys Phe Asn Lys Glu Asn Ser Asn Ala Gly Pro Glu Ile Val Leu
20 25 30

Leu Pro Asn Leu Asn Ala Ala Gln Leu Cys Ala Phe Ile Gln Ser Leu
35 40 45

Glu Asp Asp Pro Ser Gln Ser Ala Asn Leu Leu Ala Glu Ala Lys Lys
50 55 60

Leu Asn Asp Ala Gln Ala Pro Lys Val Asp Val Asp Asn Lys Phe Asn
65 70 75 80

Lys Glu Asn Ser Asn Ala Gly Pro Glu Ile Val Leu Leu Pro Asn Leu
85 90 95

Asn Ala Ala Gln Leu Cys Ala Phe Ile Gln Ser Leu Glu Asp Asp Pro
100 105 110

Ser Gln Ser Ala Asn Leu Leu Ala Glu Ala Lys Lys Leu Asn Asp Ala
115 120 125

Gln Ala Pro Lys Glu Gln Lys Leu Ile Ser Glu Glu Asp Leu
130 135 140

<210> 80

<211> 142

<212> PRT

<213> Artificial

<400> 80

Met Gly Ser Ser His His His His His His Tyr Tyr Leu Glu Val Asp
1 5 10 15

Asn Lys Phe Asn Lys Glu His Arg Cys Ala His Tyr Glu Ile Thr Val
20 25 30

Leu Pro Asn Leu Asn Pro Arg Gln Cys Arg Ala Phe Ile Ser Ser Leu
35 40 45

Arg Asp Asp Pro Ser Gln Ser Ala Asn Leu Leu Ala Glu Ala Lys Lys
50 55 60

Leu Asn Asp Ala Gln Ala Pro Lys Val Asp Val Asp Asn Lys Phe Asn
65 70 75 80

Lys Glu His Arg Cys Ala His Tyr Glu Ile Thr Val Leu Pro Asn Leu
85 90 95

Asn Pro Arg Gln Cys Arg Ala Phe Ile Ser Ser Leu Arg Asp Asp Pro
100 105 110

Ser Gln Ser Ala Asn Leu Leu Ala Glu Ala Lys Lys Leu Asn Asp Ala
115 120 125

Gln Ala Pro Lys Glu Gln Lys Leu Ile Ser Glu Glu Asp Leu
130 135 140

<210> 81

<211> 142

<212> PRT

<213> Artificial

<400> 81

```
Met Gly Ser Ser His His His His His His Tyr Tyr Leu Glu Val Asp
1               5                  10                  15

Asn Lys Phe Asn Lys Glu Pro Lys Gln Ala Thr Trp Glu Ile Val Arg
            20                  25                  30

Leu Pro Asn Leu Asn Arg Gln Thr Glu Ala Ala Phe Ile Arg Ser Leu
        35                  40                  45

Leu Asp Asp Pro Ser Gln Ser Ala Asn Leu Leu Ala Glu Ala Lys Lys
    50                  55                  60

Leu Asn Asp Ala Gln Ala Pro Lys Val Asp Val Asp Asn Lys Phe Asn
65                  70                  75                  80

Lys Glu Pro Lys Gln Ala Thr Trp Glu Ile Val Arg Leu Pro Asn Leu
                85                  90                  95

Asn Arg Gln Thr Glu Ala Ala Phe Ile Arg Ser Leu Leu Asp Asp Pro
            100                 105                 110

Ser Gln Ser Ala Asn Leu Leu Ala Glu Ala Lys Lys Leu Asn Asp Ala
        115                 120                 125

Gln Ala Pro Lys Glu Gln Lys Leu Ile Ser Glu Glu Asp Leu
    130                 135                 140
```

INTERNATIONAL SEARCH REPORT

International Application No
PCT/SE2005/000159

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C07K14/31 A61K38/16 G01N33/68 C07K19/00

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)
IPC 7 C07K G01N A61K

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 practical, search terms used)
EPO-Internal, WPI Data, PAJ, BIOSIS, Sequence Search

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 97/36614 A (TERMAN, DAVID, S) 9 October 1997 (1997-10-09) the entire document, particularly pages 1, 4,5,11,16,25-27, table 1, and the claims	1-81
X	WO 03/080655 A (AMERSHAM BIOSCIENCES AB; HOBER, SOPHIA) 2 October 2003 (2003-10-02) the entire document, particularly pages 9-13; tables 1-3; example 4; claims 1-21; figures 1,3,5, sequences 1-4	1-60,66
X	EP 0 343 227 A (CREATIVE BIOMOLECULES, INC) 29 November 1989 (1989-11-29) page 2; claims 1-6; figures 1A,1B	1-60,66
X	EP 0 550 771 A (ORIENTAL YEAST CO., LTD) 14 July 1993 (1993-07-14) page 2; figure 1	1-60,66

☐ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document 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
25 May 2005	06/06/2005

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, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Schmidt, Harald

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Form PCT/ISA/210 (second sheet) (January 2004)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE2005/000159

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.b of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application and necessary to the claimed invention, the international search was carried out on the basis of:

   a. type of material

   [X] a sequence listing

   [X] table(s) related to the sequence listing

   b. format of material

   [X] in written format

   [X] in computer readable form

   c. time of filing/furnishing

   [ ] contained in the international application as filed

   [ ] filed together with the international application in computer readable form

   [X] furnished subsequently to this Authority for the purpose of search

2. [X] In addition, in the case that more than one version or copy of a sequence listing and/or table relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

Fujirebio Diagnostics, Inc. v. Quanterix Corp.
EX-1045
7696

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box II.1

Although claims 67-71,77,80 and 81 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
Although claims 72-76 are directed to a diagnostic method practised on the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE2005/000159

Box II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

see FURTHER INFORMATION sheet PCT/ISA/210

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

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

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

Fujirebio Diagnostics, Inc. v. Quanterix Corp.
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(54) Title: ANTIBODIES SPECIFIC FOR SOLUBLE AMYLOID BETA PEPTIDE PROTOFIBRILS AND USES THEREOF

(57) Abstract: The invention pertains to the development of antibodies that specifically bind amyloid beta protein (Abeta) in its protofibril conformation. The invention also comprises methods of using anti-Abeta protofibril antibodies to diagnose or treat Alzheimer's disease, Down's syndrome Lewybody dementia, vascular dementia, and other neurodegenerative disorders. Furthermore, the invention pertains to the use of anti-Abeta protofibril antibodies to screen and identify substances that will modulate protofibril activity or formation in vitro or in vivo. The invention also pertains to methods for synthesising pure Abeta protofibril antigens as well as to a method for stabilising Abeta protofibrils antigens as well as to a method for stabilising Abeta protofibrils.

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Antibodies specific for soluble amyloid beta peptide protofibrils and uses thereof

1. FIELD OF INVENTION

This invention pertains to the diagnosis, prevention and treatment of neurodegenerative diseases, in particular Alzheimer's disease, and other similar disease. More precisely, to antibodies that specifically bind amyloid beta protein (Aβ) in its protofibril conformation.

2. BACKGROUND OF THE INVENTION

Alzheimer's disease (AD) is a progressive and irreversible neurodegenerative disorder causing cognitive, memory and behavioural impairments. It is the most common cause of dementia in the elderly population affecting roughly 5% of the population above 65 years and 20% above 80 years of age. AD is characterized by an insidious onset and progressive deterioration in multiple cognitive functions. The neuropathology involves both extracellular and intracellular argyrophillic proteineous deposits. The extracellular deposits, referred to as neuritic plaques, mainly consist in amyloid beta protein (Aβ) surrounded by dystrophic neurites (swollen, distorted neuronal processes). Aβ within these extracellular deposits are fibrillar in their character with a β-pleated sheet structure. Aβ in these deposits can be stained with certain dyes e.g. Congo Red and display a fibrillar ultrastructure. These characteristics, adopted by Aβ in its fibrillar structure of neuritic plaques, are the definition of the generic term amyloid. The classic intracellular AD pathologic lesion is the neurofibrillary tangle (NFT) which consists of filamentous structures called paired helical filaments (PHFs) composed of twisted strands of hyperphosphorylated microtubule-associated protein tau. Frequent neuritic plaques and neurofibrillary tangle deposits in the brain are diagnostic criteria for AD, as carried out post mortem. AD brains also display macroscopic brain atrophy, nerve cell loss, local inflammation (microgliosis and astrocytosis) and often congophilic amyloid angiopathy (CAA) in cerebral vessel walls.

Two forms of Aβ peptides, Aβ40 and Aβ42, are the dominant species in AD neuritic plaques (*Masters 1985*), while Aβ40 is the prominent species in cerebrovascular amyloid associated with AD (*Glenner 1984*). Enzymatic activities allow Aβ to be

continuously formed from a larger protein called the amyloid precursor protein (APP) in both healthy and AD afflicted subjects in all cells of the body. Two major APP processing events through β- and γ-secretase activities enables Aβ production, while a third enzyme called α-secretase activities prevents Aβ generation by cleavage inside the Aβ sequence (*Selkoe, 1994*; US5604102). The Aβ42 is a forty two amino acid long peptide i.e. two amino acids longer at the C-terminus, as compared to Aβ40. Aβ42 is more hydrophobic, and does more easily aggregate into larger structures of Aβ peptides such as Aβ dimers, Aβ tetramers, Aβ oligomers, Aβ protofibrils or Aβ fibrils. Aβ fibrils are hydrophobic and insoluble, while the other structures are all less hydrophobic and soluble. All these higher molecular structures of Aβ peptides are individually defined based on their biophysical and structural appearance e.g. in electron microscopy, and their biochemical characteristics e.g. by analysis with size-exclusion chromatography/western blot. These Aβ peptides, particularly Aβ42, will gradually assemble into a various higher molecular structures of Aβ during the life span. AD, which is a strongly age-dependent disorder, will occur earlier in life if this assembly process occurs more rapidly. This is the core of the "amyloid cascade hypothesis" of AD which claims that APP processing, the Aβ42 levels and their assembly into higher molecular structures is a central cause of AD. All other neuropathology of AD brain and the symptoms of AD such as dementia are somehow caused by Aβ or assembled forms thereof.

Aβ can exist in different lengths i.e. 1-39,1-40,1-42 and 1-43 and fragments sizes i.e. 1-28, 3-40/42, 11-40/42, 17-40/42 and 25-35. All these peptides can aggregate and form soluble intermediates and insoluble fibrils, each molecular form having a unique structural conformation and biophysical property. Monomeric Aβ1-42 for example, is a 42 amino acid long soluble and non-toxic peptide, that is suggested to be involved in normal synapse functions. Under certain conditions, the Aβ1-42 can aggregate into dimers, trimers, tetramers, pentamers up to 12-mer and higher oligomeric forms, all with its distinct physicochemical property such as molecular size, EM structure and AFM (atomic force microscopy) molecular shape. An example of a higher molecular weight soluble oligomeric Aβ form is the protofibril *(Hartley 1999, Walsh 1999)*, which has an apparent molecular weight >100 kDa and a curvelinear structure of 4-11 nm in diameter and < 200 nm in length. It has

recently been demonstrated that soluble oligomeric Aβ peptides such as Aβ protofibrils impair long-term potentiation (LTP) (*Hartley, 1999),* a measure of synaptic plasticity that is thought to reflect memory formation in the hippocampus (*Walsh 2001*). Furthermore, oligomeric Arctic Aβ peptides display much more profound inhibitory effect than wtAβ on LTP in the brain, likely due to their strong propensity to form Aβ protofibrils (*Klyubin 2003).*

There are also other soluble oligomeric forms described in the literature that are distinctly different from protofibrils. One such oligomeric form is ADDL (Amyloid Derived Diffusible Ligand) *(Lambert 1998).* AFM analysis of ADDL revealed predominantly small globular species of 4.7-6.2 nm along the z-axis with molecular weights of 17-42 kDa. *(Stine 1996).* Another form is called ASPD (Amyloidspheroids). ASPD are spherical oligomers of Aβ1-40. Toxicity studies showed that spherical ASPD >10 nm were more toxic than lower molecular forms (*Hoshi* 2003). The Aβ fibril as the main neurotoxic species is inconsistent with the poor correlation between neuritic plaque density and AD dementia score and also with the modest signs of neurodegeneration in current APP transgenic mice. Soluble neurotoxic Aβ-intermediate species and their appropriate subcellular site of formation and distribution could be the missing link that will better explain the amyloid hypothesis. This idea has gained support from recent discovery of the Arctic (E693) APP mutation, which causes early-onset AD (*US 2002/0162129 A1; Nilsberth et al., 2001*). The mutation is located inside the Aβ peptide sequence. Mutation carriers will thereby generate variants of Aβ peptides e.g. Arctic Aβ40 and Arctic Aβ42. Both Arctic Aβ40 and Arctic Aβ42 will much more easily assemble into higher molecular structures (protofibrils) that are soluble and non-fibrillar. Thus the pathogenic mechanism of the Arctic mutation suggests that the soluble higher molecular protofibrils are causing AD.

2.1 Diagnosis of Alzheimer's disease

2.1.1 Clinical diagnosis

The clinical diagnosis of Alzheimer's disease (AD) is difficult to make, especially in early stages of the disease. Today, the diagnosis is based on a typical medical history combined with the exclusion of other causes of dementia. Clinical centres

with high specialization can have a diagnostic accuracy of 85-90% compared with the neuropathological diagnosis. In the early stages of the disease the clinical picture is vague and definite diagnostic markers have not yet been identified (*McKhann 1984).* The development of biochemical diagnostic markers is important for a number of reasons: to support the clinical diagnosis, to allow clinicians to give adequate information to patients and their relatives, to initiate pharmacological treatment and care-giving, and in various aspects of clinical research.

2.1.2 Amyloid β-peptide

Pathogenic mutations in the APP and presenilin (PS) genes have been discovered in families with early-onset AD inherited as a dominant trait (*Hardy 1992*). The effects of some of these mutations are now fairly well understood. The Swedish AD mutation *(Mullan 1992; Axelman 1994; Lannfelt 1994)* has revealed one pathogenic mechanism for the development of AD. When a cDNA construct with this mutation was transfected into human cell-lines it gave rise to approximately six times higher release of soluble Aβ *(Citron 1992, Cai 1993).* Furthermore, fibroblasts from individuals with the Swedish mutation secreted three times more Aβ into the media compared to fibroblasts from non-carriers *(Johnston 1994).* Overproduction of Aβ therefore seemed to be an important factor in the disease pathogenesis in this Swedish family. Thus, it was expected that Aβ levels measured in cerebrospinal fluid (CSF) from family members would differentiate carriers from non-carriers of the mutation. However, no difference was found in levels of total Aβ between the groups (14.5±3.3 ng/ml versus 14.9±2.3 ng/ml) (*Lannfelt 1995).* One explanation for this result may be that Aβ is cleared from CSF by aggregating to amyloid in the brain. However, there was a strong correlation between duration of dementia and decreasing Aβ levels. These measurements were done with antibodies recognizing soluble monomeric Aβ. With protofibril specific monoclonal antibodies more accurate measurements of the toxic species will be possible.

2.1.3 Aß in plasma

Aß is found in a soluble form in plasma and other tissues *(Seubert 1992),* and not as previously presumed, only in the brains of AD cases. Aβ plasma levels in members of the Swedish mutation family revealed that both Aβ40 and Aβ42 were 2-3 times increased in mutation-carriers *(Scheuner 1996).* The proportion of Aβ42 of total Aβ was approximately 10% in both groups, which is in agreement with

experiments performed in cell cultures with the Swedish mutation. Mutation-carriers below the age of expected onset of the disease had the same levels of Aß as already affected cases. This indicates that APP mismetabolism may play an important role early in the pathogenesis of the disease.

2.1.4 Aß42 in CSF in Alzheimer's disease

ELISAs specifically measuring Aß40 and Aß42 in CSF in AD cases have given different results. Some researchers *(Pirttilä 1994; Motter 1995)* have found decreased Aß42 in AD, while one group have found elevated levels in cases early in the disease progression. The most demented cases in one study had all very low levels of Aß42. In conclusion, Aß42 is most likely increased early in the disease process and levels of Aß42 and Aß40 decreased during progression of the disease. The development of accurate biochemical markers of early AD is important especially when efficient pharmacological treatments will be available in the future. Pharmacological therapy should most likely be initiated at an early stage of disease, before severe brain damage has occurred. A therapy making it possible to prevent the progression of the disease to its later stages would therefore be much desired.

2.2 Prevention and treatment of Alzheimer's disease

Antibodies that are specific towards different conformations of Aβ, such as Aβ fibrils (O´*Nuallain 2002),* micellar Aβ (*Kayed 2003),* ADDL (M93, M94) (*Lambert 2001)* have also been described.

Several pre-clinical studies in transgenic animal models have shown decreased plaque burden and improvements in memory function after active or passive immunization with antibodies raised against fibrils (*Shenk 1999, Janus 2000, Morgan 2000, Weiner 2000, Sigurdsen 2001).* Since fibrils are present in pathological deposits occurring late in the Alzheimer disease process, the Shenk antibodies may only be used to slow the progression of Alzheimer's disease when it has already reached its later stages.

Recently, a phase II clinical trial in Alzheimer patients with mild to moderate dementia was performed by ELAN Pharmaceuticals with their vaccine AN 1792, which is an aggregated preparation of human wtAβ42. The study had to be stopped

due to side effects in 5% of the patients. The side effect was considered to be due to T-lymphocyte-induced meningioencephalitis (*Nicholl 2003).* The drug targets of the ELAN vaccine were insoluble fibrils found in plaques inside the brain and deposits on the brain blood vessel walls (Congophilic Amyloid Angiopathy, CAA), which are common features of Alzheimer's disease. Thus, an immune response towards insoluble fibrils could be responsible for the invasive inflammation in the brain blood vessel walls leading to meningioencephalitis.

WO02/203911 disclose the discovery of the Arctic mutation in a Swedish family leading to early onset of Alzheimer's disease (55.6 years). The Arctic mutation (Glu>Gly), which is located at position 22 in the beta amyloid peptide (Aß), in combination with various experiments led to the insight that the Aß peptide was much more prone to oligomerize and form protofibrils compared to wild type Aβ40. The discovery indicated for the first time that the protofibril is a central component in the disease process, and that AD could be treated by reducing the amount of protofibrils in the brain. This unique property of the Arctic mutation could then be used to generate protofibrils. WO02/203911 then suggested that said protofibrils could be used to immunize a mouse or other animal, in order to generate antibodies, after which any protofibril-specific monoclonal antibody could identified be screening. Said antibodies should then be specific towards an Aβ peptide of SEQ ID No 1 (page 7, third paragraph), i.e. a peptide carrying the Arctic mutation and having a protofibril conformation.

Thus, in view of the prior art techniques for preventing and treating Alzheimer's disease, there is a need for a technique that enables earlier detection of markers of Alzheimer's disease. If said markers could be prevented without causing negative side-effects, this would be a means to prevent and treat Alzheimer's disease at an early stage. Any treatment of Alzheimer's disease that would reduce the amount of protofibrils in the brain of AD patients, would be of significant therapeutic value.

3. DESCRIPTION OF THE INVENTION

Protofibrils occur early in Alzheimer's disease, when little brain damage has occurred. An antibody treatment that reduce protofibril levels in the brain will save the brain from neuronal destruction and hence be more advantageous for Alzheimer

patients. The present invention describes the development of such therapeutic protofibril specific antibodies.

According to one aspect, the present invention relates to antibodies that have the property to bind both wild type Aβ42/40 and Aβ42/40arc protofibrils (conformation-specific antibodies) and which are suitable for development into pharmaceuticals for Alzheimer's disease, targeting wild type Aβ42/40 protofibrils. Current ideas in the field of Alzheimer's disease research is that Aβ fibrils are the main cause of the disease. Hence, the approach of the present invention contradicts the general opinion within the field.

According to another aspect, the present invention also relates to a composition comprising said antibody and optionally a carrier or excipient.

To immunise and screen for conformation-specific anti-protofibril antibodies, it is necessary to produce pure Aβ42arc and Aβ42 protofibrils (>95% degree of purity). In order to obtain said pure protofibril preparation, it is also necessary to be able to stabilise the protofibrils, such that they stay protofibrils and do not separate into monomers or aggregate into fibrils. It is also necessary to find a solvent that made it possible to separate the protofibrils by column chromatography. Another necessity is the ability to test said purity before immunisation. The above-mentioned abilities are essential, since the degree of purity of the antigen (protofibrils) decide the possibility to produce conformation-specific anti-protofibril antibodies, which in turn decide the possibility to screen for antibodies with useful specificity. Should the preparation for example contain 50% Aβ monomer and 50% Aβ protofibril, it is only possible to claim that the antibody binding said preparation may bind both forms or only one. All of said necessary features are provided by the present invention.

The above-mentioned features were not possible when WO02/203911 was filed. The HPLC analysis method disclosed it the present application or any other analysis method had not been developed, wherefore it was not possible to determine whether the Aβ42arc peptide actually formed protofibrils, probably due to the lack of binding to protofibrils. It was only a hypothesis at the time. The major reasons why WO02/203911 could not analyse and purify protofibril preparation to a degree of

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purity of >95%. Also, to the fact that they did not know how to stabilise protofibrils. In that connection, it should also be mentioned that protofibrils are very difficult to handle in comparison with for example Aβ40 peptides. Aß42, but in particular the Aß42Arc peptide, is extremely difficult to handle, since they stick to test tubes and columns and aggregate into fibrils. The latter problems has been solved by the present invention by the provision of methods to stabilise the protofibrils involving low temperatures and selected solvents. WO02/203911 also had no means for testing the purity of the protofibrils before immunisation. This is possible with the present invention by the provision of a new HPLC (size exclusion) method. In addition to this, WO02/203911 did not provide any means to check the protofibril specificity of the screened antibodies. The present invention makes this possible by the provision of a new Elisa method. WO02/203911 could only produce impure and transient Aβ40arc protofibrils, not useful for monoclonal development of protofibril specific antibodies (WO02/203911 only describes analysis of Aß40 and Aß40Arc peptides, see Example 3).

The antibodies according to the present invention are particularly suitable for treating Alzheimer's disease since they will: i) target and eliminate the most toxic Aß form (protofibril), ii) provide an opportunity to treat the disease early since protofibrils develops early in the disease process, iii) avoid side-effects since the will not significantly cross-react with Aß fibrils present in the blood vessel wall of the brain (CAA= Congophilic Amyloidogenic Angiopathy).

The antibodies according to the present invention may be human or humanised, monoclonal or polyclonal. The present invention also relates to biologically active fragments of said antibodies, with the proviso that they still have the claimed properties.

The Aβ protofibril specific antibodies according to the present invention can also be used to quantitate wild type Aβ42/40 protofibrils in biological fluids, thus the antibodies will be suitable for clinical diagnosis at an early stage of the disease and suitable as a biomarker to monitor efficacy in clinical studies.

Furthermore, the invention describes procedures to generate wild type Aβ42 and Aβ42arc protofibrils as antigens for immunization and for reagents to screen for antibodies that binds Aβ42arc and wild type Aβ42 protofibrils.
Antigen (protofibril) purity is very important to determine in antibody screening experiments, since it affects antibody specificity determinations by ELISA.

To asses the purity of antigen (Aβ42arc and wild type Aβ42 protofibrils) preparation, a size exclusion HPLC method has been developed. The HPLC method invented uses a detergent, with low optical interference in chromatography. The detergent has the advantage that it eliminates interactions of Aβ42arc and wild type Aβ42 protofibrils with the column matrix, preventing loss of material and material from adhering to the column, and erroneous estimations of protofibril purity. Furthermore, the detergent is compatible with Aβ protofibrils and does not solubilize them into Aβ monomers or alter their biological profile. Polysorbate (Tween) has been found to be a suitable detergent, in particular Polysorbate-80 (Tween-80). Other detergents with similar properties are also useful.

The antibody according to the present cross-reacts less than 50% with Aß fibrils. Said antibody can detect both wild type Aß protofibrils and Arctic Aß protofibrils in an ELISA in a concentration range of 1000-10 ng/ml (see Example 5 and Figures 4A-C). In an optimised ELISA or by other detection systems with higher sensitivity, for example proximity ligation, said detection level could probably be brought down to a detection level of 1-0.1 ng/ml.

The present invention provides a method to raise antibodies that are specific to amyloid β (Aβ) protofibrils (high molecular weight soluble non-fibrillar Aβ oligomers). The antibodies are raised against Aβ in its protofibril conformation. These antibodies will be administered to AD patients to reduce protofibril levels in the brain, which will be of significant therapeutic value.
The lowering of protofibril levels might occur through the elimination of the antigen when bound to an antibody, through microglia-mediated phagocytosis. This is a well documented biological process, which occurs through the binding of the antibody's constant region (Fc) to an Fc receptor on microglial cells. Binding induces phagocytosis (internalisation and destruction) of the antibody and its

bound antigen (protofibril) leading to reduced levels of protofibrils in the brain. Other non-Fc receptor mediated processes might also occur to eliminate the antigen (protofibril).

Another aspect of the invention pertains to a method to synthesize Aβ protofibrils which are to be used as antigen for immunization. The synthesis can be made from wild type Aβ (wtAβ) or alternatively, from non-wild type, mutated or modified forms of Aβ. In a preferred embodiment of the invention, but not limited to said embodiment, the synthesis is initiated by dissolving wtAβ1-42 in a dissociating agent, for example NaOH, to achieve a homogenous Aβ solution, of about 50-500 uM in peptide concentration, but not limited to this concentration. Alternative agents with dissociating capacity are for example dimethylsulfoxide, DMSO; hexafluoroisopropanol, HFIP; trifluoroacetic acid, TFA. Wild type protofibrils can also be made from wtAβ peptides of different lengths i.e. wtAβ1-39, wtAβ1-40, wtAβ1-43 or N-terminal truncated forms of these peptides. The truncation can be 1-10 amino acids, giving wtAβ forms such as: wtAβ2-42, wtAβ3-42, wtAβ4-42, wtAβ5-42 and so on. The dissolved wtAβ1-42 peptide is subsequently neutralized by PBS or similar physiological buffer and incubated at higher temperature, preferably 37° C, for a period of time sufficient for protofibril-formation to occur, for example over night. This will yield wild type Aβ protofibrils. The invention also provide a molecular size (molecular weight) determination method to asses protofibril formation and purity, preferably, but not limited to, a size-exclusion chromatographic method (SEC).

The method of synthesizing pure wild type (wtAβ42) protofibril antigen, comprises the steps of dissolving an wtAβ42 by using a dissociating agent, such as NaOH (pH>10), dimethylsulfoxide, DMSO; hexafluoroisopropanol, HFIP; trifluoroacetic acid and TFA, to achieve a monodisperse solution, neutralizing the solution by PBS (pH 7-8) or similar biocompatible buffers, to achieve a physiological solution, incubating the neutralized wtAβ42 peptide solution at an elevated protein concentration between 1-1000 uM, preferably 440 uM, for 6 hours or longer at 20-40 °C, preferably 37 °C, to form Aβ42wt protofibrils, diluting the protofibrils to approximately 1-500uM, preferably 50uM centrifuging at sufficient speed and time to sediment wtAβ42 fibrils, which normally takes 5 minutes at 17.000 x g at +4°C,

assessing the purity of the protofibril preparation by HPLC to control that the purity is >95%, using for example size exclusion HPLC, using a physiological buffer such as PBS at neutral pH as running buffer, including a detergent, such as Polysorbate (Tween), or similar detergent, to avoid sticking of the protofibrils to the column matrix and dissociation.

Another aspect of the invention pertains to the synthesis of non-wild type Aβ protofibrils using Aβ1-42, Aβ1-41, Aβ1-40 or Aβ1-39 or N-terminal truncated forms (1-10 truncations) with either the Arctic (G22E) mutation (US 09/899,815), the Dutch mutation (E22Q), the Flemish (A21G)mutation, the Italian (E22K) mutation, the Iowa (D23N) mutation, and combinations thereof. In a preferred embodiment of the invention Aβ42arc peptide (i.e., comprising the arctic mutation) is used. The method to make Aβ42arc protofibrils is similar to that for wtAβ42 protofibrils except that Aβ42arc protofibrils are not incubated at 37° C over night since they are spontaneously formed after the neutralization step.

The method of synthesizing pure Aβ42arc protofibril antigen comprises the steps of dissolving an Aβ42arc peptide by using a dissociating agent, such as NaOH (pH>10), dimethylsulfoxide, DMSO; hexafluoroisopropanol, HFIP; trifluoroacetic acid and TFA to achieve a monodisperse solution, neutralizing the solution by PBS (pH 7-8) or similar biocompatible buffers, to achieve a physiological solution, stabilizing the spontaneously formed protofibrils by keeping them at below 20° C, preferably at 0-5 ° C, centrifuging the protofibrils, at the same temperature as in the stabilising step, at a sufficient speed and time to sediment Aβ42arc fibrils, which normally takes 5 minutes at 17.000 x g at +4°C, assessing the purity of the protofibril preparation by HPLC to control that the purity is >95%, using for example size exclusion HPLC, using a physiological buffer such as PBS at neutral pH as running buffer, including a detergent, such as Polysorbate (Tween), or similar detergent, to avoid sticking of the protofibrils to the column matrix and dissociation.

Another aspect of the invention pertains to the development of antibodies that cross-react with wild type Aß42 protofibrils after immunization with Aß42arc protofibrils.

Another aspect of the invention pertains to a method to stabilize Aβ protofibril and where stabilization can be assessed by size-exclusion chromatography (SEC). Aβ protofibrils elutes after 12-13 minutes in a uniform peak on a Superdex 75 or similar size-exclusion column. Conformation stability can also be assessed by staining with Congo Red and (electron microscopy), where protofibrils attain a curve-linear structure of 6-10 nm in diameter and <200 nm in length. Lowered temperature has a significant effect on Aβ protofibril conformational stability. Samples should preferably be kept below 20° C, preferably below 5° C, and most between 0° C and 5° C. Furthermore, agents that decrease the polarity or surface tension or increase viscosity have stabilizing effects on Aβ protofibril conformation. For example, 10-50% glycerol or 0.6–5% Polysorbate (Tween), have stabilizing effects on Aβ protofibrils. These agents and treatments can be added to the Aβ protofibril preparation preferably after the neutralization step in the method to synthesize Aβ protofibrils described above. Addition of these agents before this step is also possible. Increased stability of protofibrils is advantageous when developing monoclonal antibodies and when Aβ protofibrils are used as reagents in immunoassays, such as ELISA, radio-immunoassay (RIA), Western blotting or dot blotting.

The method of stabilizing the Aβ protofibrils includes mixing them with an agent that decreases the solvent polarity or one that lowers the surface tension, such as glycerol or Polysorbate (Tween), or a combination of said agents. The stabilisation can also be achieved by keeping the protofibrils at a temperature below 20° C, preferably 0-5 °C. Said methods can also be combined.

The invention further pertains to the use of anti-Aβ protofibril specific antibodies for determinations of Aβ protofibrils in human and animal tissues, for example, cerebrospinal fluid, blood, serum, urine and brain tissue, but is not limited to these tissues, providing for a possible diagnostic method for Alzheimer's disease. Suitable methods for assaying Aβ protofibrils in these tissues as well as in cell cultures using an anti-Aβ protofibril antibody are immunoassays such as ELISA, RIA, Western blotting dot blotting or proximity ligation. The method would be suitable to

follow treatment efficacy (protofibril reduction) in clinical trials and suitable as a diagnostic test for Alzheimer's disease or Down's syndrome.

The method of diagnosing or monitoring Alzheimer's disease (AD) or Down's syndrome comprises the steps of labelling the antibody according to the present invention with an agent that can generate a measurable signal, administering said antibody according to a subject having or suspected of having AD or Down's syndrome, measuring the amount of protofibrils bound to the antibody by measuring the signal generated by the agent.

The invention further pertains to the use of an anti-Aβ protofibril antibody in imaging for detection, localization and quantitation of Aβ protofibrils in human and animal tissues. The anti-Aβ protofibril antibody could be label with a radioactive ligand such as I^{131}, C^{14}, H^{3} or $Gallium^{68}$, but it is not limited to these radioisotopes, for detection purposes. In addition to labelling with radioactive markers, DNA, fluorescent molecules, enzymes which converts a substrate such that its absorbance can be measured, could also be used. The method will be suitable as a diagnostic tool for Alzheimer's disease or Down's syndrome, Lewybody dementia, vascular dementia, and other neurodegenerative disorders.

A further aspect of the invention pertains to the use of an anti-Aβ protofibril antibody for the prevention or treatment of Alzheimer's disease. Pre-clinical studies in transgenic animal models have demonstrated effects on plaque burden (*Bard* 2000), reversal of memory deficits (*Dodard 2002)* and drainage of Aβ from CNS after anti-Aβ antibody treatment. However, the antibodies used in these studies have not been specific to Aβ protofibrils. Administration of an anti-Aβ protofibril antibody, with no or little cross-reactivity towards Aβ monomers and fibrils would be particular suitable for treatment and prevention of Alzheimer's disease. Firstly, it would not bind significantly to fibrillar forms of Aβ and avoid interaction with fibrillar deposits which are prevalent in blood vessel walls, avoiding sever immunoreactions with concomitant serious brain inflammation and encephalitis, which was encountered in the ELAN vaccination study (see at the end of the Background) with their vaccine AN-1792 (*Nicoll 2004).* Secondly, an anti-Aβ protofibril antibody with low Aβ1-40 and Aβ1-42 monomer cross-reactivity would

not bind and interfere with their normal biological functions, thus avoiding side effects.

In said method of prevention or treatment of Alzheimer's disease or Down's a subject having or suspected of having Alzheimer's disease, Down's syndrome, Lewybody dementia, vascular dementia, and other neurodegenerative disorders, is administered the antibody or composition according to the present invention.

The invention also provides a technique whereby anti-Aβ protofibril antibodies can be used to identify and select for epitopes present on Aβ protofibrils but less on Aβ monomers and Aβ fibrils or other Aβ conformational forms. The method is general and is applicable to other amyloids forming protofibrils such as, but not limited to, islet amyloid protein peptide (IAPP, amylin) associated with Type–2 diabetes ,prion protein (PrP), alpha-synuclein (Parkinson). Considering the central role of amyloid beta (Aβ) protofibrils in Alzheimer's disease, these antibodies can be used to diagnose or treat Alzheimer's disease. There is a need for a method that specifically can determine protofibrils in different human and animal tissues such as cerebral spinal fluid (CSF), plasma, blood, urine and brain tissue, but is not limited to these tissues. Such a method could be used as a diagnostic method for Alzheimer's disease but also for similar diseases that forms amyloid protofibrils including Parkinson (alpha-synuclein) (*Sthilerman 2002)*, Type-2 diabetes (Islet amyloid polypeptide, IAPP) (*WO03/092619* A2), and Creutzfeldt-Jacob Disease and the corresponding animal disease called mad cow disease (Prion protein) (*DeMarco 2004)*, but is not limited to these diseases.

According to another aspect, the present invention relates to the use of anti-Aβ protofibril antibodies for *in vitro* or *in vivo* screening of substances that inhibit or modulate Aβ protofibril levels and/or activity in cell cultures or animal models, being potential Alzheimer drugs. Suitable screening systems would be, but are not limited to, cell cultures (HEC or neuroblastoma cells) or for example the Thy-1 APPSweArc transgenic mouse model, (Nilsson L. et al. Swedish patent application 0400707-6, 2004) that expresses human Amyloid Precursor Protein (APP) with the Arctic mutation (E693G) providing increased production of Aβ protofibrils. Alzheimer drug candidates can be administered to these cell cultures or transgenic

animal models and their effects on Aβ protofibril levels measured by an immunoassay, using an anti-Aβ protofibril antibody as reagent. Such method would be ideally suitable for identifying potent Alzheimer drug candidates.

Said method for *in vitro* or *in vivo* screening of substances that inhibit or modulate Aβ protofibril levels and/or activity in cell cultures or animal models, comprises the steps of administering potential drug candidates to a cell culture or an animal model, administering the antibody according to any the present invention, labelled with an agent that can generate a measurable signal, to said cell culture or animal model, evaluating the effect of said drug candidates by measuring the amount of protofibrils bound to the antibody by measuring the signal generated by the agent.

According to another aspect, the present invention relates to a method of detecting Aβ protofibrils in vitro, comprising labelling the according to the present invention with an agent that can generate a measurable signal, contacting said antibody or the composition comprising the antibody with a biological sample suspected of containing soluble protofibrils, measuring the amount of protofibrils bound to the antibody by measuring the signal generated by the agent. Said method may be an immunoassay. The biological samples tested may be selected from plasma, CSF, brain and other tissues of animal or human origin. The labelling includes labelling with radioactive markers, DNA, fluorescent molecules, enzymes which converts a substrate such that its absorbance can be measured.

All percentages in the description relates to percent volume, unless stated otherwise.

4. DESCRIPTION OF THE DRAWINGS

Figure 1. Size exclusion chromatography (SEC) of Human Monomer Aβ40, Protofibril Aβ42Arc, Protofibril wtAβ42 and Fibril wtAβ42 preparations

Human Aβ monomers and Aβ protofibrils are eluting in 19-20 minutes and at 12-13 minutes, respectively. The protofibril preparations (B and C) are essentially free (<3%) from contamination of other conformational Aβ forms. The analysis of the fibril preparation (D) was done after centrifugation at 17.900 x g for 5 minutes at

room temp. The supernatant was analysed demonstrating the almost complete absence of any soluble Aβ forms in the fibril preparation.

Figure 2. Stability study of protofibrils
The stability of Protofibril Aβ42Arc and Protofibril wt42 preparations was measured by SEC. A conversion of protofibrils to fibrils was assessed by a decrease in the protofibril peak area (decreased absorbance (AU) at 214 nm elution time 12-13 minutes). The addition of 10-50% glycerol, 0.6% Tween-20 or storage at 0-5° C, all increased the protofibril stability significantly.

Figure 3. Titration of mouse serum after immunization with protofibril wtAβ42
Mice were immunized with a protofibril wtAβ42 preparation in Freund complete adjuvant (first injection) and incomplete Freund adjuvant (5-6 boosters). Blood was collected and the antibody titers against Aβ protofibrils were determined. Mouse #2 and #4 showed the highest titers.

Figure 4. Sandwich-ELISA method using anti-mAb 258 for Aβ protofibril determination
mAb 258 was coated over night in a microtiterplate. Preparations of wtAβ42 protofibrils, Aβ42Arc protofibrils, wtAβ40 monomers and wtAβ42 fibrils were added in decreasing concentrations and incubated for 1 hour at +4° C. Secondary anti-Aβ antibody (6E10) was added. Finally, a detection ALP (alkaline phosphatase) conjugated antibody (anti-IgG) was added to each well. Detection was achieved by adding ALP substrate. The colour formation was measured in at 405 nm and 492 nm.

mAb 258 bound wtAβ42 protofibrils and slightly less Aβ42Arc protofibrils.
No binding was observed to wtAβ40 monomers. The slight binding to wtAβ42 fibrils is probably due to a slight contamination with wtAβ42 protofibrils (see Figure 1 D).

5. EXAMPLES
The following examples are provided for illustration and are not intended to limit the invention to the specific examples provided.

Example: 1 *Synthesis of human amyloid beta peptide (Aβ) of different conformations*

Synthetic human wild type or mutant forms of Aβ1-42 or Aβ1-40 peptides were purchased from Polypeptide Laboratories GmbH. The peptides had been synthesized by a standard solid phase peptide synthesis procedure. The peptides were subsequently purified with RP-HPLC to a purity in the range of 90-95%. Alternative vendors producing Aβ peptides with similar purity are possible to use as well.

Synthesis of human wild type Aβ1-40 monomers

A synthetic wtAβ1-40 peptide was dissolved in 1 volumes of 10 mM NaOH pH>10, and 1 volume of cold 2 x PBS (pH 7-8) to a concentration of 50 uM. The wtAβ1-40 monomer preparation was centrifuged at 17.900 x g at +4° C for 5 minutes prior to analysis (Figure 1).

Synthesis of human wild type Aβ1-42 protofibrils

A synthetic wtAβ1-42 peptide was dissolved in 9 volumes of 10 mM NaOH pH>10, vortexed for 2 minutes and diluted with 1 volume of 10 x PBS (pH 7-8). The final peptide concentration was at this point 443 uM. The peptide was further incubated over night at 37° C. After the overnight incubation, the peptide was further diluted with PBS to 50 uM. The sample was centrifuged for 17.900 x g for 5 minutes prior to analysis and immunisation.

Synthesis of human Aβ1-42Arc protofibrils

A synthetic Aβ1-42Arc (E22G) peptide was dissolved in 1 volumes of 10 mM NaOH pH>10 and 1 volume cold 2 x PBS (pH 7-8) to a final peptide concentration of 50 uM. The Aβ1-42Arc protofibrils were formed immediately and the Aβ1-42Arc protofibrils were stabilized by keeping the solution at 0-4° C before analysis or immunization. The sample was centrifuged at 17.000 x g for 5 minutes at +4°C prior to analysis. Alternative ways to stabilize the Aβ1-42Arc protofibrils were to add glycerol to a final concentration of 5-50 % or to add Tween-20 to a final concentration of 0.6%.

Synthesis of human wild type Aβ1-42 fibrils

A synthetic wtAβ1-42 peptide was dissolved in 1 volumes of double distilled water, vortexed for 2 minutes and diluted with 1 volume of 2 x PBS (pH 7-8) and vortexed

again for 2 minutes. The final peptide concentration at this point was 50 uM. The wtAβ1-42 protofibril preparation was incubated at 37° C, for 48 hours before analysis. The sample was centrifuged at 17.900 x g for 5 minutes prior to SEC analysis. The supernatant was analysed after centrifugation. No centrifugation was performed when Aβ1-42 fibril preparations were analysed by ELISA or dot blotting.

Analysis of Aβ preparation by Size Exclusion Chromatography (SEC)
A Merck Hitachi D-7000 HPLC LaChrom system, having a diod array detector (DAD) model L-7455 and a model L-7100 pump, was used for the chromatographic analysis of protofibril preparations in combination with a Superdex 75 PC3.2/30 column (Amersham Pharmacia Biotech, Uppsala, Sweden). The chromatographic system separates Aβ monomers from protofibrils, which are eluting at the void volume of the column. The column was equilibrated with 50 mM Na_2HPO_4 · NaH_2PO_4 (pH 7.4) with 0.15 M NaCl (PBS) and 0.6% Tween 20 and eluted with the same buffer at a flow rate of 0.08 ml/min (pressure was 5-6 bars) at ambient temperature (22°C). Ten (10 ul) of a 50 uM-100 uM Aβ sample was subjected analysis using a wavelength scan between 200-400 nm. Tween-20 was added to the sample to give a final concentration of 0.6% prior to chromatography. Data was extracted from measurements at 214 and 280 nm. Peak areas were integrated using Merck Hitachi model D-7000 Chromatography Data Station Software. Figure 1 shows chromatograms of wtAβ1-40 monomer, wtAβ1-42 protofibril, Aβ1-42Arc protofibril and wtAβ1-42 fibril preparations.

Each preparation was essentially free (< 3%) of other contaminating conformational Aβ forms except the wtAβ1-40 monomer preparation which contained 6.4 % protofibrils (elution time 12-13 minutes).

Example 2: *Protofibrils specific monoclonal antibody development.*
A standard procedure was used for monoclonal development. Mice were injected with wtAβ1-42 protofibril preparation. Alternatively, Aβ1-42Arc protofibrils could be used. Every wtAβ1-42 protofibril batch was assayed by SEC and Congo Red (binds β-sheet structures in proteins) before immunisation to ascertain protofibril purity and that the preparation contained β-sheet structure. The procedure used to immunise mice was a standard protocol involving s.c injections in the presence of

Freund adjuvant. Each of six mice were immunized with 10 ug wtAβ1-42 protofibrils and subsequently boostered with 30 ug. The sample was mixed 1:1 with Freund complete adjuvant prior to the first injection. For subsequent 5 booster injections incomplete Freund adjuvant was used. Mouse n was not immunized (control). Mice were bleed and blood collected for antibody titer determinations (Figure 3). Mice were given one more booster (30ug) and then sacrificed and the spleen collected for hybridom development. The hybridom preparation method used was according to standard procedure (*Harlow 1988*).

Example 3: *Screening for wtAβ1-42 protofibril specific antibodies*
An ELISA method was developed where by hybridom supernatants were screened for antibodies that bind Aβ1-42 protofibrils. Hybridom
supernatant #258 showed high protofibril specificity (Figure 3). The hybridom #258 was reseeded and screened again to ascertain a homogenous cell line. The monoclonal antibody that was produced from the #258 hybridom was defined as monoclonal antibody 258 (mAb258). Alternatively, screening (binding to Aß protofibrils but not to Aß monomers or fibrils) can be performed against an antibody phage display library, where the library has been made from RNA isolated from animals immunized with protofibril.

Example 4. *Characterization of mAb 258 by Western blot analysis.*
The aim of the experiment was to determine if mAb 258 cross-reacts with wild type human amyloid precursor protein (wtAPP) or mutated human APP,
APPswe, APP swe-arc (*Nilsberth 2001, Mullan 1992*) all of which contains uncleaved Aβ1-42. Cells were transfected with plasmids expressing human wtAPP, APPswe and APPswe-arc. Cells were subsequently harvested and solubilized and cellular proteins separated by SDS-PAGE and transferred to nitrocellulose filter papers and subsequently incubated with either mAb 258 or mAb 6E10. Specific binding of these antibodies to separated cellular proteins was detected by incubating the filter papers in a secondary anti-mouse IgG/IgM antibody solution and subsequent developed by incubation with Pierce super signal (art nr 34080-P) and a light sensitive film.
mAb 258 showed no binding to wtAPP, APPswe or APPswe-arc nor to wtAβ40 monomer. The commercial mAb 6E10 bound all these forms.

Example 5: *Sandwich ELISA for protofibril characterization and determinations*
The specificity of mAb 258 was determined by a sandwich-ELISA.
An 96-well ELISA plate was coated with mAb 258 over night at +4°C. After coating, wells were blocked with BSA for 1 hour at room temp. Aβ samples i.e wtAβ40 monomers, wtAβ42 protofibrils, wtAβ42-Arc protofibrils wtAβ42 fibrils, were added to the microtiterplate in 5 x dilutions starting with 10 ug/ml. Samples were incubated for 1 hour at +4°C, after which 10 ng/well of a commercial secondary antibody, 6E10 (Signet) was added and incubated for 1 hour at room temp. Detection was achieved by incubation with a commercial ALP-conjugated anti-IgG antibody for 1 hour at room temp. and subsequent incubation with the substrate at room temp. for one hour according to the manufacturer's procedure. Samples were read in a microtiterplate reader (Spectra max 190 Molecular Devices, Sunyvale, USA) at 405 nm and 492 nm. Figure 4A.
mAb 258 showed little or no cross-reactivity towards wtAβ40 monomers or wtAβ42 fibrils. Concentrations of wtAβ42 protofibrils down to 10 ng/ml were measurable.

Binding of an anti-Aβ42arc protofibril specific antibody (7E4)(Aβ42arc in protofibril conformation was used as a antigen during immunization) was measured in the sandwich-ELISA coated with increasing concentrations of wtAβ42 protofibrils. Detection was achieved by either an Aβ -specific mAb (1C3) as a secondary detection antibody (Figure 4B) or by the commercial mAb 6E10, as secondary antibody (Figure 4C). Strong binding of mAb 7E4 was achieved to wtAβ42 protofibrils. Concentration levels as low as 2-5 ng/ml of wtAβ42 protofibrils were measurable. The monoclonal antibodies 4E11 and 10F7show less strong binding to wtAβ42 protofibrils (Figure 4B and 4C).

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CLAIMS

1. An antibody specific for soluble amyloid beta peptide (Aβ) protofibrils and/or biologically active fragments thereof.

2. The antibody according to claim 1, wherein said antibody is specific for wild type Aß protofibrils.

3. The antibody according to claim 2, wherein said antibody is specific for human wild type Aß protofibrils.

4. The antibody according to claim 3, wherein said antibody is specific for human wild type Aß42 protofibrils.

5. The antibody according to any of claims 1-4, wherein said antibody cross-reacts less than 50% with Aß fibrils.

6. The antibody according to claims any of claims 1-5, wherein said antibody can detect both wild type Aß protofibrils and Arctic Aß protofibrils in an ELISA in the concentration range of 1000-10 ng/ml.

7. The antibody according to claims any of claims 1-5, wherein said antibody can detect both wild type Aß protofibrils and Arctic Aß protofibrils in an ELISA in the concentration range of 1000-0.1 ng/ml.

8. The antibody according to any of claims 1-7, wherein said antibody is polyclonal.

9. The antibody according to any of claims 1-7, wherein said antibody is monoclonal (mAb).

10. The antibody according to any of claims 1-7, wherein said antibody is mAb 7E4, mAb 4E11 or mAb 10F7.

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11. The antibody according to any of claims 1-10, wherein said antibody has been raised against protofibrils comprising Aβ selected from the group consisting of Aβ 1-39, 1-40, 1-42, 1-43, and combinations thereof.

12. The antibody according to any of claims 1-10, wherein said antibody has been raised against protofibrils comprising Aβ selected from the group consisting of Aβ 1-39, 1-40, 1-42, 1-43 truncated at the N-terminal end with up to ten (10) amino acids, and combinations thereof.

13. The antibody according to claims 1-12, wherein said antibody has been raised against protofibrils comprising said Aβ peptides containing one or several mutations selected from the group consisting of the Arctic (G22E mutation, the Dutch mutation (E22Q), the Flemish (A21G) mutation, the Italian (E22K) mutation, the Iowa (D23N) mutation, and combinations thereof.

14. The antibody according to claims 1-13, wherein said antibody is human or has been humanized.

15. A composition comprising one or more antibodies according to any of claims 1-14 and optionally a carrier or excipient.

16. A method of detecting Aβ protofibrils *in vitro*, comprising:
-labelling the antibody according to any of claims 1-14 or in the composition according to claim 15 with an agent that can generate a measurable signal,
-contacting said antibody or the composition comprising the antibody with a biological sample suspected of containing soluble protofibrils,
-measuring the amount of protofibrils bound to the antibody by measuring the signal generated by the agent.

17. The method according to claim 16, wherein said detection method is an immunoassay.

18. The method according to any of claims 16-17, wherein said biological sample is selected from plasma, CSF, brain and other tissues of animal or human origin.

19. The method according to any of claims 16-17, wherein the labelling includes labelling with radioactive markers, DNA, fluorescent molecules, enzymes which converts a substrate such that its absorbance can be measured.

20. Use of the antibody according to any of claims 1-14 or the composition according to claim 15 for prevention or treatment of Alzheimer's disease or Down's syndrome in a subject of having or suspected of having Alzheimer's disease, Down's syndrome, Lewybody dementia, vascular dementia, and other neurodegenerative disorders.

21. A method for *in vitro* or *in vivo* screening of substances that inhibit or modulate Aβ protofibril levels and/or activity in cell cultures or animal models, comprising the steps of:

-administering potential drug candidates to a cell culture or an animal model,

-administering an antibody according to any of claims 1-8 or in the composition according to claim 9, labelled with an agent that can generate a measurable signal, to said cell culture or animal model

-evaluating the effect of said drug candidates by measuring the amount of protofibrils bound to the antibody by measuring the signal generated by the agent.

22. A method of synthesizing pure Aβ42arc protofibril antigen, comprising the steps of:

-dissolving an Aβ42arc peptide by using a dissociating agent, to achieve a monodisperse solution,

-neutralizing the solution by PBS (pH 7-8) or similar biocompatible buffers, to achieve a physiological solution,

-stabilizing the spontaneously formed protofibrils by keeping them at below 20° C,
-centrifuging the protofibrils, at the same temperature as in the stabilising step, at a sufficient speed and time to sediment Aβ42 arc fibrils,
-assessing the purity of the protofibril preparation by HPLC to control that the purity is >95%.

23. The method according to claim 22, wherein the dissociating agent is selected from NaOH (pH>10), dimethylsulfoxide, DMSO; hexafluoroisopropanol, HFIP; trifluoroacetic acid and TFA.

24. The method according to claim 22 or 23, wherein the temperature is the stabilising step is 0-5 ° C.

25. The method according to any of claims 22-24, wherein the purity assessment is achieved by using size exclusion HPLC, using a physiological buffer at neutral pH as running buffer, including a detergent, to avoid sticking of the protofibrils to the column matrix and dissociation.

26. The method according to claim 25, wherein the detergent in the purifying step is Polysorbate (Tween) or similar detergent.

27. A method to synthesize pure wild type Aβ42 protofibril antigen, comprising the steps of:
-dissolving an Aβ42arc peptide by using a dissociating agent, to achieve a monodisperse solution,
-neutralizing the solution by PBS (pH 7-8) or similar biocompatible buffers, to achieve a physiological solution,
-incubating the neutralized Aβ42wt peptide solution at an elevated protein concentration between 1-1000 uM, for 6 hours or longer at 20-40 °C, to form Aβ42wt protofibrils,
–diluting the protofibrils to 1-500uM,
-centrifuging at sufficient speed and time to sediment Aβ42 wt fibrils,

-assessing the purity of the protofibril preparation by HPLC to control that the purity is >95%.

28. The method according to claim 27, wherein the dissociating agent is selected from NaOH (pH>10), dimethylsulfoxide, DMSO; hexafluoroisopropanol, HFIP; trifluoroacetic acid and TFA.

29. The method according to any of claims 27-28, wherein the protofibrils are diluted to approximately 50uM in the dilution step.

30. The method according to any of claims 27-29, wherein the purity assessment is achieved by using size exclusion HPLC, using a physiological buffer at neutral pH as running buffer, including a detergent, to avoid sticking of the protofibrils to the column matrix and dissociation.

31. The method according to claim 30, wherein the detergent in the purifying step is Polysorbate (Tween) or similar detergent.

32. A method of diagnosing or monitoring Alzheimer's disease (AD) or Down's syndrome, comprising the steps of:

-labelling the antibody according to any of claims 1-14 or in the composition according to claim 15 with an agent that can generate a measurable signal,

-administering said antibody to a subject having or suspected of having AD or Down's syndrome,

-measuring the amount of protofibrils bound to the antibody by measuring the signal generated by the agent.

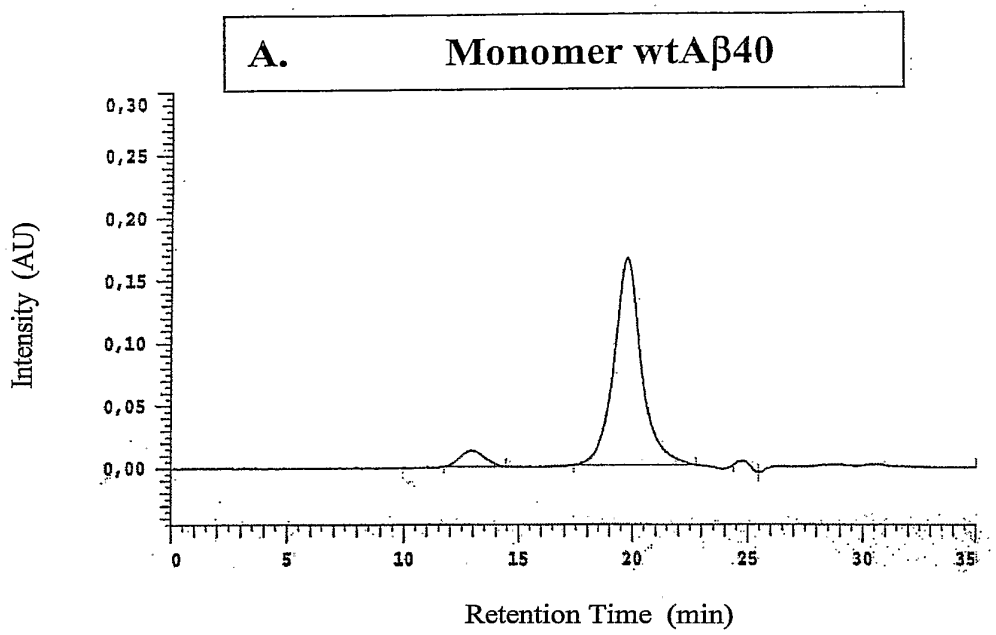
33. A method of prevention or treatment of Alzheimer's disease (AD) or Down's syndrome comprising the step of administering the antibody thereof according to any of claims 1-14 or the composition according to claim 15 to a subject having or suspected of having AD or Down's syndrome.

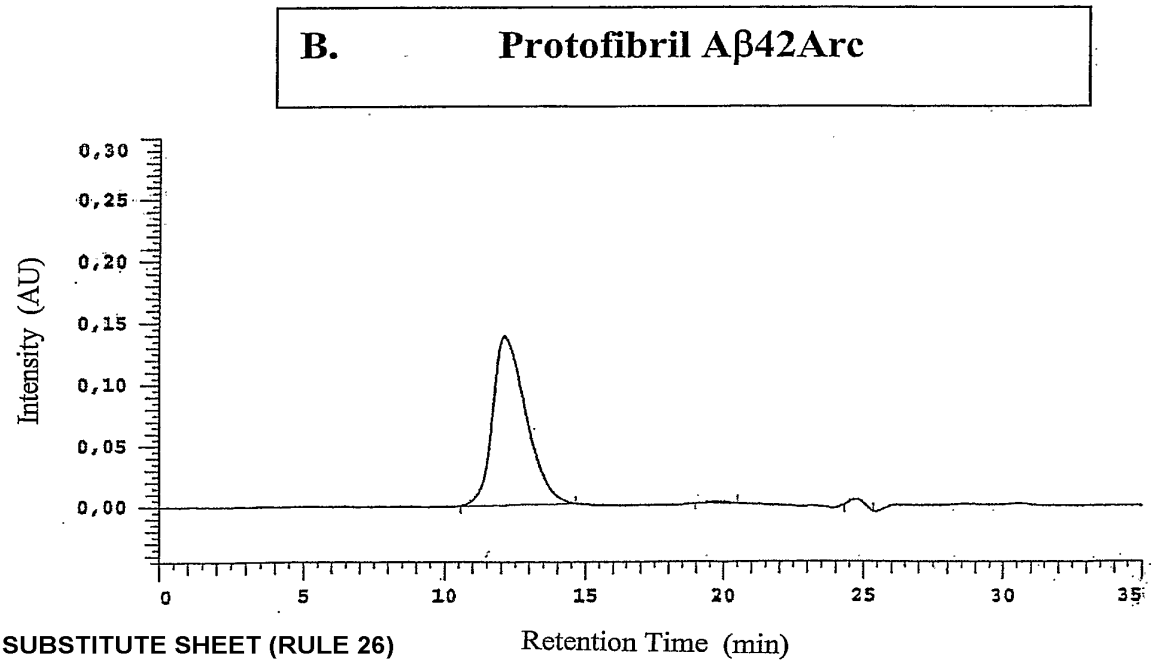
34. A method of stabilizing Aβ protofibrils by mixing them with an agent that decreases the solvent polarity or one that lowers the surface tension, or a combination of said agents, and/or keeping the protofibrils at a temperature below 20° C.

35. The method according to claim 34, wherein said agent is glycerol or Polysorbate (Tween), or combinations thereof.

36. The method of stabilizing Aβ protofibrils according to claim 34 or 37, wherein the Aβ protofibrils are kept at a temperature between 0-5 °C.

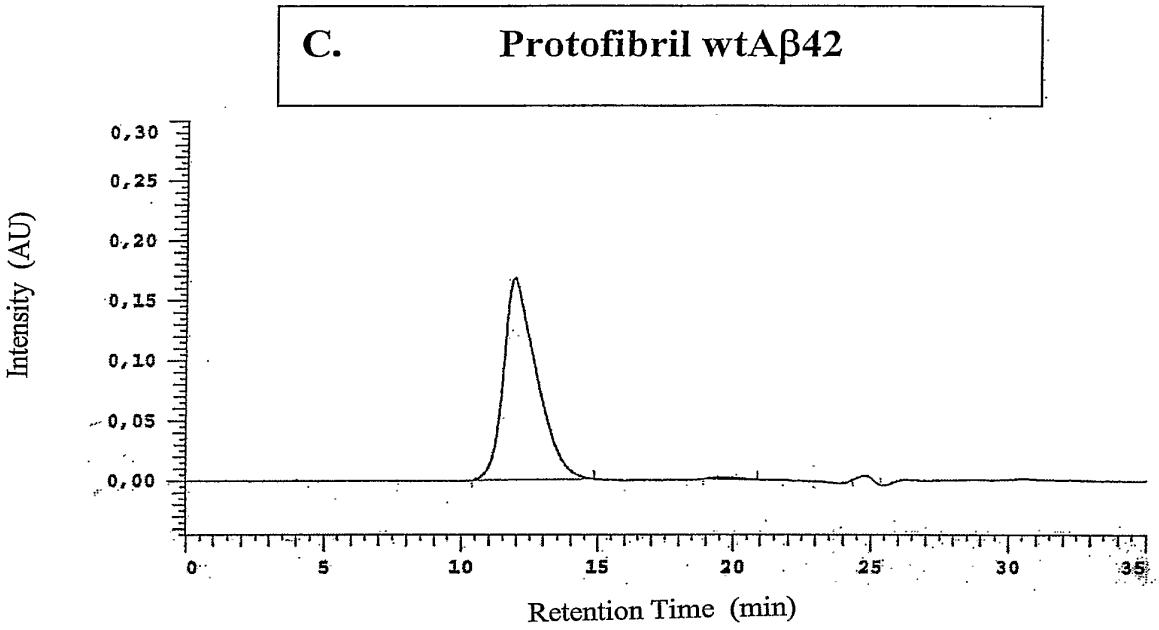
FIG. 1

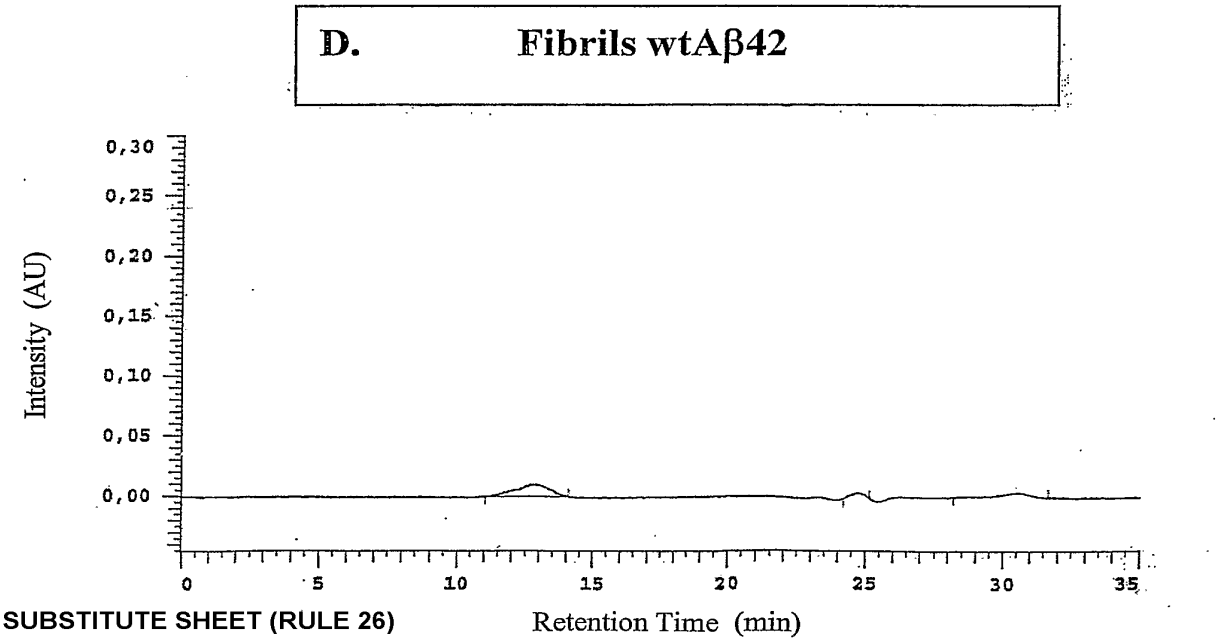




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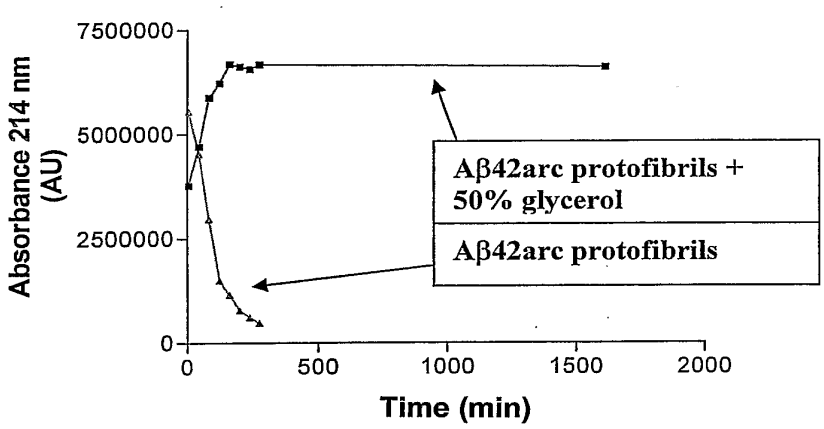
FIG. 1 (cont.)

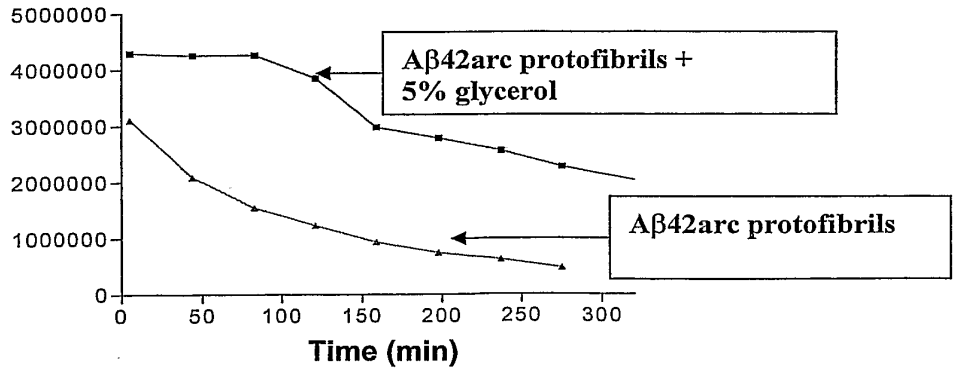




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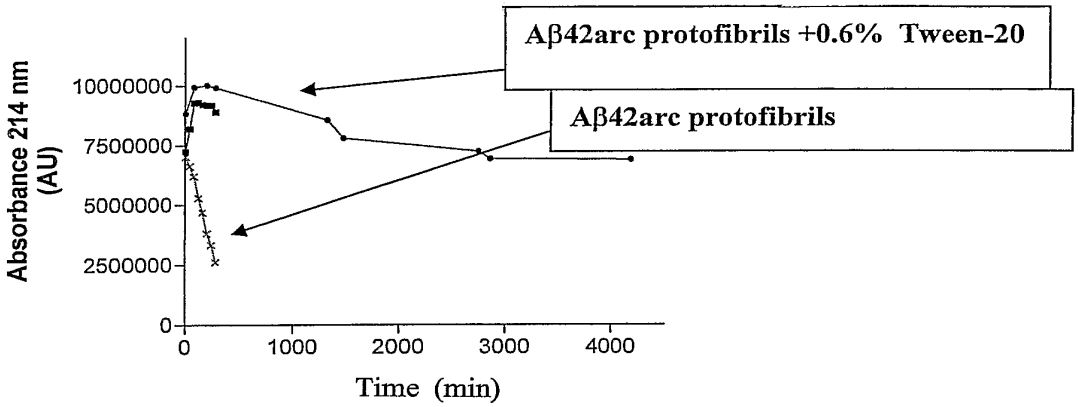
FIG. 2

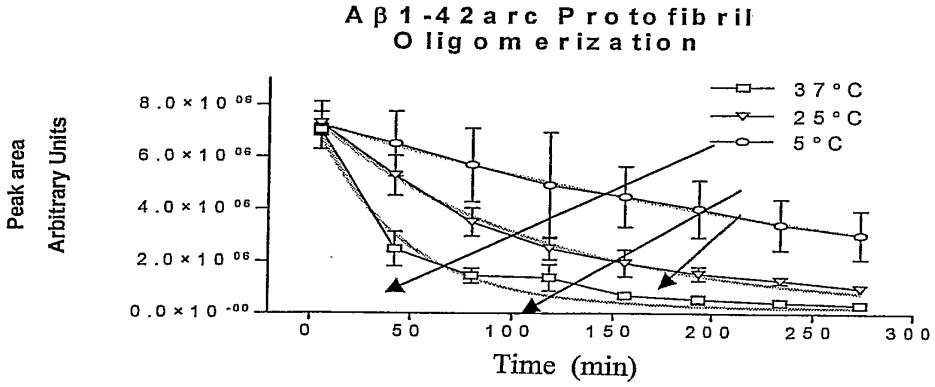




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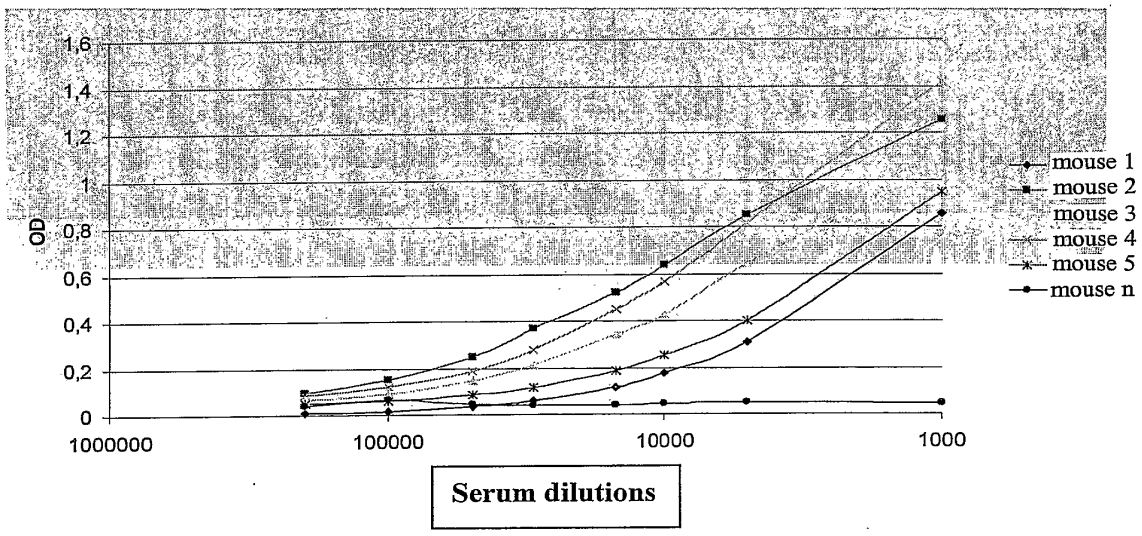
FIG. 2 (cont.)





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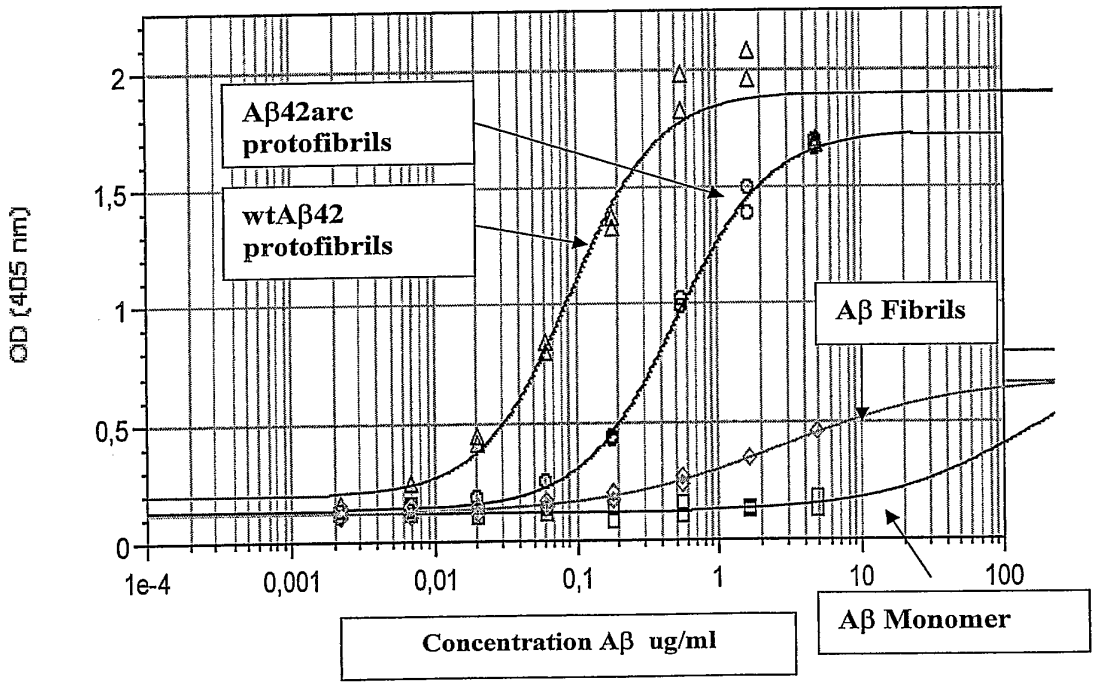
FIG. 3



Mouse *n*, non-immunized mouse (control)

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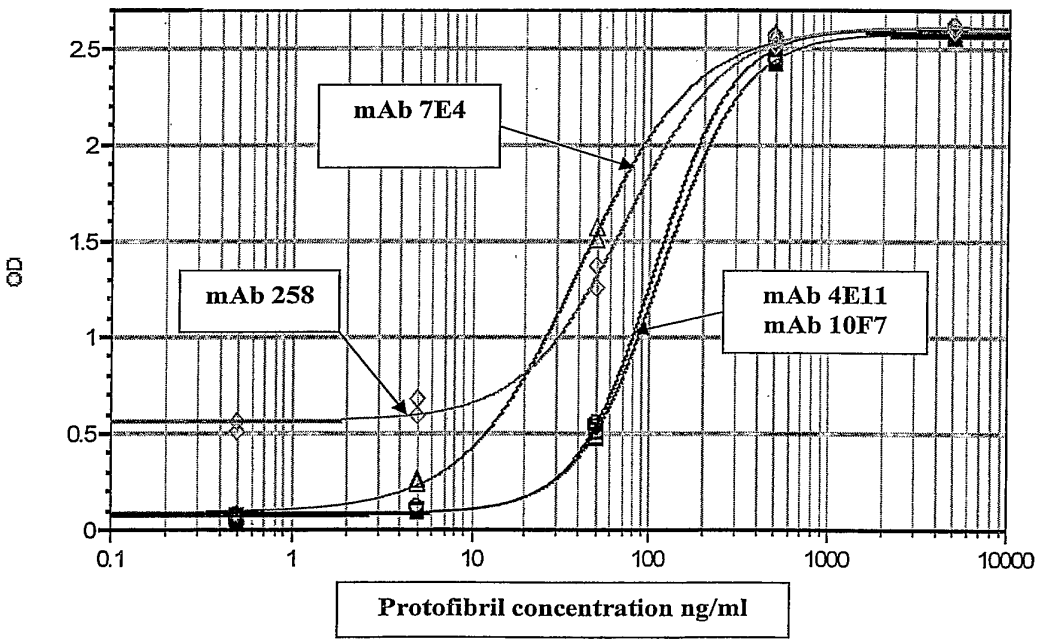
FIG. 4, A



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FIG. 4 B

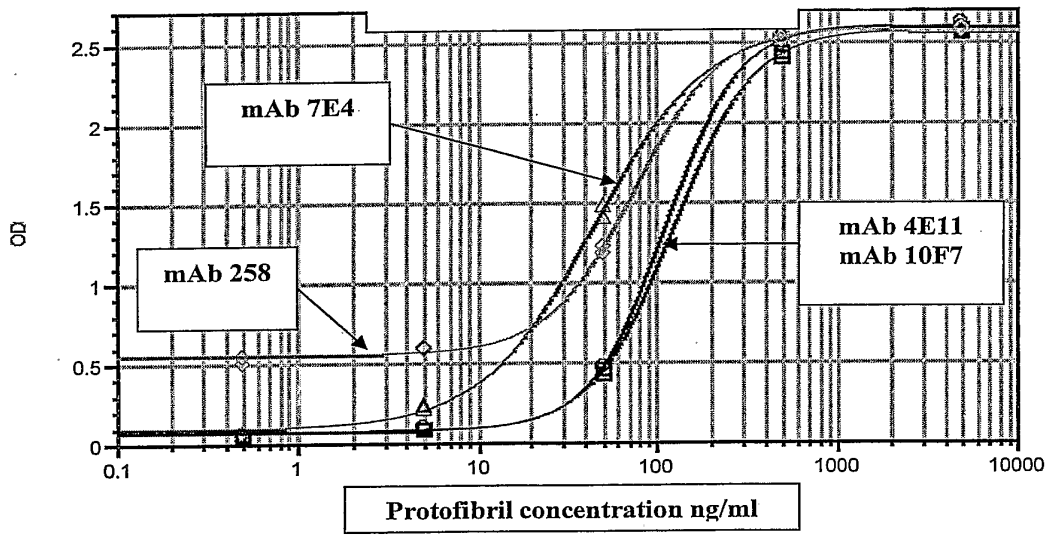
Titration by a sandwich-ELISA of 4 monoclonal antibodies (258, 7E4, 4E11 and 10F7) against wtAβ42 protofibril concentration.



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FIG. 4 C

Titration by a sandwich-ELISA of 4 monoclonal antibodies (258, 7E4, 4E11 and 10F7) against wtAβ42 protofibril concentration.



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INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE 2005/000993

A. CLASSIFICATION OF SUBJECT MATTER

IPC7: C07K 16/18, A61K 39/395, A61P 25/28, C07K 14/47
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)

IPC7: C07K, A61K, A61P, C07K

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

SE,DK,FI,NO classes as above

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

EPO-INTERNAL, WPI DATA, PAJ, BIOSIS, MEDLINE, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	KAYED, RAKEZ ET AL, "Common Structure of Soluble Amyloid Oligomers Implies Common Mechanism of Pathogenesis", Science, 18 April 2003, volume 300, page 486 - page 489, page 486, column 2, paragraphs 1-2; page 487, column 1; figures 1-2	1-10,14-15
	--	
Y	WO 0203911 A2 (LANNFELT, LARS), 17 January 2002 (17.01.2002), page 8, line 6 - line 16	1-21,32-33
	--	

[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
"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
28 Sept 2005	04-10-2005
Name and mailing address of the ISA/ Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86	Authorized officer Terese Sandström/EÖ Telephone No. +46 8 782 25 00

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

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE 2005/000993

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	LASHUEL, HILAL A. ET AL, "Mixtures of Wild-type and a Pathogenic (E22G) Form of A Beta 40 in Vitro Accumulate Protofibrils, Including Amyloid Pores", J. Mol. Biol., 2003, volume 332, page 795 - page 808, page 805, column 1, last 2, paragraph 5, column 2, paragraph 2	22-24,27-29, 34,36
Y		1-21,32-33
	--	
X	YE, CHIAN P. ET AL, "Protofibrils of amyloid Beta-protein inhibit specific K+ currents in neocortical cultures", Neurobiology of Disease, 2003, volume 13, page 177 - page 190, page 178, column 1, last paragraph	27-29
Y		1-21,32-33
	--	
X	WARD, ROBIN V. ET AL, "Fractionation and characterization of oligomeric, protofibrillar and fibrillar forms of Beta-amyloid peptide", 2000, vol. 348, page 137 - page 144, page 138, column 1, paragraphs 2-3	27-29
Y		1-21,32-33
	--	
X	US 6054114 A (PETER T. LANSBURY, JR. ET AL), 25 April 2000 (25.04.2000), column 103, last paragraph - column 104, paragraph 2	27-29
Y		1-21,32-33
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A	CHROMY, BRETT A. ET AL, "Self-Assembly of A Beta 1-42 into Globular Neurotoxins", Biochemistry, 2003, vol. 42, page 12749 - page 12760	1-21,32-33
	--	

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE 2005/000993

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 03089460 A1 (THE GOVERING COUNCIL OF THE UNIVERSITY OF TORONTO), 30 October 2003 (30.10.2003), page 17, line 1 - line 4; page 69, line 10 - line 15	1-21,32-33
	--	
A	US 20030187011 A1 (HILAL A. LASHUEL ET AL), 2 October 2003 (02.10.2003), paragraphs (0046) - (0051)	34-36
	--	
P,X	WO 2005025516 A2 (THE REGENTS OF THE UNIVERSITY OF CALIFORNIA), 24 March 2005 (24.03.2005), page 4, line 8 - page 5, line 11; page 10, line 12 - page 23	1-10,14-15
	--	

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

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE2005/000993

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

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

1. ☒ Claims Nos.: 20, 21 (partly), 32-33
because they relate to subject matter not required to be searched by this Authority, namely:

Claims 20, 32-33 and the part of claim 21 that refers to in vivo screening relate to a method of treatment of the human or animal body by surgery or by therapy, as well as diagnostic methods /Rule 39.1(iv).

.../...

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

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

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

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

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Box II

1) Nevertheless, a search has been executed for these claims. For claims 20 and 32-33, the search has been based on the alleged effects of the antibodies. For claim 21, only the part relating to the screening being performed in vitro has been searched.

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INTERNATIONAL SEARCH REPORT
Information on patent family members

31/08/2005

International application No.
PCT/SE 2005/000993

WO	0203911	A2	17/01/2002	AU	6800501 A	21/01/2002
				CA	2414772 A	17/01/2002
				EP	1309341 A	14/05/2003
				JP	2004502442 T	29/01/2004
				US	20020162129 A	31/10/2002
US	6054114	A	25/04/2000	WO	9741856 A	13/11/1997
WO	03089460	A1	30/10/2003	AU	2003218560 A	00/00/0000
				CA	2481952 A	30/10/2003
				EP	1497321 A	19/01/2005
				US	20030232758 A	18/12/2003
US	20030187011	A1	02/10/2003	NONE		
WO	2005025516	A2	24/03/2005	NONE		

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(19) World Intellectual Property Organization
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5 October 2006 (05.10.2006)

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G01N 33/53 (2006.01)

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PCT/EP2006/003363

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(74) Agent: **ARIAS SANZ, Juan**; ABG PATENTES, S.L., Orense, 68, E-28020 Madrid (ES).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, LY, MA, MD, MG, MK, MN, MW, MX, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SM, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT, LU, LV, MC, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 2006/103116 A1

(54) Title: HUMAN ANTIBODIES WITH BETA-AMYLOID PEPTIDE-BINDING CAPACITY AND THEIR APPLICATIONS

(57) Abstract: The present invention relates to isolated specific binding members, particularly antibodies or fragments, derivatives or variants therof that recognize and bind amyloid beta 1-42 protein. Methods of diagnosing, prognosing or treating diseases or disorders in which elevated levels of amyloid beta protein, including amyloid beta 1-42 are prevalent, in particular Alzheimer's disease, are disclosed. The present invention also relates to pharmaceutical compositions comprising such binding members, antibodies and mimics therof and methods of screening for novel binding members, which may or may not be antibodies.

HUMAN ANTIBODIES WITH BETA-AMYLOID PEPTIDE-BINDING CAPACITY AND THEIR APPLICATIONS

FIELD OF THE INVENTION

[0001] The present invention relates to human antibodies, fragments thereof or derivatives, with beta-amyloid 1-42 peptide-binding capacity, obtaining said antibodies and their use as therapeutic and/or diagnostic agents for Alzheimer's disease.

BACKGROUND OF THE INVENTION

[0002] Alzheimer's disease is the most frequent cause of dementia among the elderly. It is a neurodegenerative disease essentially characterized by the progressive accumulation of beta-amyloid peptide in the areas of the brain involved in cognitive functions, such as memory and language. Alzheimer's disease generally affects older people over the age of 65, although it may also occur in younger people. According to data published by the World Health Organization (WHO), Alzheimer's disease currently affects 5% of men and 6% of women over the age of 60 worldwide. Close to 400,000 people are currently affected in Spain, over 8 million people in Europe, and due to the accelerated aging of the population, the number of Alzheimer's patients is expected to double in the year 2020 and triple in the year 2050 (Selmes van den Bril, 2003).

[0003] A number of studies indicate that the APP (Amyloid Precursor Protein), the normal function of which is unknown, plays an essential role in the onset of the disease. The presence of amyloid plaques caused by the anomalous processing of the APP protein constitutes a central event in the pathogenesis of Alzheimer's disease. This protein is sequentially cleaved by means of the action of alpha, beta and gamma-secretases. In a pathological process, APP is anomalously processed, resulting in insoluble peptides, with 40-42 amino acids (beta-amyloid peptide or beta-peptides), which tend to be aggregated as fibrils and are deposited as plaques in neuronal cells. The accumulation of said plaques results in cell death of several types of neuronal cells with the subsequent cerebral deterioration. Nevertheless, beta-amyloid peptide production from APP protein processing is not the only factor influencing the existence of a beta-peptide deposit. There is evidence that factors involved in catabolism and elimination, as well as in the aggregation of the beta-amyloid peptide, are also critical (Dodel et al., 2002).

[0004] Despite having made great progress in recent years in terms of knowledge of the etiology, genetics and physiopathology of Alzheimer's disease, the therapies existing today are palliative therapies. The absence of drugs which act by preventing or significantly delaying the development of Alzheimer's disease means that new alternative therapies and new neuroprotective active ingredients that allow reducing the deposit of beta-peptides and the inflammatory response, contributing to reducing progression of the disease, are required.

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[0005] The immune system may play an important role in the development of Alzheimer's disease. Active immunotherapy studies conducted in transgenic mice capable of reproducing human Alzheimer's disease (Janus et al., 2000; Morgan et al., 2000; Schenk et al., 1999) indicate that after immunization with beta-amyloid peptide, said mice produce specific antibodies capable of disintegrating and reducing the amyloid plaques caused by the beta-peptide accumulation, as well as preventing the development of new plaques and the onset of associated symptoms. The reduction of the beta-amyloid peptide deposit observed in said *in vivo* assays was also accompanied by cognitive changes in the animal.

[0006] The results obtained in the mouse model prompted in 2001 the start of a clinical trial on active immunotherapy by vaccination in humans with beta-amyloid 1-42 peptide. After concluding phase I of the clinical trial without having observed adverse reactions, clinical phase II had to be suspended at the end of 2002 because 6% of the individuals vaccinated with beta-amyloid peptide developed reactive encephalitis (Orgogozo et al., 2003; Munch and Robinson, 2002; Schenk, 2002). Post-mortem studies performed on those individuals who exhibited an inflammatory adverse response showed, in addition to said encephalitic changes, a reduction in the number of amyloid plaques as well as signs of T cell infiltration in the central nervous system. The cellular immune response induced by the administration of beta-amyloid peptide is considered to be one of the factors involved in the occurrence of the observed adverse effects (Morgan and Gitter, 2004).

[0007] Despite having to suspend the clinical trial, the subjects participating in phase II of the clinical trial continued to be monitored (Hock et al., 2002). Most of the vaccinated individuals had developed anti beta-amyloid peptide antibodies that recognized amyloid plaques. It was observed that there was an inversely proportional relationship between the serum anti beta-amyloid antibodies in vaccinated subjects and the loss of intellectual capacity in said individuals (Hock et al., 2003), suggesting the protective role of anti beta-amyloid antibodies compared to the progression of Alzheimer's disease. An absence of correlation between serum anti beta-amyloid antibody levels present and the onset of meningoencephalitis was also detected (Orgogozo et al., 2003).

[0008] An alternative therapy to active immunization with beta-amyloid peptide for the treatment and/or prevention of Alzheimer's disease is the passive administration of specific anti beta-amyloid antibodies. Passive immunotherapy allows customizing the antibody dose and replacing one antibody with another one aimed at a different epitope, as well as to select the isotype of the antibody to be administered. Another advantage of the administration of specific antibodies compared to active immunization is that its effectiveness does not depend on there being an immune response in the recipient, which is particularly interesting in the case of elderly individuals since patients of this age group usually have a variable immune response. On the other hand, passive immunotherapy allows suspending or reducing the antibody dose administered in the event of the occurrence of adverse reactions (Morgan and Gitter, 2004).

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[0009] Several passive immunotherapy studies have been conducted in transgenic mice which reproduce human Alzheimer's disease (DeMattos et al., 2001, Bard et al., 2000, Frenkel et al. 2000). Said studies show how there is a reduction of the number of amyloid plaques after the passive administration of antibodies with beta amyloid peptide-binding capacity, as well as a reduction in progression of the disease. Said results suggest that the T cell mediated immune response is not required in eliminating beta-amyloid deposits, which generates an expectation on the therapeutic potential offered by developing specific anti beta-amyloid peptide antibodies. Therefore, the administration of monoclonal antibodies against the beta-amyloid peptide would produce positive effects similar to those obtained by means of active immunization. It would in turn prevent the occurrence of undesirable adverse effects, such as reactive encephalitis, in the absence of a T cell mediated immune response.

[0010] The positive effects exerted by anti beta-amyloid antibodies observed in the *in vivo* passive immunotherapy assays conducted in transgenic mice prompted different studies for the purpose of investigating the presence of auto-antibodies against the beta-amyloid peptide in non-immunized healthy humans and their possible protective role compared to Alzheimer's disease or other diseases in which there is a beta-amyloid peptide deposit. However said studies generated contradictory results.

[0011] On one hand, there are articles providing data which support a possible protective role of anti beta-amyloid antibodies. In one of them (Du et al., 2001), the presence of antibodies (IgG) specifically recognizing beta-amyloid peptide in non-immunized humans, both in serum and in cerebral spinal fluid was identified. The antibody titer in cerebral spinal fluid of patients with Alzheimer's was significantly lower than that detected in control individuals. In a later study (Weksler et al., 2002) it was also observed that the serum anti beta-amyloid IgG titer was reduced in elderly Alzheimer patients with respect to healthy elderly individuals (> 65 years of age) who did not exhibit a loss of intellectual capacity. Both studies provide evidence in favor of the potential protective role of the specific humoral immune response compared to the beta-amyloid peptide in Alzheimer's disease. Nevertheless, the reduced anti beta antibody levels observed in said studies could be the consequence of an increase in the beta-peptide concentration in the central nervous system. Therefore, binding of the beta-peptides to the specific antibodies would result in an apparent reduction of the anti beta-amyloid peptide antibody concentration (Weksler and Goodhardt, 2002).

[0012] On the other hand, studies have been published which contradict the hypothesis of the possible protective role of serum auto-anti beta-amyloid antibodies present in non-immunized elderly individuals. Hyman et al. (Hyman et al, 2001) found no significant differences upon comparing the total serum anti beta-amyloid immunoglobulin levels in Alzheimer's patients with the levels detected in control individuals. In another recent study (Baril et al., 2004) the levels of antibodies specific against serum beta-amyloid peptide in elderly Alzheimer's patients were compared with the control individuals of the same age range (cases: 66-86 years of age, control:

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63-82 years of age). The individuals of both groups showed low anti beta-amyloid antibody titers with variable levels. In said study, an analysis of the serum concentration of anti beta-amyloid 1-42 peptide IgG in different age groups (children: 1-10 years of age, young subjects: 20-48 years of age and elderly subjects: case and control subjects of the study) was performed. The results of said analysis show that there are no statistically significant differences in the levels of immunoglobulin aimed at the beta-amyloid 1-42 peptide between the different age groups.

[0013] It is currently accepted that beta-peptide deposition in the central nervous system takes place progressively with age and in Alzheimer's disease. Beta-amyloid peptide deposition and plaque formation begin 10-20 years before the onset of any symptom indicative of a loss of cognitive capacity suggesting the presence of dementia (Monsonego et al., 2003; Weksler and Goodhardt, 2002). Starting from this premise, healthy elderly individuals, selected for not having any altered intellectual capacity, who have taken part as control individuals in the studies mentioned, would probably have beta-amyloid peptide deposits and would probably develop Alzheimer's disease in the future. Therefore, the fact that some elderly individuals do not exhibit symptoms of dementia does not exclude those elderly individuals from developing the disease in the future. In a cohort study performed in healthy volunteers (Kawas et al., 2000), the evolution of the participating patients was monitored biennally for the purpose of determining the incidence rate of Alzheimer's disease for different age ranges. In that study, it was observed that the incidence rate increases with age from 0.08% per year for the age range between 60 and 65 years of age, to 6.48% per year in the group of individuals over the age of 85, considering the data for men and women combined, estimating that the incidence rate is doubled in about 4.4 years.

[0014] All publications, patent applications, patents and other reference material mentioned herein are incorporated by reference in their entirety. In addition, the materials, methods and examples are only illustrative and are not intended to be limiting. The citation of references herein shall not be construed as an admission that such is prior art to the present invention.

SUMMARY OF THE INVENTION

[0015] The present invention relates to specific binding members that recognize amyloid beta 1-42, more particularly, to antibodies, to functional fragments thereof or derivatives with beta-amyloid 1-42 peptide-binding capacity and to obtaining them by means of genetic engineering techniques from the genetic repertoire of immunoglobulins isolated from healthy elderly, and particularly, nonagenarian human donors. It particularly relates to antibodies, to functional fragments thereof or derivatives with beta-amyloid 1-42 peptide-binding capacity, to the nucleic acid sequences encoding for said antibodies, as well as to the uses and pharmaceutical compositions comprising these binding members and antibodies and fragments, derivatives or variants thereof.

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[0016] The present Inventors proceeded to search for and identify healthy elderly individuals between the ages of 60 to 100, and particularly over the age of 90 who show full cognitive capacity and good health in the absence of symptoms of Alzheimer's disease or another neuropathy and who had never received any type of medication for the prevention or treatment of neurodegenerative diseases, i.e. a restricted group of individuals who had surpassed the ages considered as critical for the onset of Alzheimer's disease were chosen as candidates for obtaining anti beta-amyloid 1-42 peptide antibodies. Therefore, a group of healthy nonagenarian elderly subjects was selected in whom, in the absence of immunization, the presence of serum anti beta-amyloid 1-42 peptide antibodies was analyzed by means of performing specific immunoassays, detecting the existence of antibodies against the beta-amyloid 1-42 peptide.

[0017] Surprisingly, two of the antibodies of the invention have numerous somatic mutations in their nucleotide sequence in comparison to the corresponding genomic genes (germline), indicating the existence of antigenic maturation processes against the beta-amyloid 1-42 peptide. These facts indicate a competent immune response against the beta peptide and reflect the existence of natural anti beta amyloid antibodies, which do not exhibit harmful effects in healthy elderly patients, with a potential therapeutic application in Alzheimer's disease

[0018] Thus, the present invention relates to human anti beta-amyloid 1-42 peptide recombinant monoclonal antibodies and to obtaining them by means of the isolation, cloning and expression of immunoglobulin genes isolated from a representative portion of the genetic repertoire of immunoglobulins from healthy elderly, and particularly, nonagenarian human donors selectively chosen for having full cognitive capacity and for not exhibiting symptoms of Alzheimer's or another neuropathy.

[0019] Antibodies per se are widely known in the state of the art. Antibodies structurally consist of two different chains called heavy chain (HC) and light chain (LC) proteins which are bound by means of disulfide bridges. Both have a variable region (V) and a constant region (C). Variable regions are involved in antigen recognition, whereas the HC constant region determines the isotype of the antibody and the functional properties thereof. When comparing the sequence similarity of the different domains of an antibody molecule, it has been observed that most domains belonging to antibodies of the same subclass have the same sequence. Said domains are called constant (C) domains. CL is the light chain constant domain and the heavy chain constant domains are CH_1, CH_2 and CH_3. However, one domain of each one of the chains is variable (VH and VL). Kabat et al. (Kabat et al., 1978) observed that variability is concentrated in 3 regions of the two variable domains. Said hypervariable domains or CDRs (Complementary-Determining Regions) interact in a three-dimensional manner to give rise to the antigen binding site, which antigen contains six CDR regions, three VH domain regions and three VL domain regions. Different authors have observed that heavy chain CDR3 (HCDR3) is the region having a greater degree of variability and a predominant participation in antigen-antibody interaction.

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[0020] The genes encoding HC and LC chains are located in different chromosomes, forming multigenic families in each case. The genes encoding the HC and LC variable and constant regions are separated in the genome; therefore somatic recombination thereof is first required for their expression (Tonegawa, 1983). The heavy chain variable region is encoded by three gene segments: Variability (V), Diversity (D) and Joining (J), whereas the light chain variable region consists of two gene segments: V and J. Each one of these segments consists of a variable number of genes that are grouped into families according to their degree of homology (Williams and Winter, 1993; Zachau, 1993). The segments of the heavy and light chain constant region are close to the J segments and are incorporated to the V(D)J complex by means of RNA splicing (Early et al. 1980). The existence of multiple copies of each one of the gene segments forming the HC and LC, as well as the different combination possibilities among them, contribute to generating the diversity of the antibody repertoire.

[0021] The somatic recombination of the V(D)J segments occurs during the B cell maturation process. Both the addition of nucleotides N by the terminal deoxynucleotidyl transferase enzyme (TdT) and deletions caused by exonucleases or the insertion of palindromic elements generated during the resolution of the recombinant products, contribute to the variability of the complementary-determining region (HCDR3). Said region is directly involved in antigenic recognition and contributes to the diversity of the immunoglobulin repertoire. The antibody repertoire is subsequently diversified during the antigenic response of the mature lymphocyte by means of somatic mutation. This process involves introducing isolated mutations in the genes of both the HC and LC chain variable regions generating intraclonal variables. The most frequent mutations are transitions, which entail substitutions in the amino acid sequence, resulting in the onset of new variants of the original immunoglobulin. An antigen-directed selective process then begins, which gradually enriches B cell populations with higher affinity immunoglobulins (and perhaps better kinetics as well). This phenomenon is what is known as affinity maturation. In selected clones, analyses of the somatic mutations of immunoglobulin variable chains show that the changes generally tend to be concentrated in the complementary-determinant regions (Foote and Milstein, 1991).

[0022] Within the context of the present invention, the term genetic repertoire of immunoglobulins is understood to be the group of gene segments of immunoglobulins which, once rearranged, encode for antibody synthesis.

[0023] Accordingly, a first aspect of the invention relates to a process of obtaining binding members, in particular, antibodies, fragments thereof or derivatives with beta-amyloid 1-42 peptide-binding capacity, by means of genetic engineering techniques from the genetic repertoire of immunoglobulins isolated from healthy elderly human donors. In the context of the present invention, the term elderly includes donors of 60 years of age or older, for example 70 years of age or older, 80 years of age or older or 90 years of age or older. Preferably the donor

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subjects of the invention are nonagenarian donors of 90 years of age or older, for example between about 90 to about 100 years of age.

[0024] Murine antibodies are unsuitable as clinical candidate therapeutics since mouse antibodies are not immunogenic in humans and usually trigger an immune response generating human anti-mouse antibody (HAMA). Said immunoglobulins recognize therapeutic antibodies and will prevent them from reaching their target, and they furthermore trigger a type III hypersensitivity reaction (George and Urch, 2000). The antibodies of the present invention are recombinant antibodies of human origin, minimizing the possibilities that the immune system of the individual recipient rejects them.

[0025] A preferred aspect of the invention relates to the cloning and expression of the genetic repertoire of immunoglobulins isolated from healthy elderly, and particularly, nonagenarian human donors selectively chosen for having full cognitive capacity and for not exhibiting symptoms of Alzheimer's or another neuropathy, and in whose serum the presence of anti beta-amyloid 1-42 peptide antibodies was detected.

[0026] There are several known strategies for producing monoclonal antibodies. Hybridoma technology (Kennet, McKearn and Bechtol, 1980) meant a revolution for immunology investigation, allowing antibody production resulting from the synthesis activity of a single B cell clone. Recent advances in genetic engineering have greatly facilitated the production, identification and conjugation of recombinant antibody fragments. An even more preferred aspect of the invention relates to the construction of a library containing the genetic repertoire of immunoglobulins isolated from healthy nonagenarian human donors selectively chosen for having full cognitive capacity and for not exhibiting symptoms of Alzheimer's or another neuropathy, in whose serum the presence of anti beta-amyloid 1-42 peptide antibodies was detected. Said antibody gene library must preferably be widely representative of the genetic repertoire of immunoglobulins and can be obtained, for example, by means of PCR amplification of B cell cDNA. Preferably, the selection method of this library must have an efficacy that is comparable to that of the immune system, which can be achieved by displaying the produced antibodies on the surface of microorganisms (Little et al., 1994). Some examples of microorganisms which can be used are: filamentous phages such as M13 (McCafferty et al., 1990), bacteria such as E. coli (Fuchs et al., 1991) or yeasts (Boder and Wittrup, 1997). In the surface displaying strategy there is a physical link between the antigen binding capacity and the gene encoding for said antibody. The antibody displayed on the cell surface or on the viral capsid surface is encoded in the genetic material of the selected clone, such that affinity for the antigen allows the isolatation of the microorganism carrying the gene of the antibody of interest. Within the context of the present invention, any antibody with beta-amyloid 1-42 peptide-binding capacity is understood to be an antibody of interest. Another known strategy for displaying the antigen of the antibodies of the library is Ribosome Display (Hanes J and Pluckthun A., 1997), in which DNA is transcribed into mRNA, which is purified and translated in vitro. The in vitro

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translation of mRNA is designed such that the dissociation of the mRNA-ribosome-peptide complex is prevented. Therefore, said complexes will be used for selecting, by antigen affinity, those clones encoding the antibody of interest.

[0027] The antigenic selection process consists of identifying those clones having affinity for the antigen and subsequently enriching the library in said clones by means of amplification in E. coli or another suitable microorganism. Said selection (bio-panning) can be done by means of different strategies that include but are not limited to: selection by means of the use of antigens immobilized by methods such as ELISA, immunotubes, affinity columns or BIAcore (surface plasmon reasonance) sensor chips; selection by means of the use of antigens in solution, such as for example: biotinylated antigens, and selection by markers expressed on the cell surface, both in monolayers of adhesive cells and in cells in suspension. It is generally necessary to perform several selection cycles in the antigen selection process. After the antigen selection from the constructed libraries, it is normal to obtain the clones of antibodies specific against the selecting molecules. Evaluation of their antigenic specificity can, for example, be carried out by means of enzyme immunoassay.

[0028] A second aspect of the present invention relates to an antibody, a functional fragment thereof or derivative with beta-amyloid 1-42 peptide-binding capacity.

[0029] A particular aspect of the invention relates to an antibody, functional fragment thereof or derivative with beta-amyloid 1-42 peptide-binding capacity obtained by means of genetic engineering techniques from the genetic repertoire of immunoglobulins isolated from healthy elderly, and particularly, nonagenarian human donors.

[0030] Within the context of the present invention, those antibody fragments that can be obtained by means of recombinant DNA technology and preserve antigen binding activity are understood to be functional antibody fragments. Included as functional antibody fragments are fragments of an IgG antibody molecule, such as Fab, Fab' and $F(ab')_2$. Fv fragments and derivatives thereof such as scFv (single-chain Fv) and dsFv (disulfide-stabilized Fv), which have been modified to increase stability of the recombinant Fv fragment are also included. The binding of two molecules other than scFv will give rise to bispecific scFvs. Other functional fragments that are very similar to bispecific scFvs are those called diabodies. Diabodies are antibody fragments consisting of two associated chains, in which each one of the chains comprises a light or heavy chain variable domain or bound to a linker. Changes in the length of said linker direct the formation of diabodies, triabodies or tetrabodies. Likewise, other functional fragments based on scFv fragments, such as minibodies, which are bivalent antibody fragments formed by the binding of two molecules of the fusion peptide scFv-CH_3, have also been described. Fd-like fragments (Chamow and Ashkenazy, 1999), domain antibodies (Holt et al., 2003) and other antibody fragments obtained by genetic engineering which by themselves are able to interact with the other antigen, are also understood as functional antibody fragments.

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[0031] Within the context of the present invention, any molecule comprising an antibody or a functional fragment thereof in its structure is understood as a derivative, for example: immunoconjugates, understanding as such molecules formed by an antibody or a functional antibody fragment and an effector molecule.

[0032] A particular embodiment of the present invention relates to an antibody, a functional fragment thereof or derivative with beta-amyloid 1-42 peptide-binding capacity, comprising one or more of the sequences selected from the group consisting of: the amino acid sequence defined in SEQ ID NO: 2, which corresponds to the heavy chain of the antibody AD38 (Figure 8), the amino acid sequence defined in SEQ ID NO: 4, which corresponds to the light chain of AD38 (Figure 9), or sequences that are homologous to these.

[0033] Antibody AD38 of the present invention is defined in nucleotide sequence SEQ ID NO: 9, whose corresponding amino acid sequence is defined in SEQ ID NO: 10, and said antibody is referred to hereinafter as AD38.

[0034] Within the context of the present invention, those amino acid sequences showing a degree of similarity of at least 80% with the amino acid sequences of the antibodies of the present invention, preferably those having a degree of similarity of at least 85%, more preferably those having a degree of similarity of at least 90% with the amino acid sequences of the antibodies of the present invention, and even more preferably those amino acid sequences having a degree of similarity of at least 95%, are understood as homologous sequences. Sequence analysis algorithms are known in the state of the art, such as BLAST for example, disclosed in Altschul et al., 1990.

[0035] In a further particular embodiment, the antibody, functional fragment thereof or derivative with beta-amyloid 1-42 peptide-binding capacity, comprises at least one heavy chain and one light chain, in which the heavy chain comprises the amino acid sequence defined in SEQ ID NO: 2 and the light chain comprises the amino acid sequence defined in SEQ ID NO: 4.

[0036] In a yet further particular embodiment, reference is made to a functional antibody fragment with beta-amyloid 1-42 peptide-binding capacity comprising at least one heavy chain and one light chain, in which the heavy chain consists of the amino acid sequence defined in SEQ ID NO: 2 and the light chain consists of the amino acid sequence defined in SEQ ID NO: 4, such as the single chain (scFv) recombinant antibody AD38 (SEQ ID NO: 10) for example.

[0037] Another particular embodiment of the present invention relates to an antibody, functional fragment thereof or derivative with beta-amyloid 1-42 peptide-binding capacity comprising one or more of the sequences selected from the group consisting of: the amino acid sequence defined in SEQ ID NO: 6 which corresponds to the heavy chain of antibody AD64 (Figure 10); the amino acid sequence defined in SEQ ID NO: 8 which corresponds to the light chain of AD64 (Figure 11) or sequences that are homologous to these.

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[0038] Antibody AD64 of the present invention is defined in nucleotide sequence SEQ ID NO: 11, the corresponding amino acid sequence of which is defined in SEQ ID NO: 12, and said antibody will be referred to hereinafter as AD64.

[0039] In a particular embodiment, the antibody, functional fragment thereof or derivative with beta-amyloid 1-42 peptide-binding capacity, comprises at least one heavy chain and one light chain, in which the heavy chain comprises sequence SEQ ID NO: 6, and the light chain comprises the sequence SEQ ID NO: 8.

[0040] In another particular embodiment, reference is made to a functional antibody fragment with beta-amyloid 1-42 peptide-binding capacity comprising at least one heavy chain and one light chain, in which the heavy chain consists of the amino acid sequence defined in SEQ ID NO: 6, and the light chain consists of the amino acid sequence defined in SEQ ID NO: 8, such as the single chain (scFv) recombinant antibody AD64 (SEQ ID NO: 12), for example.

[0041] Another particular embodiment relates to an antibody, functional fragment thereof or derivative with beta-amyloid 1-42 peptide-binding capacity, obtained by means of affinity maturation strategies from the antibodies of the present invention. Said affinity maturation strategies include but are not limited to: chain shuffling (Marks et al., 1992), introduction of directed mutations (Balint and Larrick, 1993) or random mutation strategies.

[0042] Within the context of the present invention, a recombinant polypeptide in which the sequences of the light and heavy chain variable domains (VL and VH) of an antibody have been linked by means of an artificial sequence are understood as scFv fragment. Therefore, for example the C-terminal end of the VH sequence is linked with the N-terminal end of the VL sequence by means of a binding peptide. The arrangement of the variable domains will affect the characteristics of the scFv. The scFv fragments have a series of advantages with respect to intact antibodies and to other functional antibody fragments: scFv fragments lack the Fc region, and therefore unwanted binding of the antibody to cells having Fc binding, such as cells of the reticular endothelial system, as well as complement fixation, is prevented. The absence of the Fc region implies an advantage in certain applications, such as directed immunotherapy and molecular imaging.

[0043] Due to their low molecular weight, scFv fragments have a fast biodistribution, accessing tissues more easily than intact antibodies and other functional fragments.Plasma elimination of scFv fragments is faster than that observed in Fab fragments and in intact antibodies. Due to the higher scFv elimination rate, the immunocomplexes formed by scFv conjugated with active ingredients or radionucleotides show lower toxicity. Due to their smaller size, the retention of scFv fragment antigen-antibody complexes in the renal glomerulus is less than that observed in Fab fragments and in intact antibodies.scFv fragments are highly versatile functional fragments since they are easily modified by means of genetic engineering. It is possible to generate other functional fragments from one scFv fragment.

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[0044] Another particular embodiment relates to an antibody, antibody fragment or derivative comprising part of any of the amino acid sequences defined in sequences SEQ ID NO: 2, SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 8 or combinations of all or part of the sequence thereof, which maintains beta-amyloid 1-42 peptide-binding capacity.

[0045] In another particular embodiment, the heavy chain variable domain CDR1, CDR2 or CDR3 of antibody AD38 defined in Figure 8, the light chain variable domain CDR1, CDR2 or CDR3 of antibody AD38 defined in Figure 9, the heavy chain variable domain CDR1, CDR2 or CDR3 of antibody AD64 defined in Figure 10, and the light chain variable domain CDR1, CDR2 or CDR3 of antibody AD64 defined in Figure 11, are understood as part of any of the amino acid sequences defined in sequences SEQ ID NO: 2, SEQ ID NO: 4, SEQ ID NO: 6 and SEQ ID NO: 8.

[0046] A third aspect of the invention relates to a nucleic acid molecule encoding the amino acid sequence of the light chain or heavy chain variable domain of an antibody with beta-amyloid 1-42 peptide-binding capacity of the present invention, and to variants of said nucleic acid molecule.

[0047] Within the context of the present invention, substantially similar sequences are understood as variants of a nucleic acid molecule. Substantially similar sequences include nucleotide sequences which, due to the degeneration of the genetic code, encode the amino acid sequence of the light chain or heavy chain variable domain of an antibody of the present invention. The amino acids are encoded by triplets consisting of three nucleotides, also called codons. Most amino acids are encoded by more than one triplet. This is called genetic code degeneration. Therefore, one or more triplets may be replaced by other triplets, but said nucleotide sequence will continue encoding for the same amino acid.

[0048] Substantially similar sequences also include nucleotide sequences encoding variants of the antibodies or antibody fragments of the invention. These nucleotide sequences homologous to a particular nucleotide sequence of the present invention will have at least a 40%, 50%, 60%, 70% identity with said sequence, preferably at least a 75%, 80%, 85%, 90% identity with said sequence, and even more preferably, it will have a sequence similarity of 95%, 96%, 97%, 98%, 99% or greater.

[0049] A further aspect of the invention relates to a nucleic acid molecule comprising any of the nucleotide sequences shown in SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 5 or SEQ ID NO: 7, active fragments thereof, or any combination thereof.

[0050] A fourth aspect of the invention relates to a vector comprising a nucleic acid molecule according to the present invention. Said vector includes but is not limited to: viral plasmids, cosmids and vectors. Said vectors preferably contain one or more regulatory regions, such as promoters, termination and secretion signal sequences allowing the nucleic acid molecules of the present invention to be expressed as a protein. Even more particularly, said vector is a viral

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vector that can be used to infect cells *in vitro* or *in vivo*. Said vector can be used in gene therapy.

[0051] A fifth aspect of the invention relates to a host cell containing at least one vector comprising a nucleic acid molecule of the present invention. Said host cells are cells that are known by any person skilled in the art, and they include but are not limited to: mammalian cells, yeast cells and prokaryotic cells such as *Escherichia coli*. Other unicellular host cells may be selected from the group consisting of Pseudomonas, Bacillus, Streptomyces, CHO, YB/20, NSO, SP2/0, R1.1, B-W, L-M, COS 1, COS 7, BSC1, BSC40, and BMT10 cells, plant cells, insect cells, and human cells in tissue culture.

[0052] In one embodiment, the unicellular host is transformed with:

(A) the DNA sequence of SEQ ID NO:1;

(B) the DNA sequence of SEQ ID NO:3;

(C) the DNA sequence of SEQ ID NO:1 and the DNA sequence of SEQ ID NO:3;

(D) a DNA sequence that hybridizes to any of the foregoing DNA sequences under standard hybridization conditions;

(E) a DNA sequence that codes on expression for an amino acid sequence encoded by any of the foregoing DNA sequences; or

(F) a fragment of any one of the foregoing DNA sequences which encodes a specific binding member which

(i) recognizes an epitope on amyloid beta 1-42, wherein said binding member is obtained from healthy elderly subjects having full cognitive capacity and who exhibit no symptoms of Alzheimer's disease or other neuropathies, for example where the subjects are 60 years of age or older, for example 70 years of age or older, 80 years of age or older or 90 years of age or older;

(ii) is an antibody having amyloid beta 1-42 binding capacity, and wherein the antibody comprises an amino acid sequence of SEQ ID NOs: 2, or 4, or an amino acid sequence that is substantially homologous to SEQ ID NOs: 2 or 4, and functional fragments, derivatives or variants thereof;

(iii) is an antibody, functional fragment or derivative thereof with beta-amyloid 1-42 peptide-binding capacity comprising one or more of the amino acid sequences selected from the group consisting of SEQ ID NO: 2, SEQ ID NO: 4 and sequences substantially homologous to either one of SEQ ID NO: 2 or SEQ ID NO: 4;

(iv) is an antibody, functional fragment thereof or derivative with beta-amyloid 1-42 peptide-binding capacity, according to claim 4, comprising at least one heavy chain and

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one light chain, wherein the heavy chain comprises the amino acid sequence of SEQ ID NO: 2 and the light chain comprises the amino acid sequence of SEQ ID NO: 4;

(v) is an antibody, functional fragment or derivative thereof with beta-amyloid 1-42 peptide-binding capacity comprising one or more of the amino acid sequences selected from the group consisting of SEQ ID NO: 6, SEQ ID NO: 8 and sequences substantially homologous to either one of SEQ ID NO: 6 or SEQ ID NO: 8;

or,

(vi) is an antibody, functional fragment thereof or derivative with beta-amyloid 1-42 peptide-binding capacity, according to the claim 6, comprising at least one heavy chain and one light chain, wherein the heavy chain comprises the amino acid sequence of SEQ ID NO: 6 and the light chain comprises the amino acid sequence of SEQ ID NO: 8,

wherein said DNA sequence is operatively linked to an expression control sequence.

[0053] In another embodiment, the unicellular host is transformed with:

(A) the DNA sequence of SEQ ID NO:5;

(B) the DNA sequence of SEQ ID NO:7;

(C) the DNA sequence of SEQ ID NO:5 and the DNA sequence of SEQ ID NO:7;

(D) a DNA sequence that hybridizes to any of the foregoing DNA sequences under standard hybridization conditions;

(E) a DNA sequence that codes on expression for an amino acid sequence encoded by any of the foregoing DNA sequences; or

(F) a fragment of any one of the foregoing DNA sequences which encodes a specific binding member which

(i) recognizes an epitope on amyloid beta 1-42, wherein said binding member is obtained from healthy elderly subjects having full cognitive capacity and who exhibit no symptoms of Alzheimer's disease or other neuropathies, for example where the subjects are 60 years of age or older, for example 70 years of age or older, 80 years of age or older or 90 years of age or older;

(ii) is an antibody having amyloid beta 1-42 binding capacity, and wherein the antibody comprises an amino acid sequence of SEQ ID NOs: 2, or 4, or an amino acid sequence that is substantially homologous to SEQ ID NOs: 2 or 4, and functional fragments, derivatives or variants thereof;

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(iii) is an antibody, functional fragment or derivative thereof with beta-amyloid 1-42 peptide-binding capacity comprising one or more of the amino acid sequences selected from the group consisting of SEQ ID NO: 2, SEQ ID NO: 4 and sequences substantially homologous to either one of SEQ ID NO: 2 or SEQ ID NO: 4;

(iv) is an antibody, functional fragment thereof or derivative with beta-amyloid 1-42 peptide-binding capacity, according to claim 4, comprising at least one heavy chain and one light chain, wherein the heavy chain comprises the amino acid sequence of SEQ ID NO: 2 and the light chain comprises the amino acid sequence of SEQ ID NO: 4;

(v) is an antibody, functional fragment or derivative thereof with beta-amyloid 1-42 peptide-binding capacity comprising one or more of the amino acid sequences selected from the group consisting of SEQ ID NO: 6, SEQ ID NO: 8 and sequences substantially homologous to either one of SEQ ID NO: 6 or SEQ ID NO: 8;

or,

(vi) is an antibody, functional fragment thereof or derivative with beta-amyloid 1-42 peptide-binding capacity, according to the claim 6, comprising at least one heavy chain and one light chain, wherein the heavy chain comprises the amino acid sequence of SEQ ID NO: 6 and the light chain comprises the amino acid sequence of SEQ ID NO: 8,

wherein said DNA sequence is operatively linked to an expression control sequence.

[0054] A sixth aspect of the invention relates to the use of an antibody, a fragment thereof including active and immunogenic fragments thereof, or derivatives thereof, for preparing a pharmaceutical composition. In a related aspect the invention also provides a binding member which :

(i) recognizes an epitope on amyloid beta 1-42, wherein said binding member is obtained from healthy elderly subjects having full cognitive capacity and who exhibit no symptoms of Alzheimer's disease or other neuropathies, for example where the subjects are 60 years of age or older, for example 70 years of age or older, 80 years of age or older or 90 years of age or older;

(ii) is an antibody having amyloid beta 1-42 binding capacity, and wherein the antibody comprises an amino acid sequence of SEQ ID NOs: 2, or 4, or an amino acid sequence that is substantially homologous to SEQ ID NOs: 2 or 4, and functional fragments, derivatives or variants thereof;

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(iii) is an antibody, functional fragment or derivative thereof with beta-amyloid 1-42 peptide-binding capacity comprising one or more of the amino acid sequences selected from the group consisting of SEQ ID NO: 2, SEQ ID NO: 4 and sequences substantially homologous to either one of SEQ ID NO: 2 or SEQ ID NO: 4;

(iv) is an antibody, functional fragment thereof or derivative with beta-amyloid 1-42 peptide-binding capacity, according to claim 4, comprising at least one heavy chain and one light chain, wherein the heavy chain comprises the amino acid sequence of SEQ ID NO: 2 and the light chain comprises the amino acid sequence of SEQ ID NO: 4;

(v) is an antibody, functional fragment or derivative thereof with beta-amyloid 1-42 peptide-binding capacity comprising one or more of the amino acid sequences selected from the group consisting of SEQ ID NO: 6, SEQ ID NO: 8 and sequences substantially homologous to either one of SEQ ID NO: 6 or SEQ ID NO: 8;

or

(vi) is an antibody, functional fragment thereof or derivative with beta-amyloid 1-42 peptide-binding capacity, according to the claim 6, comprising at least one heavy chain and one light chain, wherein the heavy chain comprises the amino acid sequence of SEQ ID NO: 6 and the light chain comprises the amino acid sequence of SEQ ID NO: 8.

for use as a medicament. Also provided is a pharmaceutical composition which comprises a binding member as described above and a pharmaceutical acceptable excipient. Further provided is the use of a binding member as described above for the manufacture of a medicament for the treatment or prevention of Alzheimer's disease, or for the preparation of a pharmaceutical composition for its use in the treatment or prevention of Alzheimer's disease.

[0055] A further particular aspect of the invention relates to a pharmaceutical composition containing an isolated specific binding member which recognizes amyloid beta 1-42, and in particular, an antibody, a fragment thereof including active and immunogenic fragments thereof, or derivatives thereof.

[0056] A yet further particular aspect of the invention relates to a pharmaceutical composition containing an antibody, a fragment thereof including active and immunogenic fragments thereof, or derivatives thereof, for use in treating or preventing Alzheimer's disease.

[0057] In one particular embodiment, the composition comprises a binding member, such as an antibody comprising one or more of the amino acid sequences set forth in SEQ ID NOs: 2, 4, 6, or 8 or amino acid sequences having substantial homology to one or more of the sequences set forth in SEQ ID NOs: 2, 4, 6 or 8.

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[0058] In another particular embodiment, the binding member is the antibody aforesaid, and particularly, a scFv.

[0059] In another particular embodiment, the pharmaceutical composition further comprises an additional agent useful for treating Alzheimer's disease, selected from the group consisting of small organic molecules, other amyloid beta antibodies, and combinations thereof. The invention contemplates and includes therefore, compositions comprising at least one binding member of the invention, in combination with one or more other agents as aforesaid.

[0060] A seventh aspect of the invention relates to the use of an antibody, fragment thereof or derivative of the present invention for detecting the beta-amyloid 1-42 peptide *in vitro*. The use of antibodies as affinity reagents is of particular interest. Specific monoclonal antibody production will be obvious for any person skilled in the art. The use of specific antibodies in *in vitro* immunodetection techniques is also widely known. Said techniques include but are not limited to: ELISA, Western Blot, immunofluorescence, immunohistochemistry or flow cytometry.

[0061] An eighth aspect of the invention relates to the use of an antibody, fragments thereof or derivatives thereof, for detecting the beta-amyloid 1-42 peptide *in vivo*. Further provided by the invention is the use of an antibody, fragments thereof or derivatives thereof in the preparation of a composition for the *in vivo* detection of the beta-amyloid 1-42 peptide. The *in vivo* detection of the beta-amyloid 1-42 peptide can be carried out by means of molecular imaging techniques. Labeled monoclonal antibodies constitute specific detectable contrasts according to the presence of binding to their ligand (Weissleder, 2002). Molecular imaging techniques allow conducting in vivo specific detection studies. In animal experiments, it allows studying the evolution of a pathology or the effect of a drug on the animal without necessarily having to sacrifice it. In human beings, it allows the pharmacological study or study of the pathology in different tissues in a non-invasive and virtually painless manner. The use of a labeled antibody which specifically binds to the beta-amyloid 1-42 peptide would allow very early detection (before the onset of clinical symptoms characteristic of Alzheimer's disease) of the presence of beta-amyloid 1-42 peptide deposits, as well as the study of the evolution of the disease over time and of treatment efficacy.

[0062] A ninth aspect of the invention relates to the use of an antibody, a fragment thereof including active and immunogenic fragments thereof, or derivatives thereof for identifying a compound that is able to compete with said antibody, fragment or derivative for binding to the beta-amyloid 1-42 peptide.

[0063] In a preferred aspect of the invention, said compound able to compete with the antibody, fragment or derivative of the present invention is a peptide or a peptoid. Within the context of the present invention, a compound that is analogous to a peptide in which the peptide bonds have been replaced by peptide-like bonds, such as: Carba [psi] (CH_2-CH_2), Depsi [psi] (CO-OR), Hydroxyethylene [psi] (CHOH-CH_2), Ketomethylene [psi] (CH-CH_2), Methylene-oxy CH_2-O-,

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Reduced CH_2-NH, Thiomethylene CH_2-S-, Thiopeptide CS-NH, N-modified -NRCO-, is understood as a peptoid.

[0064] In an even more preferred aspect of the invention, said compound able to compete with the antibody, fragment or derivative of the present invention is a mimotope. Within the context of the present invention, a peptide molecule the structure of which imitates that of the epitope recognized by the antibodies, fragments or derivatives of the present invention, is understood as a mimotope. The amino acid sequence of said mimotope does not necessarily have to present sequence similarity with the epitope; nevertheless, the mimotope will adopt a conformation that imitates that of the epitope (Steward et al., 1995).

[0065] In an especially preferred aspect of the invention, said compound able to compete with the antibody, fragment or derivative of the present invention is a synthetic peptide based on the sequence of the heavy chain CDR3 region of the antibodies of the present invention.

[0066] A tenth aspect of the invention provides a process of obtaining antibodies or functional fragments thereof with beta-amyloid 1-42 peptide-binding capacity from the genetic repertoire of immunoglobulins isolated from healthy donors, comprising the steps of:

A) purifying B cells from, for example, a blood sample or a bone marrow sample, said sample being obtained from a subject fulfilling the following criteria;
   (i) being 60 years of age or older;
   (ii) having full cognitive capacity and good health;
   (iii) having no symptoms of Alzheimer's disease or another neuropathy;
   (iv) not having previously received any medication for the prevention or treatment of neurodegenerative diseases; and
   (v) possessing detectable anti beta-amyloid 1-42 peptide antibodies in the blood serum;
B) obtaining the immunoglobulin gene repertoire for said anti beta-amyloid 1-42 peptide antibodies from said B cells;
C) using said repertoire to express said antibodies.

[0067] The method for obtaining the immunoglobulin gene repertoire for the anti beta-amyloid 1-42 peptide antibodies from the B cells in step B) can be carried out by the following steps:

(i) obtaining mRNA from the said B cells;
(ii) obtaining cDNA from the mRNA of step (i); and;
(iii) using a primer extension reaction to amplify from said cDNA the fragments corresponding to the heavy chains (HC) and the kappa light chains (LC) of said anti beta-amyloid 1-42 peptide antibodies.

In a preferred embodiment the B cells are peripheral blood B cells obtained from a blood sample.

[0068] An eleventh aspect of the invention provides a method of preparing a specific binding member capable of binding an epitope on amyloid beta 1-42, which method comprises:

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a) providing a starting repertoire of nucleic acids encoding a VH domain which lacks a CDR3 encoding region;

b) combining said repertoire with a donor nucleic acid encoding an amino acid sequence substantially as set out in SEQ ID NOs: 2, 4, 6 or 8 such that said donor nucleic acid is inserted into the missing CDR3 region, so as to provide a product repertoire of nucleic acids encoding a VH domain;

c) expressing the nucleic acids of said product repertoire; and

d) selecting a specific binding member which recognizes amyloid beta 1-42; and

e) recovering said binding member or the nucleic acid encoding it.

[0069] A twelfth aspect of the invention provides a method of treating or preventing the progression of Alzheimer's disease in a subject, or for ameliorating the symptoms associated with Alzheimer's disease, or for diagnosing or screening a subject for the presence of Alzheimer's disease or for determining a subject's risk for developing Alzheimer's disease, which method comprises administering to said subject an effective amount of a specific binding member or antibody or fragment, derivative or variant thereof, and pharmaceuticals containing the binding member.

[0070] Other objects and advantages will become apparent to those skilled in the art from a review of the following description which proceeds with reference to the following illustrative drawings.

BRIEF DESCRIPTION OF THE FIGURES

[0071] Figures 1A-1D show the results obtained in the ELISA assays carried out using the beta-amyloid 1-42 peptide at a concentration of 5 µg/ml as a coating and anti IgG+IgM+IgA-HRP as a secondary antibody to detect the existence of serum anti beta-amyloid 1-42 peptide antibodies in donors Vj2, Vj3, Vj4 and Vj5. The serial dilutions of assayed serum are shown on the X-axis and optical density at 450 nm is shown on the Y-axis.

[0072] Figures 2A and 2B show the results obtained in the ELISA assays using the beta-amyloid 1-42 peptide at a concentration of (1 µg/ml) as a coating and anti IgG (H+L)-HRP as a secondary antibody, compared to the different dilutions of serums Vj2 and Vj5. The serial dilutions of assayed serum are shown on the X-axis and optical density at 492 nm is shown on the Y-axis. Figure 2A shows the titration curve of the serum from donor Vj2, and Figure 2B shows the titration curve of the serum from donor Vj5.

[0073] Figures 3A and 3B show the photograph of a 2% agarose gel electrophoresis of the PCR products obtained in the first amplification of the genetic human immunoglobulin repertoire for the construction of an antibody fragment (Fab) library. Figure 3A shows the gamma-1 heavy chains and Figure 3b shows the kappa chains. Figure 3C shows the photograph of a 2% agarose gel electrophoresis of the PCR products obtained in the second amplification using the purified PCR products from the first amplification as a mold.

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[0074] Figures 4A and 4B show the photograph of a 2% agarose gel electrophoresis of the PCR products obtained in the first amplification of the genetic human immunoglobulin repertoire for the construction of a single chain fragment (scFv) library. Figure 4A shows the kappa chains and Figure 4B shows the gamma heavy chains. Figure 4C shows the photograph of a 2% agarose gel electrophoresis of the PCR products obtained in the second amplification, carried out in triplicate, using the purified PCR products from the first amplification as a mold.

[0075] Figure 5 shows the HCDR3 sequences of several clones chosen randomly from the antibody fragment (Fab) library and from the single chain antibody fragment (scFv) library, which have been sequenced for the purpose of checking the diversity of the obtained libraries.

[0076] Figure 6 shows the degree of enrichment obtained in the antigen selection step of the single chain fragment library against the beta-amyloid 1-42 peptide immobilized in solid phase (ELISA). The graph represents the progress of each selection cycle as the percentage of antibodies eluted in each round (CFU eluted/ml); final enrichment degree (x 3.9) was determined considering the negative control (c-).

[0077] Figures 7A and 7B show the binding profile of the recombinant antibodies AD38 and AD64, respectively, by means of specific immunoassaying (ELISA) against the beta-amyloid 1-42 peptide.

[0078] Figure 8 shows the nucleotide sequence of the heavy chain of recombinant antibody AD38 and its corresponding amino acid sequence, pointing out the hypervariable regions or CDRs.

[0079] Figure 9 shows the nucleotide sequence of the light chain of recombinant antibody AD38 and its corresponding amino acid sequence, pointing out the hypervariable regions or CDRs.

[0080] Figure 10 shows the nucleotide sequence of the heavy chain of recombinant antibody AD64 and its corresponding amino acid sequence, pointing out the hypervariable regions or CDRs.

[0081] Figure 11 shows the nucleotide sequence of the light chain of recombinant antibody AD64 and its corresponding amino acid sequence, pointing out the hypervariable regions or CDRs.

DETAILED DESCRIPTION OF THE INVENTION

[0082] Before the present methods and treatment methodology are described, it is to be understood that this invention is not limited to particular methods, and experimental conditions described, as such methods and conditions may vary. It is also to be understood that the terminology used herein is for purposes of describing particular embodiments only, and is not intended to be limiting, since the scope of the present invention will be limited only in the appended claims.

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[0083] As used in this specification and the appended claims, the singular forms "a", "an", and "the" include plural references unless the context clearly dictates otherwise. Thus, for example, references to "the method" includes one or more methods, and/or steps of the type described herein and/or which will become apparent to those persons skilled in the art upon reading this disclosure and so forth.

[0084] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the invention, the preferred methods and materials are now described. All publications mentioned herein are incorporated herein by reference in their entirety.

[0085] In accordance with the present invention there may be employed conventional molecular biology, microbiology, and recombinant DNA techniques within the skill of the art. Such techniques are explained fully in the literature. See, e.g., Sambrook et al, "Molecular Cloning: A Laboratory Manual" (1989); "Current Protocols in Molecular Biology" Volumes I-III [Ausubel, R. M., ed. (1994)]; "Cell Biology: A Laboratory Handbook" Volumes I-III [J. E. Celis, ed. (1994))]; "Current Protocols in Immunology" Volumes I-III [Coligan, J. E., ed. (1994)]; "Oligonucleotide Synthesis" (M.J. Gait ed. 1984); "Nucleic Acid Hybridization" [B.D. Hames & S.J. Higgins eds. (1985)]; "Transcription And Translation" [B.D. PHames & S.J. Higgins, eds. (1984)]; "Animal Cell Culture" [R.I. Freshney, ed. (1986)]; "Immobilized Cells And Enzymes" [IRL Press, (1986)]; B. Perbal, "A Practical Guide To Molecular Cloning" (1984).

Definitions

[0086] The terms used herein have the meanings recognized and known to those of skill in the art, however, for convenience and completeness, particular terms and their meanings are set forth below.

[0087] An "isolated" or "purified" polypeptide or protein, e.g., an "isolated antibody," refers to a protein, polypeptide or antibody that is purified to a state beyond that in which it exists in nature. For example, the "isolated" or "purified" polypeptide or protein, e.g., an "isolated antibody," refers to a protein that is separated from at least one component of cellular material or other contaminating proteins from the cell or tissue source from which the protein is derived, or separated from at least one component of the chemical precursors or other chemicals when chemically synthesized. For example, an isolated protein can be substantially free of other proteins, other cellular material, or chemical precursors. In some embodiments, the preparation of antibody protein having less than about 50% of non-antibody protein (also referred to herein as a "contaminating protein"), or of chemical precursors, is considered to be "substantially free." In other embodiments, 40%, 30%, 20%, 10% and more preferably 5% (by dry weight), of non-antibody protein, or of chemical precursors is considered to be substantially free. When the

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antibody protein or biologically active portion thereof is recombinantly produced, it is also preferably substantially free of culture medium, e.g., the culture medium represents less than about 30%, 20%, more preferably less than about 10%, and most preferably less than about 5% of the volume or mass of the protein preparation. Proteins or polypeptides referred to herein as "recombinant" are proteins or polypeptides produced by the expression of recombinant nucleic acids. Proteins can be purified by standard methods (including, e.g., ion exchange and affinity chromatography) to provide preparations in which a particular protein is at least 5, 10, 20, 25, 50, 75, 80, 90, 95, 98, 99% pure relative to other proteins or relative to other biologically active components.

[0088] The phrase "pharmaceutically acceptable" refers to molecular entities and compositions that are physiologically tolerable and do not typically produce an allergic or similar untoward reaction, such as gastric upset, dizziness and the like, when administered to a human. Preferably, as used herein, the term "pharmaceutically acceptable" means approved by a regulatory agency of the Federal or a state government or listed in the U.S. Pharmacopeia or other generally recognized pharmacopeia for use in animals, and more particularly in humans.

[0089] The term "carrier" refers to a diluent, adjuvant, excipient, or vehicle with which the compound is administered. Such pharmaceutical carriers can be sterile liquids, such as water and oils, including those of petroleum, animal, vegetable or synthetic origin, such as peanut oil, soybean oil, mineral oil, sesame oil and the like. Water or aqueous solution saline solutions and aqueous dextrose and glycerol solutions are preferably employed as carriers, particularly for injectable solutions. Suitable pharmaceutical carriers are described in "Remington's Pharmaceutical Sciences" by E.W. Martin.

[0090] The phrase "therapeutically effective amount" is used herein to mean an amount sufficient to prevent, and preferably reduce by at least about 25 percent, more preferably by at least 50 percent, most preferably by at least 90 percent, a clinically significant change in a feature of pathology such as for example, elevated blood pressure, fever, or white cell count, as may attend its presence and activity. As related to the present invention, the term may also mean an amount sufficient to ameliorate or reverse one or more symptoms associated with Alzheimer's disease. In particular, a "therapeutically effective amount" of the treatment may result in amelioration, reduction or elimination of at least one of the following symptoms: memory impairment, persistent sadness or anxiety, feelings of emptiness, hopelessness, pessimism, guilt, worthlessness, helplessness, a loss of interest or pleasure in hobbies and activities that were once enjoyed, decreased energy, or fatigue, difficulty concentrating, remembering, or making decisions, insomnia, early-morning awakening, or oversleeping, appetite and/or weight loss or overeating and weight gain, thoughts of death or suicide and suicide attempts, restlessness, irritability, and persistent physical symptoms that do not respond to treatment, such as headaches, digestive disorders, and chronic pain.

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[0091] "Treatment" or "treating" refers to therapy, prevention and prophylaxis and particularly refers to the administration of medicine or the performance of medical procedures with respect to a patient, for either prophylaxis (prevention) or to cure or reduce the extent of or likelihood of occurrence of the infirmity or malady or condition or event in the instance where the patient is afflicted.

[0092] "Subject" or "patient" refers to a mammal, preferably a human, in need of treatment for a condition, disorder or disease.

[0093] An "antibody" is any immunoglobulin, including antibodies and fragments or derivatives thereof, that binds a specific epitope. Such an antibody that binds a specific epitope is said to be "immunospecific". The term encompasses "polyclonal", "monoclonal", and "chimeric" antibodies, the last mentioned described in further detail in U.S. Patent Nos. 4,816,397 and 4,816,567. Commonly used carriers that are chemically coupled to peptides include bovine or chicken serum albumin, thyroglobulin, and other carriers known to those skilled in the art. The coupled peptide is then used to immunize the animal (e.g, a mouse, rat or rabbit). The "chimeric antibody" refers to a molecule in which different portions are derived from different animal species, such as those having a human immunoglobulin constant region and a variable region derived from a murine mAb. (See, e.g., Cabilly et al., U.S. Patent No. 4,816,567; and Boss et al., U.S. Patent No. 4,816,397.). The antibody may be a human or a humanized antibody. The antibody may be a single chain antibody. (See, e.g., Curiel et al., U.S. Patent No. 5,910,486 and U.S. Patent No. 6,028,059). The antibody may be prepared in, but not limited to, mice, rats, rabbits, goats, sheep, swine, dogs, cats, or horses. More particularly, the present invention provides for the isolation of antibodies from healthy elderly human, who do not exhibit any of the symptoms associated with Alzheimer's disease, such as, for example, loss of memory or irritability. As used herein, the term "single-chain antibody" refers to a polypeptide comprising a V_H region and a V_L region in polypeptide linkage, generally linked via a spacer peptide (e.g., [Gly-Gly-Gly-Gly-Ser] $_x$), and which may comprise additional amino acid sequences at the amino- and/or carboxy- termini. For example, a single-chain antibody may comprise a tether segment for linking to the encoding polynucleotide. As an example, a scFv (single chain fragment variable) is a single-chain antibody. Single-chain antibodies are generally proteins consisting of one or more polypeptide segments of at least 10 contiguous amino acids substantially encoded by genes of the immunoglobulin superfamily (e.g., see The Immunoglobulin Gene Superfamily, A. F. Williams and A. N. Barclay, in Immunoglobulin Genes, T. Honjo, F. W. Alt, and T. H. Rabbitts, eds., (1989) Academic Press: San Diego, Calif., pp.361-387, most frequently encoded by a rodent, non-human primate, avian, porcine, bovine, ovine, goat, or human heavy chain or light chain gene sequence. A functional single-chain antibody generally contains a sufficient portion of an immunoglobulin superfamily gene product so as to retain the property of binding to a specific target molecule, typically a receptor or antigen (epitope). For a review of scFv see Pluckthun in The Pharmacology of Monoclonal Antibodies,

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vol. 113, Rosenburg and Moore eds. Springer-Verlag, N.Y., pp. 269-315 (1994). One particularly advantageous approach has been the use of so-called single-chain fragment variable (scFv) libraries (Marks et al. (1992) Biotechnology 10: 779; Winter G and Milstein C (1991) Nature 349: 293; Clackson et al. (1991) op.cit.; Marks et al. (1991) J. Mol. Biol. 222: 581; Chaudhary et al. (1990) Proc. Natl. Acad. Sci. (USA) 87: 1066; Chiswell et al. (1992) TIBTECH 10: 80; McCafferty et al. (1990) op.cit.; and Huston et al. (1988) Proc. Natl. Acad. Sci. (USA) 85: 5879). Various embodiments of scFv libraries displayed on bacteriophage coat proteins have been described.

[0094] Antibodies are naturally "bivalent antibodies", however, the term also includes diabodies. The term "diabodies" refers to small antibody fragments with two antigen-binding sites, which fragments comprise a heavy chain variable domain (V_M) connected to a light chain variable domain (V_L) in the same polypeptide chain (V_H-V_L). By using a linker that is too short to allow pairing between the two domains on the same chain, the domains are forced to pair with the complementary domains of another chain and create two antigen-binding sites. Diabodies are described more fully in, for example, (Holliger, P., et al. (1993) Proc. Natl. Acad. Sci. USA 90:6444-6448; Poljak, R. J., et al. (I 994) Structure 2:1121-1123); EP 404,097; WO 93/11161). "Multivalent" antibodies include two or more binding domains which may all be of the same specificity or may have multiple specificities.

An "antibody combining site" is that structural portion of an antibody molecule comprised of heavy and light chain variable and hypervariable regions that specifically binds antigen.

[0095] The phrase "antibody molecule" in its various grammatical forms as used herein contemplates both an intact immunoglobulin molecule and an immunologically active portion of an immunoglobulin molecule.

[0100] Exemplary antibody molecules are intact immunoglobulin molecules, substantially intact immunoglobulin molecules and those portions of an immunoglobulin molecule that contains the paratope, including those portions known in the art as Fab, Fab', $F(ab')_2$ and F(v), which portions are preferred for use in the therapeutic methods described herein.

[0101] Fab and $F(ab')_2$ portions of antibody molecules are prepared by the proteolytic reaction of papain and pepsin, respectively, on substantially intact antibody molecules by methods that are well-known. See for example, U.S. Patent No. 4,342,566 to Theofilopolous et al. Fab' antibody molecule portions are also well-known and are produced from $F(ab')_2$ portions followed by reduction of the disulfide bonds linking the two heavy chain portions as with mercaptoethanol, and followed by alkylation of the resulting protein mercaptan with a reagent such as iodoacetamide. An antibody containing intact antibody molecules is preferred herein.

[0102] The phrase "monoclonal antibody" in its various grammatical forms refers to an antibody having only one species of antibody combining site capable of immunoreacting with a particular antigen. A monoclonal antibody thus typically displays a single binding affinity for any antigen with which it immunoreacts. A monoclonal antibody may therefore contain an antibody molecule

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having a plurality of antibody combining sites, each immunospecific for a different antigen; e.g., a bispecific (chimeric) monoclonal antibody.

[0103] "Screening" or "Diagnosis" refers to diagnosis, prognosis, monitoring, characterizing, selecting patients, including participants in clinical trials, and identifying patients at risk for or having a particular disorder or clinical event or those most likely to respond to a particular therapeutic treatment, or for assessing or monitoring a patient's response to a particular therapeutic treatment.

[0104] "Amyloid" describes various types of protein aggregations that share specific traits when examined microscopically. Amyloid is typically identified by a change in the fluorescence intensity of planar aromatic dyes such as Thioflavin T or Congo Red. This is generally attributed to the environmental change as these dyes intercolate between beta-strands. The amyloid fold is characterized by a cross-beta sheet quaternary structure, that is, a monomeric unit contributes a beta strand to a beta sheet which spans across more than one molecule. While amyloid is usually identified using fluorescent dyes, stain polarimetry, circular dichroism, or FTIR (all indirect measurements), the "gold-standard" test to see if a structure is amyloid is by placing a sample in an X-ray diffraction beam; there are two characteristic scattering bands produced at 4 and 10 angstroms each, corresponding to the interstrand distances in the beta sheet structure. The amyloid protein disclosed in the present application refers to amyloid beta, as described below.

[0105] "Amyloid beta", "Abeta", "Aß", "beta-amyloid" or "amyloid beta peptide" is a physiological product normally released from the amyloid beta protein precursor (βAPP or APP) through β and γ secretase cleavage and consists of two 40 and 42 amino acid peptides, usually abbreviated as Aβ40 or amyloid beta 1-40 and Aβ42 or amyloid beat 1-42, respectively (Selkoe, D. (2002), J. Clin. Invest. 110:1375-1381. The 42 amino acid amyloid beta peptide (Aß42) is more hydrophobic & "sticky" (and hence aggregates more readily) than the 40 amino acid amyloid beta peptide (Aß40), and as such may play a greater role in the pathogenesis of Alzheimer's disease, due to its increased tendency to form insoluble fibrils and increased neurotoxicity. Thus, under certain circumstances, as in Alzheimer's disease (AD), brain levels of these peptides increase dramatically, which can lead to the oligomerization of the peptides and eventually to the formation of insoluble fibrillar aggregates, which deposit in senile plaques. The term "amyloid beta40" may be used interchangeably with "Abeta40", Aβ40 and Abeta 1-40, and "amyloid beta42" is used interchangeably with "Abeta42", Aβ42 and Abeta 1-42. The sequences of these Abeta peptides and their relationship to the APP precursor are illustrated in FIG. 1 of Hardy et al., TINS 20, 155-158 (1997).

[0106] "Fragment" refers to either a protein or polypeptide comprising an amino acid sequence of at least 4 amino acid residues (preferably, at least 10 amino acid residues, at least 15 amino acid residues, at least 20 amino acid residues, at least 25 amino acid residues, at least 40 amino acid residues, at least 50 amino acid residues, at least 60 amino residues, at least 70

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amino acid residues, at least 80 amino acid residues, at least 90 amino acid residues, at least 100 amino acid residues, at least 125 amino acid residues, or at least 150 amino acid residues) of the amino acid sequence of a parent protein or polypeptide, or a nucleic acid comprising a nucleotide sequence of at least 10 base pairs (preferably at least 20 base pairs, at least 30 base pairs, at least 40 base pairs, at least 50 base pairs, at least 50 base pairs, at least 100 base pairs, at least 200 base pairs) of the nucleotide sequence of the parent nucleic acid. Any given fragment may or may not possess a functional activity of the parent nucleic acid or protein or polypeptide.

[0107] Examples of binding fragments encompassed within the term "antigen-binding fragment" of an antibody include (i) a Fab fragment, a monovalent fragment consisting of the VL, VH, CL and CH1 domains; (ii) a $F(ab')_2$ fragment, a bivalent fragment comprising two Fab fragments linked by a disulfide bridge at the hinge region; (iii) a Fd fragment consisting of the VH and CH1 domains; (iv) a Fv fragment consisting of the VL and VH domains of a single arm of an antibody, (v) a dAb fragment (Ward et al. (1989) Nature 341:544-46), which consists of a VH domain; and (vi) an isolated complementarity determining region (CDR). Camelid antibodies, and camelized antibodies can also be used. Such antibodies, e.g., can include CDRs from just one of the variable domains described herein. Furthermore, although the two domains of the Fv fragment, VL and VH, are coded for by separate genes, they can be joined, using recombinant methods, by a synthetic linker that enables them to be made as a single protein chain in which the VL and VH regions pair to form monovalent molecules (known as single chain Fv (scFv); see, e.g., Bird et al. (1988) Science 242:423-26; Huston et al. (1988) Proc. Natl. Acad. Sci. U.S.A. 85:5879-83). Such single chain antibodies are also intended to be encompassed within the term "antigen-binding fragment" of an antibody. These antibody fragments are obtained using conventional techniques known to those skilled in the art, and the fragments are evaluated for function in the same manner as are intact antibodies.

[0108] "Combination therapy" refers to the use of the agents of the present invention with other active agents or treatment modalities, in the manner of the present invention for treatment of Alzheimer's disease (AD). These other agents or treatments may include drugs such as antidepressants including those that are standardly used to treat other various depressive disorders. The agents of the invention may also be combined with corticosteroids, non-steroidal anti-inflammatory compounds, or other agents useful in treating inflammation or alleviating pain. The combined use of the agents of the present invention with these other therapies or treatment modalities may be concurrent, or given sequentially, that is, the two treatments may be divided up such that the antibody or fragment thereof of the present invention may be given prior to or after the other therapy or treatment modality.

[0109] "Slow release formulation" refers to a formulation designed to release a therapeutically effective amount of a drug or other active agent such as a polypeptide or a synthetic compound over an extended period of time, with the result being a reduction in the number of treatments

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necessary to achieve the desired therapeutic effect. In the matter of the present invention, a slow release formulation would decrease the number of treatments necessary to achieve the desired effect in terms of amelioration of, reduction in, or reversal of at least one symptom of AD.

[0110] As used herein, "probe" refers to a labeled oligonucleotide primer, which forms a duplex structure with a sequence in the target nucleic acid, due to complementarity of at least one sequence in the probe with a sequence in the target region.

[0111] A "variant" (v) of polynucleotides or polypeptides, as the term is used herein, are polynucleotides or polypeptides that are different from a reference polynucleotide or polypeptide, respectively. Variant polynucleotides are generally limited so that the nucleotide sequence of the reference and the variant are closely related overall and, in many regions, identical. Changes in the nucleotide sequence of the variant may be silent. That is, they may not alter the amino acid sequence encoded by the polynucleotide. Where alterations are limited to silent changes of this type a variant will encode a polypeptide with the same amino acid sequence as the reference. Alternatively, changes in the nucleotide sequence of the variant may alter the amino acid sequence of a polypeptide encoded by the reference polynucleotide. Such nucleotide changes may result in amino acid substitutions, additions, deletions, fusions, and truncations in the polypeptide encoded by the reference sequence. Variant polypeptides are generally limited so that the sequences of the reference and the variant are that are closely similar overall and, in many regions, identical. For example, a variant and reference polypeptide may differ in amino acid sequence by one or more substitutions, additions, deletions, fusions, and truncations, which may be present or absent in any combination. Such variants can differ in their amino acid composition (e.g. as a result of allelic or natural variation in the amino acid sequence, e.g. as a result of alternative mRNA or pre-mRNA processing, e.g. alternative splicing or limited proteolysis) and in addition, or in the alternative, may arise from differential post-translational modification (e.g., glycosylation, acylation, phosphorylation, isoprenylation, lipidation).

[0112] "Derivative" refers to either a protein or polypeptide that comprises an amino acid sequence of a parent protein or polypeptide that has been altered by the introduction of amino acid residue substitutions, deletions or additions, or a nucleic acid or nucleotide that has been modified by either introduction of nucleotide substitutions or deletions, additions or mutations. The derivative nucleic acid, nucleotide, protein or polypeptide possesses a similar or identical function as the parent polypeptide.

[0113] A "homologue" refers to polypeptides having the same or conserved residues at a corresponding position in their primary, secondary or tertiary structure. The term also extends to two or more nucleotide sequences encoding the homologous polypeptides.

[0114] The term "standard hybridization conditions" refers to salt and temperature conditions substantially equivalent to 5 x SSC and 65°C for both hybridization and wash. However, one

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skilled in the art will appreciate that such "standard hybridization conditions" are dependent on particular conditions including the concentration of sodium and magnesium in the buffer, nucleotide sequence length and concentration, percent mismatch, percent formamide, and the like. Also important in the determination of "standard hybridization conditions" is whether the two sequences hybridizing are RNA-RNA, DNA-DNA or RNA-DNA. Such standard hybridization conditions are easily determined by one skilled in the art according to well known formulae, wherein hybridization is typically 10-20°C below the predicted or determined T_m with washes of higher stringency, if desired.

[0115] Procedures using such conditions of high stringency are as follows. Prehybridization of filters containing DNA is carried out for 15 minutes to overnight at 65°C in buffer composed of 6X SSC, 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.02% PVP, 0.02% Ficoll, 0.02% BSA, and 500 µg/ml denatured salmon sperm DNA. Filters are hybridized for 48 h at 65°C in prehybridization mixture containing 100 µg/ml denatured salmon sperm DNA and 5-20 X 10^6 cpm of ^{32}P-labeled probe. Washing of filters is done at 37°C for 1 h in a solution containing 2X SSC, 0.01% PVP, 0.01% Ficoll, and 0.01% BSA. This is followed by a wash in 0.1X SSC at 50°C for 45 min before autoradiography. Other conditions of high stringency that may be used are well known in the art.

[0116] "AD38" refers to an antibody to amyloid beta 1-42 designated as Colección Española de Cultivos Tipo (CECT) accession number 7167. The amino acid sequence for AD38 is found in SEQ ID NO: 10. The nucleic acid encoding AD38 can be found in SEQ ID NO: 9. The heavy chain of AD38, which is a functional antibody fragment that retains beta amyloid 1-42 binding capacity, has the amino acid sequence set forth in SEQ ID NO: 2. The nucleic acid encoding the heavy chain of AD38 is set forth in SEQ ID NO: 1. The light chain of AD38, which is a functional antibody fragment that retains beta amyloid 1-42 binding capacity, has the amino acid sequence set forth in SEQ ID NO: 4. The nucleic acid encoding the light chain of AD38 is set forth in SEQ ID NO: 3.

[0117] "AD64" refers to an antibody to amyloid beta 1-42 designated as CECT accession number 7168. The amino acid sequence for AD64 is found in SEQ ID NO: 12. The nucleic acid encoding AD64 can be found in SEQ ID NO: 11. The heavy chain of AD64, which is a functional antibody fragment that retains beta amyloid 1-42 binding capacity, has the amino acid sequence set forth in SEQ ID NO: 6. The nucleic acid encoding the heavy chain of AD64 is set forth in SEQ ID NO: 5. The light chain of AD64, which is a functional antibody fragment that retains beta amyloid 1-42 binding capacity, has the amino acid sequence set forth in SEQ ID NO: 8. The nucleic acid encoding the light chain of AD38 is set forth in SEQ ID NO: 7.

[0118] The term "nucleic acid molecule", as used herein, is intended to include DNA molecules and RNA molecules. A nucleic acid molecule may be single-stranded or double-stranded, but preferably is double-stranded DNA.

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[0119] The term "vector," as used herein, is intended to refer to a nucleic acid molecule capable of transporting another nucleic acid to which it has been linked. One type of vector is a "plasmid", which refers to a circular double stranded DNA loop into which additional DNA segments may be ligated. Another type of vector is a viral vector, wherein additional DNA segments may be ligated into the viral genorne. Certain vectors are capable of autonomous replication in a host cell into which they are introduced (e.g., bacterial vectors having a bacterial origin of replication and episomal mammalian vectors). Other vectors (e.g., non-episomal mammalian vectors) can be integrated into the genome of a host cell upon introduction into the host cell, and thereby are replicated along with the host genome. Moreover, certain vectors are capable of directing the expression of genes to which they are operatively linked. Such vectors are referred to herein as "recombinant expression vectors" (or simply, "expression vectors"). In general, expression vectors of utility in recombinant DNA techniques are often in the form of plasmids. In the present specification, "plasmid" and "vector" may be used interchangeably as the plasmid is the most commonly used form of vector. However, the invention is intended to include such other forms of expression vectors, such as viral vectors (e.g., replication defective retroviruses, adenoviruses and adeno-associated viruses), which serve equivalent functions.

[0120] "Operably linked" when describing the relationship between two polynucleotide sequences, means that they are functionally linked to each other. For example, a promoter is operably linked to a coding sequence if it controls the transcription of the sequence. As a regulatory sequence commonly used promoter elements as well as enhancers may be used. Generally, such expression regulation sequences are derived from genes that are expressed primarily in the tissue or cell type chosen. Preferably, the genes from which these expression regulation sequences are obtained are expressed substantially only in the tissue or cell type chosen, although secondary expression in other tissue and/or cell types is acceptable if expression of the recombinant DNA in the transgene in such tissue or cell type is not detrimental to the transgenic animal.

[0121] "Complementary" is understood in its recognized meaning as identifying a nucleotide in one sequence that hybridizes (anneals) to a nucleotide in another sequence according to the rule A→T, U and C→G (and vice versa) and thus "matches" its partner for purposes of this definition. Enzymatic transcription has measurable and well known error rates (depending on the specific enzyme used), thus within the limits of transcriptional accuracy using the modes described herein, in that a skilled practitioner would understand that fidelity of enzymatic complementary strand synthesis is not absolute and that the amplicon need not be completely matched in every nucleotide to the target or template RNA.

[0122] A "reporter gene" refers to a gene whose phenotypic expression is easy to monitor and is used to study promoter activity in different tissues or developmental stages. Recombinant DNA constructs are made in which the reporter gene is attached to a promoter region of particular interest and the construct transfected into a cell or organism. As used herein, a

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"reporter"gene is used interchangeably with the term "marker gene" and is a nucleic acid that is readily detectable and/or encodes a gene product that is readily detectable such as green fluorescent protein (as described in U.S. Patent No. 5,625,048 issued April 29, 1997, and WO 97/26333, published July 24, 1997, the disclosures of each are hereby incorporated by reference herein in their entireties), or red fluorescent protein, or yellow fluorescent protein, or wheat germ agglutinin (WGA) or a WGA-type molecule or luciferase.

[0123] The primers herein are selected to be "substantially" complementary to different strands of a particular target DNA sequence. This means that the primers must be sufficiently complementary to selectively hybridize with their respective strands. Therefore, the primer sequence need not reflect the exact sequence of the template. For example, a non-complementary nucleotide fragment may be attached to the 5' end of the primer, with the remainder of the primer sequence being complementary to the strand. Alternatively, non-complementary bases or longer sequences can be interspersed into the primer, provided that the primer sequence has sufficient complementarity with the sequence of the strand to selectively hybridize therewith and thereby form the template for the synthesis of the extension product.

[0124] Two DNA sequences are "substantially homologous" or have "substantial homology" when at least about 75% (preferably at least about 80%, and most preferably at least about 90 or 95%) of the nucleotides match over the defined length of the DNA sequences. Sequences that are substantially homologous can be identified by comparing the sequences using standard software available in sequence data banks, or in a Southern hybridization experiment under, for example, stringent conditions as defined for that particular system. Defining appropriate hybridization conditions is within the skill of the art. See, e.g., Maniatis et al., supra; DNA Cloning, Vols. I & II, supra; Nucleic Acid Hybridization, supra.

[0125] Two amino acid sequences are "substantially homologous" or show "substantial homology" when at least about 70% of the amino acid residues (preferably at least about 80%, and most preferably at least about 90 or 95%) are identical, or represent conservative substitutions.

[0126] The term "CDR" refers to the complementarity determining region or hypervariable region amino acid residues of an antibody which are responsible for antigen-binding. The hypervariable region or CDRs of the human IgG subtype of antibody comprise amino acid residues from residues 24-34 (L1), 50-56 (L2) and 89-97 (L3) in the light chain variable domain and 31-35 (H1), 50-65 (H2) and 95-102 (H3) in the heavy chain variable domain as described by Kabat et al., Sequences of Proteins of Immunological Interest, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, Md. (1991)] and/or those residues from a hypervariable loop (i.e., residues 26-32 (L1), 50-52 (L2) and 91-96 (L3) in the light chain variable domain and 26-32 (H1), 53-55 (H2) and 96-101 (H3) in the heavy chain variable domain as described by

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[Chothia et al., J. Mol. Biol. 196: 901-917 (1987)]. Framework or FR residues are those variable domain residues other than and bracketing the hypervariable regions.

[0127] The term "specific binding" refers to antibody binding to a predetermined antigen. Typically, the antibody binds with a dissociation constant (K_D) of 10^{-7} M or less, and binds to the predetermined antigen with a K_D that is at least twofold less than its K_D for binding to a non-specific antigen (e.g., BSA, casein, or any other specified polypeptide) other than the predetermined antigen. The phrases "an antibody recognizing an antigen" and "an antibody specific for an antigen" are used interchangeably herein with the term "an antibody which binds specifically to an antigen". As used herein "highly specific" binding means that the relative K_D of the antibody for the specific target epitope is at least 10-fold less than the K_D for binding that antibody to other ligands.

[0128] The affinity or avidity of an antibody for an antigen can be determined experimentally using any suitable method. (See, for example, Berzofsky, et al., "Antibody-Antigen Interactions," In Fundamental Immunology, Paul, W. E., Ed., Raven Press: New York, N.Y. (1984); Kuby, Janis Immunology, W. H. Freeman and Company: New York, N.Y. (1992); and methods described herein). The measured affinity of a particular antibody-antigen interaction can vary if measured under different conditions (e.g., salt concentration, pH). Thus, measurements of affinity and other antigen-binding parameters (e.g., K.sub.D, IC50) are preferably made with standardized solutions of antibody and antigen, and a standardized buffer, such as the buffer described herein.

[0129] A "binding member" as used in the context of the present invention relates primarily to antibodies, and fragments thereof, but may also refer to other non-antibody molecules that bind to the amyloid beta 1-42 protein.

[0130] A DNA "coding sequence" is a double-stranded DNA sequence which is transcribed and translated into a polypeptide in vivo when placed under the control of appropriate regulatory sequences. The boundaries of the coding sequence are determined by a start codon at the 5' (amino) terminus and a translation stop codon at the 3' (carboxyl) terminus. A coding sequence can include, but is not limited to, prokaryotic sequences, cDNA from eukaryotic mRNA, genomic DNA sequences from eukaryotic (e.g., mammalian) DNA, and even synthetic DNA sequences. A polyadenylation signal and transcription termination sequence will usually be located 3' to the coding sequence.

[0131] Transcriptional and translational control sequences are DNA regulatory sequences, such as promoters, enhancers, polyadenylation signals, terminators, and the like, that provide for the expression of a coding sequence in a host cell.

[0132] A "promoter sequence" is a DNA regulatory region capable of binding RNA polymerase in a cell and initiating transcription of a downstream (3' direction) coding sequence. For purposes of defining the present invention, the promoter sequence is bounded at its 3' terminus by the transcription initiation site and extends upstream (5' direction) to include the minimum

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number of bases or elements necessary to initiate transcription at levels detectable above background. Within the promoter sequence will be found a transcription initiation site (conveniently defined by mapping with nuclease S1), as well as protein binding domains (consensus sequences) responsible for the binding of RNA polymerase. Eukaryotic promoters will often, but not always, contain "TATA" boxes and "CAT" boxes. Prokaryotic promoters contain Shine-Dalgarno sequences in addition to the -10 and -35 consensus sequences.

[0133] An "expression control sequence" is a DNA sequence that controls and regulates the transcription and translation of another DNA sequence. A coding sequence is "under the control" of transcriptional and translational control sequences in a cell when RNA polymerase transcribes the coding sequence into mRNA, which is then translated into the protein encoded by the coding sequence.

[0134] A "signal sequence" can be included before the coding sequence. This sequence encodes a signal peptide, N-terminal to the polypeptide, that communicates to the host cell to direct the polypeptide to the cell surface or secrete the polypeptide into the media, and this signal peptide is clipped off by the host cell before the protein leaves the cell. Signal sequences can be found associated with a variety of proteins native to prokaryotes and eukaryotes.

[0135] The term "oligonucleotide," as used herein in referring to the probe of the present invention, is defined as a molecule comprised of two or more ribonucleotides, preferably more than three. Its exact size will depend upon many factors which, in turn, depend upon the ultimate function and use of the oligonucleotide.

[0136] A "small molecule" refers to a composition that has a molecular weight of less than 3 kilodaltons (kDa), and preferably less than 1.5 kilodaltons, and more preferably less than about 1 kilodalton. Small molecules may be nucleic acids, peptides, polypeptides, peptidomimetics, carbohydrates, lipids or other organic (carbon-containing) or inorganic molecules. As those skilled in the art will appreciate, based on the present description, extensive libraries of chemical and/or biological mixtures, often fungal, bacterial, or algal extracts, may be screened with any of the assays of the invention to identify compounds that modulate a bioactivity. A "small organic molecule" is an organic compound (or organic compound complexed with an inorganic compound (e.g., metal)) that has a molecular weight of less than 3 kilodaltons, and preferably less than 1.5 kilodaltons, and more preferably less than about 1 kDa.

[0137] Blood-brain barrier: As used herein, the term "blood-brain barrier" or "BBB" refers to that obstacle to biological transport of drugs, ions, peptides, proteins and toxins that is formed by the membrane properties, structure and tight junctions of brain capillary endothelial cells.

[0138] By "scaffold" is meant a protein used to select or design a protein framework with specific and favorable properties, such as binding. When designing proteins from the scaffold, amino acid residues that are important for the framework's favorable properties are retained, while others residues may be varied. Such a scaffold has less than 50% of the amino acid residues that vary between protein derivatives having different properties and greater than or

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equal to 50% of the residues that are constant between such derivatives. Most commonly, these constant residues confer the same overall three-dimensional fold to all the variant domains, regardless of their properties.

[0139] "Diseases characterized by amyloid deposits" include Alzheimer's disease (AD), both late and early onset. In both diseases, the amyloid deposit comprises a peptide termed Aβ, which accumulates in the brain of affected individuals. Examples of some other diseases characterized by amyloid deposits are SAA amyloidosis, hereditary Icelandic syndrome, multiple myeloma, and spongiform encephalopathies, including mad cow disease, Creutzfeldt Jakob disease, sheep scrapie, and mink spongiform encephalopathy (see Weissmann et al., Curr. Opin. Neurobiol. 7, 695-700 (1997); Smits et al., Veterinary Quarterly 19, 101-105 (1997); Nathanson et al., Am. J. Epidemiol. 145, 959-969 (1997)). The peptides forming the aggregates in these diseases are serum amyloid A, cystantin C, IgG kappa light chain respectively for the first three, and prion protein for the others.

[0140] "Diagnosis" or "screening" refers to diagnosis, prognosis, monitoring, characterizing, selecting patients, including participants in clinical trials, and identifying patients at risk for or having a particular disorder or clinical event or those most likely to respond to a particular therapeutic treatment, or for assessing or monitoring a patient's response to a particular therapeutic treatment.

[0141] The term "affinity maturation strategies" is described by Gonzales-Fernandez et al., "Low antigen dose favors selection of somatic mutants with hallmarks of antibody affinity maturation," Immunology, 93:149-153 (1998).

General Description

[0142] Amyloid beta 1-42 binding agents, e.g., anti-amyloid beta 1-42 antibodies and antigen-binding fragments thereof, pharmaceutical compositions thereof, nucleic acids encoding the aforesaid antibodies, as well as vectors and host cells containing the aforesaid nucleic acid sequences, are disclosed. More particularly, the present invention relates to the isolation and identification of anti-amyloid beta 1-42 antibodies from healthy elderly individuals who show full cognitive capacity and good health in the absence of symptoms of Alzheimer's disease or another neuropathy and who had never received any type of medication for the prevention or treatment of neurodegenerative diseases. More particularly, these patients were a restricted group of individuals who had surpassed the ages considered as critical for the onset of Alzheimer's disease and were chosen as candidates for obtaining anti beta-amyloid 1-42 peptide antibodies. Therefore, a group of healthy nonagenarian elderly subjects was selected in whom, in the absence of immunization, the presence of serum anti beta-amyloid 1-42 peptide antibodies was analyzed by means of performing specific immunoassays, detecting the existence of antibodies against the beta-amyloid 1-42 peptide.

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[0143] More particularly, two of the antibodies of the invention have numerous somatic mutations in their nucleotide sequence in comparison to the corresponding genomic genes (germline), indicating the existence of antigenic maturation processes against the beta-amyloid 1-42 peptide. These facts indicate a competent immune response against the beta peptide and reflect the existence of natural anti beta amyloid antibodies, which do not exhibit harmful effects in healthy elderly patients, with a potential therapeutic application in Alzheimer's disease

[0144] Accordingly, the present invention also relates to human anti beta-amyloid 1-42 peptide recombinant and/or monoclonal antibodies and to obtaining them by means of the isolation, cloning and expression of immunoglobulin genes isolated from a representative portion of the genetic repertoire of immunoglobulins from healthy elderly, and particularly, nonagenarian human donors selectively chosen for having full cognitive capacity and for not exhibiting symptoms of Alzheimer's or another neuropathy.

[0145] Another aspect of the invention provides a method of treating and/or preventing the progression of Alzheimer's disease or the symptoms associated with Alzheimer's disease, such as, but not limited to, memory loss and irritability, comprising administering a therapeutically effective amount of an antibody to amyloid beta 1-42, which may reduce intracellular and/or extracellular amyloid beta levels in the brain. In addition, the invention provides a method of treating or preventing the progression of Alzheimer's disease or the symptoms associated with Alzheimer's disease, such as, but not limited to, memory loss and irritability, comprising administering an agent that prevents or interferes with amyloid beta-induced neurotoxicity.

[0146] Methods of obtaining the aforesaid antibodies, as well as methods for modulating one or more amyloid beta 1-42-associated activities using antibodies that bind to amyloid beta, e.g., human amyloid beta 1-42, are also disclosed. Anti-amyloid beta 1-42 antibodies can be used to mitigate amyloid beta 1-42-mediated disorders, e.g., Alzheimer's disease. Anti-amyloid beta 1-42 antibodies can be used alone or in combination with other therapies used to treat the same or another related disease, such as amyloidosis and other neurodegeneratve disorders for which amyloid beta is thought to play a role.

Anti-Human Amyloid beta 1-42 Antibodies

[0147] Antibodies that are capable of binding to, neutralizing and/or inhibiting one or more amyloid beta 1-42 -associated activities, are useful for treating amyloid beta 1-42 -mediated diseases, such as Alzheimer's disease.

[0148] In one embodiment, the anti-amyloid beta 1-42 antibodies disclosed herein are isolated or purified.

[0149] The antibodies of the invention may be used for treating and/or preventing the progression of Alzheimer's disease or to decrease the symptoms associated with Alzheimer's disease, such as for example, memory loss, irritability or anxiety. While not wishing to be bound by theory, the anti-amyloid beta 1-42 antibodies may be administered in a therapeutically

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effective amount to reduce intracellular and/or extracellular amyloid beta levels in the brain or may interfere with amyloid beta-induced neurotoxicity.

[0150] In one particular embodiment, the anti-amyloid-beta 1-42 antibodies of the invention are human antibodies. In another particular embodiment, the antibodies are polyclonal or monoclonal antibodies. In yet another particular embodiment, the antibodies are single chain antibodies and fragments thereof.

[0151] Conservative substitutions may be contemplated in the antibodies of the invention. These typically include the substitution of one amino acid for another with similar characteristics, e.g., substitutions within the following groups: valine, glycine; glycine, alanine; valine, isoleucine, leucine; aspartic acid, glutamic acid; asparagine, glutamine; serine, threonine; lysine, arginine; phenylalanine, and tyrosine.

[0152] Antibodies, also known as immunoglobulins, are typically tetrameric glycosylated proteins composed of two light (L) chains of approximately 25 kDa each and two heavy (H) chains of approximately 50 kDa each. Two types of light chain, termed lambda and kappa, may be found in antibodies. Depending on the amino acid sequence of the constant domain of heavy chains, immunoglobulins can be assigned to five major classes: A, D, E, G, and M, and several of these may be further divided into subclasses (isotypes), e.g., IgG1, IgG2, IgG3, IgG4, IgA1, and IgA2. Each light chain includes an N-terminal variable (V) domain (VL) and a constant (C) domain (CL). Each heavy chain includes an N-terminal V domain (VH), three or four C domains (CHs), and a hinge region. The CH domain most proximal to VH is designated as CHI. The VH and VL domains consist of four regions of relatively conserved sequences called framework regions (FR1, FR2, FR3, and FR4), which form a scaffold for three regions of hypervariable sequences (complementarity determining regions, CDRs). The CDRs contain most of the residues responsible for specific interactions of the antibody with the antigen. CDRs are referred to as CDR1, CDR2, and CDR3. Accordingly, CDR constituents on the heavy chain are referred to as H1, H2, and H3, while CDR constituents on the light chain are referred to as L1, L2, and L3. CDR3 is typically the greatest source of molecular diversity within the antibody-binding site. H3, for example, can be as short as two amino acid residues or greater than 26 amino acids.

[0153] The subunit structures and three-dimensional configurations of different classes of immunoglobulins are well known in the art. For a review of the antibody structure, see Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory, eds. Harlow et al., 1988. One of skill in the art will recognize that each subunit structure, e.g., a CH, VH, CL, VL, CDR, FR structure, comprises active fragments, e.g., the portion of the VH, VL, or CDR subunit the binds to the antigen, i.e., the antigen-binding fragment, or, e.g., the portion of the CH subunit that binds to and/or activates, e.g., an Fc receptor and/or complement. The CDRs typically refer to the Kabat CDRs, as described in Sequences of Proteins of Immunological Interest, US Department of Health and Human Services (1991), eds. Kabat et al. Another standard for characterizing the antigen binding site is to refer to the hypervariable loops as described by

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Chothia. See, e.g., Chothia, D. et al. (1992) J. Mol. Biol. 227:799-817; and Tomlinson et al. (1995) EMBO J. 14:4628-4638. Still another standard is the AbM definition used by Oxford Molecular's AbM antibody modeling software. See, generally, e.g., Protein Sequence and Structure Analysis of Antibody Variable Domains. In: Antibody Engineering Lab Manual (Ed.: Duebel, S. and Kontermann, R., Springer-Verlag, Heidelberg). Embodiments described with respect to Kabat CDRs can alternatively be implemented using similar described relationships with respect to Chothia hypervariable loops or to the AbM-defined loops.

[0154] Antibody diversity, in a natural system, is created by the use of multiple germline genes encoding variable regions and a variety of somatic events. The somatic events include recombination of variable gene segments with diversity (D) and joining (J) gene segments to make a complete VH region, and the recombination of variable and joining gene segments to make a complete VL region. The recombination process itself can be imprecise, resulting in the loss or addition of amino acids at the V(D)J junctions. These mechanisms of diversity occur in the developing B cell prior to antigen exposure. After antigenic stimulation, the expressed antibody genes in B cells undergo somatic mutation. Based on the estimated number of germline gene segments, the random recombination of these segments, and random VH-VL pairing, up to 1.6×10^7 different antibodies could be produced (Fundamental Immunology, 3rd ed. (1993), ed. Paul, Raven Press, New York, N.Y.). When other processes that contribute to antibody diversity (such as somatic mutation) are taken into account, it is thought that upwards of 10^{10} different antibodies could be generated (Immunoglobulin Genes, 2nd ed. (1995), eds. Jonio et al., Academic Press, San Diego, Calif.). Because of the many processes involved in generating antibody diversity, it is unlikely that independently derived monoclonal antibodies with the same antigen specificity will have identical amino acid sequences.

[0155] Thus, this disclosure provides, inter alia, antibodies, and antigen-binding fragments thereof, that bind to amyloid beta 1-42. The antigen-binding fragments, e.g., structures containing a CDR, will generally be an antibody heavy or light chain sequence, or an active fragment thereof, in which the CDR is placed at a location corresponding to the CDR of naturally occurring VH and VL. The structures and locations of immunoglobulin variable domains may be determined as described in Sequences of Proteins of Immunological Interest, U.S. Department of Health and Human Services (1991), eds. Kabat et al.

[0156] Another aspect of the invention provides for methods of treating and/or preventing the progression of Alzheimer's disease or decreasing one or more symptoms associated with Alzheimer's disease, comprising administering a therapeutically effective amount of an antibody that binds to amyloid beta 1-42. In so doing, one may reduce brain amyloid beta levels. Such reduction of brain amyloid beta levels may be the result of treatment with an antibody, which either prevents the production or generation of amyloid beta (such production or generation occurring through the cleavage of beta amyloid precursor protein or APP), or which prevents the aggregation of amyloid beta fibrils and deposition in cerebral parenchymal tissues and blood

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vessels, or by preventing the formation of the oligomeric forms of amyloid beta, amyloid beta 1-40 or amyloid beta 1-42, or which increases the degradation of amyloid beta, or which increases the clearance of amyloid beta from the brain, or which facilitates the peripheral metabolism and clearance of amyloid beta. In another aspect the method of treating or preventing Alzheimer's disease comprises administering an agent, such as an antibody that might prevent or interfere with amyloid beta induced neurotoxicity. In one particular embodiment, the amyloid beta is amyloid beta 1-42 or fragments thereof. In another particular embodiment of the present invention, it is envisioned that amyloid beta, including amyloid beta 1-42 and oligomeric forms may be damaging (eg. neurotoxic) even if not deposited. That is, the present invention contemplates that amyloid beta 1-42 may be neurotoxic and can lead to neuronal damage or alterations in neurotransmitter levels or perturbations in neurotransmission through interference with signaling systems, the result of which is Alzheimer's disease and the symptoms associated with Alzheimer's disease.

[0157] Antibody molecules (including antigen-binding fragments) disclosed herein, i.e., antibody molecules that bind to amyloid beta 1-42, include, but are not limited to, human single chain (svFv) recombinant antibodies designated as AD38 (SEQ ID NO: 10) (CECT accession number 7167) and AD64, (SEQ ID NO: 12) (CECT accession number 7168), and variants or derivatives thereof. These antibody molecules and their fragments thereof may be useful in preventing or treating the progression of Alzheimer's disease and one or more symptoms associated with Alzheimer's disease.

[0158] The amino acid sequence of the heavy chain of AD38 (CECT accession number 7167) is set forth in SEQ ID NO: 2, whereas the light chain amino acid sequence of AD38 is shown in SEQ ID NO: 4. The nucleic acid sequence encoding a single chain (scFv) recombinant antibody designated as AD38 (CECT accession number 7167) is set forth in SEQ ID NO: 9. The nucleic acid sequences encoding the heavy or light chain fragments from the antibody designated as AD38 (CECT accession number 7167) are set forth in SEQ ID NOs: 1 and 3, respectively.

[0159] The amino acid sequences of the heavy chain of AD64 (CECT accession number 7168) is set forth in SEQ ID NO: 6 and the light chain amino acid sequence of AD64 is set forth in SEQ ID NO: 8. The nucleic acid sequence encoding a single chain (scFv) recombinant antibody designated as AD64 (CECT accession number 7168) is set forth in SEQ ID NO: 11. The nucleic acid sequences encoding the heavy or light chain fragments from the antibody designated as AD64 (CECT accession number 7168) are set forth in SEQ ID NOs: 5 and 7, respectively.

[0160] In a further particular embodiment, the heavy chain variable domain CDR1, CDR2 or CDR3 of antibody AD38 as defined in Figure 8, the light chain variable domain CDR1, CDR2 or CDR3 of antibody AD38 as defined in Figure 9, the heavy chain variable domain CDR1, CDR2 or CDR3 of antibody AD64 as defined in Figure 10, and the light chain variable domain CDR1,

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CDR2 or CDR3 of antibody AD64 as defined in Figure 11, are understood as part of any of the amino acid sequences defined in sequences SEQ ID NO: 2, SEQ ID NO: 4, SEQ ID NO: 6 and SEQ ID NO: 8. It is to be understood that the CDR regions of any of the above-noted antibodies may be genetically engineered into any other protein or antibody molecule or scaffold protein, as described herein, for increasing their stability, e.g. their half-life, or for targeting to the brain or for decreasing their immunogenicity if necessary.

[0161] The CDRs contain most of the residues responsible for specific interactions with the antigen, and are contained within the VH and VL domains, i.e., the heavy chain variable region and the light chain variable region, respectively. Exemplary antibodies include at least one CDR comprising an amino acid sequence selected from the amino acid sequences set forth in SEQ ID NOs: 2, 4, 6, and 8, or selected residues, particularly amyloid beta 1-42 contact residues from such CDRs. Such antibodies may also bind to amyloid beta 1-42 and, e.g., interfere with the formation of a functional amyloid beta 1-42 signaling complex. An antibody may include one or more CDRs of the VL chain or one or more CDRs of the VH chain.

[0162] Also described above, an antigen-binding fragment may be an Fv fragment, which includes VH and VL domains. Thus, an Fv fragment of AD38 or AD64 antibodies may constitute an antibody described herein. It may bind to amyloid beta 1-42 and interfere with the formation of amyloid beta 1-42 or interfere with the neurotoxic properties of amyloid beta 1-42. Other fragments include the Fv fragment, e.g., scFv fragments, Fab fragments, and F(ab')2 fragments of the amyloid beta 1-42 antibodies described herein or of an antibody that includes one or more CDRs having an amino acid sequence selected from the amino acid sequences set forth in SEQ ID NOs: 2, 4, 6, or 8.

[0163] Other such antibody molecules may be produced by methods known to those skilled in the art. For example, the sequences described herein may be used to isolate the antigenic portion of the beta amyloid protein for which the antibodies are specific and afterwards monoclonal antibodies specific for that region of the beta amyloid protein or amyloid beta 1-42 may be produced by generation of hybridomas in accordance with known methods. Hybridomas formed in this manner are then screened using standard methods, such as enzyme-linked immunosorbent assay (ELISA) and surface plasmon resonance (Biacore.TM.) analysis, to identify one or more hybridomas that produce an antibody that specifically binds with amyloid beta 1-42, and neutralizes or inhibits one or more of the amyloid beta 1-42 activities. Recombinant amyloid beta 1-42, naturally occurring amyloid beta 1-42 (i.e., the processed mature form of amyloid beta 1-42), any variants thereof, and antigenic peptide fragments of amyloid beta 1-42 may be used as the immunogen.

[0164] According to the invention, techniques described for the production of single chain antibodies (U.S. Patent Nos. 5,476,786 and 5,132,405 to Huston; U.S. Patent 4,946,778) can be adapted to produce other e.g., amyloid beta-specific single chain antibodies. An additional embodiment of the invention utilizes the techniques described for the construction of Fab

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expression libraries (Huse et al., Science, 246:1275-1281 (1989)) to allow rapid and easy identification of monoclonal Fab fragments with the desired specificity for amyloid beta 1-42, or its derivatives, or analogs.

[0165] An antigenic peptide fragment of amyloid beta 1-42 can comprise at least 7 continuous amino acid residues and encompasses an epitope such that an antibody raised against the peptide forms a specific immune complex with amyloid beta 1-42. Preferably, the antigenic peptide comprises at least 10 amino acid residues, more preferably at least 15 amino acid residues, even more preferably at least 20 amino acid residues, even more preferably at least 30 amino acid residues and most preferably at least 40 amino acid residues.

[0166] As an alternative to preparing monoclonal antibody-secreting hybridomas, a monoclonal antibody may be identified and isolated by screening a recombinant combinatorial immunoglobulin library (e.g., an antibody phage display library) with amyloid beta 1-42, including variants and/or portions thereof, to thereby isolate immunoglobulin library members that bind to amyloid beta 1-42. Techniques and commercially available kits for generating and screening phage display libraries are known to those skilled in the art. Additionally, examples of methods and reagents particularly amenable for use in generating and screening antibody display include those described in U.S. Pat. No. 5,658,727, U.S. Pat. No. 5,667,988, and U.S. Pat. No. 5,885,793.

[0167] Polyclonal sera and antibodies described herein may be produced by immunizing a suitable subject with amyloid beta 1-42, its variants and/or portions thereof. The antibody titer in the immunized subject may be monitored over time by standard techniques, such as an Enzyme Linked immunosorbent Assay (ELISA), using immobilized amyloid beta 1-42 or other marker proteins (e.g., FLAG). Antibodies may be isolated from an animal or culture media. A variety of methods can be used to purify antibodies including well-known techniques, such as use of protein A chromatography to obtain an IgG fraction.

[0168] Certain embodiments comprise the VH and/or VL domain of the Fv fragment of the anti-amyloid beta 1-42 antibody. Fragments of antibodies e.g., Fab, F(ab')2, Fd, and dAb fragments, may be produced by cleavage of the antibodies or by recombinant engineering. For example, immunologically active Fab and F(ab')2 fragments may be generated by treating the antibodies with an enzyme such as pepsin.

[0169] Further embodiments comprise one or more complementarity determining regions (CDRs) of any of these VH and VL domains.

[0170] The VH and VL domains described herein, in certain embodiments, can be germlined, i.e., the framework regions (FRs) of these domains may be changed using conventional molecular biology techniques to match human germline genes or the consensus amino acid sequences of human germline gene products, at one or more positions (e.g., at least 70, 80, 85, 90, 95, 97, 98, or 99% of framework positions). In other embodiments, the framework sequences remain diverged from the germline.

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[0171] Human germline sequences, for example, are disclosed in Tomlinson, I. A. et al. (1992) J. Mol. Biol. 227:776-798; Cook, G. P. et al. (1995) Immunol. Today Vol. 16 (5): 237-242; Chothia, D. et al. (1992) J. Mol. Biol. 227:799-817; and Tomlinson et al. (1995) EMBO J. 14:4628-4638. The V BASE directory provides a comprehensive directory of human immunoglobulin variable region sequences (compiled by Tomlinson, I. A. et al. MRC Centre for Protein Engineering, Cambridge, UK). These sequences can be used as a source of human sequence, e.g., for framework regions and CDRs. Consensus human framework regions can also be used, e.g., as described in U.S. Pat. No. 6,300,064.

[0172] Additionally, chimeric, humanized, and single-chain antibodies described herein, comprising both human and nonhuman portions, may be produced using standard recombinant DNA techniques. Humanized antibodies may also be produced using transgenic mice that express human heavy and light chain genes, but are incapable of expressing the endogenous mouse immunoglobulin heavy and light chain genes.

[0173] Additionally, the antibodies described herein also include those that bind to amyloid beta 1-42, interfere with the formation or function of amyloid beta 1-42 signaling, and have mutations in the constant regions of the heavy and light chains. It is sometimes desirable to mutate and inactivate certain fragments of the constant region. For example, mutations in the heavy constant region can be made to produce antibodies with reduced binding to the Fc receptor (FcR) and/or complement; such mutations are well known in the art.

[0174] Exemplary antibodies can include variants of the sequences provided that retain antigen-binding ability. Such variants may be derived from the provided sequences using techniques well known in the art. Amino acid substitutions, deletions, or additions, can be made in either the FRs or in the CDRs. While changes in the framework regions are usually designed to improve stability and reduce immunogenicity of the antibody, changes in the CDRs are usually designed to increase affinity of the antibody for its target. Such affinity-increasing changes are typically determined empirically by altering the CDR region and testing the antibody. Such alterations can be made according to the methods described in Antibody Engineering, 2nd. ed. (1995), ed. Borrebaeck, Oxford University Press.

[0175] An exemplary method for making a VH domain, which is an amino acid sequence variant of a VH domain, comprises a step of adding, deleting, substituting or inserting one or more amino acids in the amino acid sequence of the VH domain, optionally combining the VH domain with one or more VL domains, and testing the VH domain or VH/VL combination or combinations for specific binding to amyloid beta 1-42, and (preferably) testing the ability of such antigen-binding domain to modulate one or more amyloid beta 1-42 associated activities or signaling. An analogous method may be employed in which one or more sequence variants of a VL domain disclosed herein are combined with one or more VH domains.

[0176] A further aspect of the invention provides a method of preparing an antigen-binding fragment that specifically binds to amyloid beta 1-42. The method comprises: (a) providing a

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starting repertoire of nucleic acids encoding a VH domain that either includes a CDR3 to be replaced or lacks a CDR3 encoding region; (b) combining the repertoire with a donor nucleic acid encoding a donor CDR, such that the donor nucleic acid is inserted into the CDR3 region in the repertoire so as to provide a product repertoire of nucleic acids encoding a VH domain; (c) expressing the nucleic acids of the product repertoire; (d) selecting a specific antibody or antigen-binding fragment specific for amyloid beta 1-42; and (e) recovering the specific antibody or antigen-binding fragment or nucleic acid encoding it.

[0177] Moreover, the present invention provides for a process of obtaining antibodies or functional fragments thereof with beta-amyloid 1-42 peptide-binding capacity from the genetic repertoire of immunoglobulins isolated from healthy elderly, and particularly, nonagenarian donors, the process comprising the steps of:

A) purifying B cells from a blood sample or from a bone marrow sample, said sample being obtained from a subject fulfilling the following criteria;
   (i) being 60 years of age or older;
   (ii) having full cognitive capacity and good health;
   (iii) having no symptoms of Alzheimer's disease or another neuropathy;
   (iv) not having previously received any medication for the prevention or treatment of neurodegenerative diseases; and
   (v) possessing detectable anti beta-amyloid 1-42 peptide antibodies in the blood serum;
B) obtaining the immunoglobulin gene repertoire for said anti beta-amyloid 1-42 peptide antibodies from said B cells;
C) using said repertoire to express said antibodies.

[0178] In a particular embodiment, the method for obtaining the immunoglobulin gene repertoire for the anti beta-amyloid 1-42 peptide antibodies from the B cells in step B) is carried out by the following steps:

(i) obtaining mRNA from the said B cells;
(ii) obtaining cDNA from the mRNA of step (i); and;
(iii) using a primer extension reaction to amplify from said cDNA the fragments corresponding to the heavy chains (HC) and the kappa light chains (LC) of said anti beta-amyloid 1-42 peptide antibodies.

In a preferred embodiment, the B cells are peripheral blood B cells obtained from a blood sample.

Other methodologies for obtaining the immunoglobulin gene repertoire from immune cells, for example peripheral blood B cells, are known in the art .

[0179] In another embodiment, an analogous method may be employed in which a VL CDR3 (i.e., L3) described herein is combined with a repertoire of nucleic acids encoding a VL domain, which either includes a CDR3 to be replaced or lacks a CDR3-encoding region. A coding

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sequence of a CDR may be introduced into a repertoire of variable domains lacking a CDR (e.g., CDR3), using recombinant DNA technology. For example, Marks et al. (Bio/Technology (1992) 10:779-83) describes methods of producing repertoires of antibody variable domains in which consensus primers directed at or adjacent to the 5' end of the variable domain area are used in conjunction with consensus primers to the third framework region of human VH genes to provide a repertoire of VH variable domains lacking a CDR3. The repertoire may be combined with a CDR3 of a particular antibody. Using analogous techniques, the CDR3-derived sequences may be shuffled with repertoires of VH or VL domains lacking a CDR3, and the shuffled complete VH or VL domains combined with a cognate VL or VH domain to provide specific antigen-binding fragments. The repertoire may then be displayed in a suitable host system such as the phage display system of WO 92/01047, so that suitable antigen-binding fragments can be selected. Analogous shuffling or combinatorial techniques are also disclosed by Stemmer (Nature (1994) 370:389-91). A further alternative is to generate altered VH or VL regions using random mutagenesis of one or more selected VH and/or VL genes to generate mutations within the entire variable domain. See, e.g., Gram et al. Proc. Nat. Acad. Sci. U.S.A. (1992) 89:3576-80.

[0180] Another method that may be used is to direct mutagenesis to CDR regions of VH or VL genes. Such techniques are disclosed by, e.g., Barbas et al. (Proc. Nat. Acad. Sci. U.S.A. (1994) 91:3809-13) and Schier et al. (J. Mol. Biol. (1996) 263:551-67). Similarly, one or more, or all three CDRs may be grafted into a repertoire of VH or VL domains, or even some other scaffold (such as a fibronectin domain). The resulting protein is evaluated for ability to bind to amyloid beta 1-42.

[0181] In one particular embodiment, a binding protein that binds to a target is modified, e.g., by mutagenesis, to provide a pool of modified binding proteins. The modified binding proteins are then evaluated to identify one or more altered binding proteins which have altered functional properties (e.g., improved binding, improved stability, lengthened stability in vivo). In one implementation, display library technology is used to select or screen the pool of modified binding proteins. Higher affinity binding proteins are then identified from the second library, e.g., by using higher stringency or more competitive binding and washing conditions. Other screening techniques can also be used.

[0182] In some embodiments, the mutagenesis is targeted to regions known or likely to be at the binding interface. If, for example, the identified binding proteins are antibodies, then mutagenesis can be directed to the CDR regions of the heavy or light chains as described herein. Further, mutagenesis can be directed to framework regions near or adjacent to the CDRs, e.g., framework regions, particular within 10, 5, or 3 amino acids of a CDR junction. In the case of antibodies, mutagenesis can also be limited to one or a few of the CDRs, e.g., to make step-wise improvements.

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[0183] In another embodiment, mutagenesis is used to make an antibody more similar to one or more germline sequences. One exemplary germlining method can include: identifying one or more germline sequences that are similar (e.g., most similar in a particular database) to the sequence of the isolated antibody. Then mutations (at the amino acid level) can be made in the isolated antibody, either incrementally, in combination, or both. For example, a nucleic acid library that includes sequences encoding some or all possible germline mutations is made. The mutated antibodies are then evaluated, e.g., to identify an antibody that has one or more additional germline residues relative to the isolated antibody and that is still useful (e.g., has a functional activity). In one embodiment, as many germline residues are introduced into an isolated antibody as possible.

[0184] In yet another embodiment, mutagenesis is used to substitute or insert one or more germline residues into a CDR region. For example, the germline CDR residue can be from a germline sequence that is similar (e.g., most similar) to the variable region being modified. After mutagenesis, activity (e.g., binding or other functional activity) of the antibody can be evaluated to determine if the germline residue or residues are tolerated. Similar mutagenesis can be performed in the framework regions.

[0185] Selecting a germline sequence can be performed in different ways. For example, a germline sequence can be selected if it meets a predetermined criteria for selectivity or similarity, e.g., at least a certain percentage identity, e.g., at least 75, 80, 85, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, or 99.5% identity. The selection can be performed using at least 2, 3, 5, or 10 germline sequences. In the case of CDR1 and CDR2, identifying a similar germline sequence can include selecting one such sequence. In the case of CDR3, identifying a similar germline sequence can include selecting one such sequence, but may including using two germline sequences that separately contribute to the amino-terminal portion and the carboxy-terminal portion. In other implementations more than one or two germline sequences are used, e.g., to form a consensus sequence.

[0186] The invention also provides methods of mutating antibodies to optimize their affinity, selectivity, binding strength or other desirable property. A mutant antibody refers to an amino acid sequence variant of an antibody. In general, one or more of the amino acid residues in the mutant antibody is different from what is present in the reference antibody. Such mutant antibodies necessarily have less than 100% sequence identity or similarity with the reference amino acid sequence. In general, mutant antibodies have at least 75% amino acid sequence identity or similarity with the amino acid sequence of either the heavy or light chain variable domain of the reference antibody. Preferably, mutant antibodies have at least 80%, more preferably at least 85%, even more preferably at least 90%, and most preferably at least 95% amino acid sequence identity or similarity with the amino acid sequence of either the heavy or light chain variable domain of the reference antibody. One method of mutating antibodies involves "affinity maturation" using phage display.

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[0187] "Affinity maturation" using phage display refers to a process described in Lowman et al., Biochemistry 30(45): 10832-10838 (1991), see also Hawkins et al., J. Mol Biol. 254: 889-896 (1992). While not strictly limited to the following description, this process can be described briefly as involving mutation of several antibody hypervariable regions in a number of different sites with the goal of generating all possible amino acid substitutions at each site. The antibody mutants thus generated are displayed in a monovalent fashion from filamentous phage particles as fusion proteins. Fusions are generally made to the gene III product of M13. The phage expressing the various mutants can be cycled through several rounds of selection for the trait of interest, e.g. binding affinity or selectivity. The mutants of interest are isolated and sequenced. Such methods are described in more detail in U.S. Pat. No. 5,750,373, U.S. Pat. No. 6,290,957 and Cunningham, B. C. et al., EMBO J. 13(11), 2508-2515 (1994).

[0188] Any cloning procedure used by one of skill in the art can be employed to make the expression vectors used in such affinity maturation/phage display procedures. For example, one of skill in the art can readily employ known cloning procedures to fuse a nucleic acid encoding an antibody hypervariable region to a nucleic acid encoding a phage coat protein. See, e.g., Sambrook et al., Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory, N.Y., 1989; Sambrook et al., Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory, N.Y., 2001.

[0189] In another embodiment, an antibody or fragment thereof has CDR sequences that differ only insubstantially from those of the antibodies described herein. Insubstantial differences include minor amino acid changes, such as substitutions of 1 or 2 out of any of typically 5-7 amino acids in the sequence of a CDR, e.g., a Chothia or Kabat CDR. Typically an amino acid is substituted by a related amino acid having similar charge, hydrophobic, or stereochemical characteristics. Such substitutions would be within the ordinary skills of an artisan. Unlike in CDRs, more substantial changes in structure framework regions (FRs) can be made without adversely affecting the binding properties of an antibody. Changes to FRs include, but are not limited to, humanizing a nonhuman-derived framework or engineering certain framework residues that are important for antigen contact or for stabilizing the binding site, e.g., changing the class or subclass of the constant region, changing specific amino acid residues which might alter an effector function such as Fc receptor binding (Lund et al. (1991) J. Immunol. 147:2657-62; Morgan et al. (1995) Immunology 86:319-24), or changing the species from which the constant region is derived. Antibodies may have mutations in the CH2 region of the heavy chain that reduce or alter effector function, e.g., Fc receptor binding and complement activation. For example, antibodies may have mutations such as those described in U.S. Pat. Nos. 5,624,821 and 5,648,260. Antibodies may also have mutations that stabilize the disulfide bond between the two heavy chains of an immunoglobulin, such as mutations in the hinge region of IgG4, as disclosed in the art (e.g., Angal et al. (1993) Mol. Immunol. 30:105-08).

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[0190] The amyloid beta 1-42 binding proteins can be in the form of intact antibodies, fragments of antibodies, e.g., Fab, F(ab')2, Fd, dAb, and scFv fragments, and intact antibodies and fragments that have been mutated either in their constant and/or variable region (e.g., mutations to produce chimeric, partially humanized, or fully humanized antibodies, as well as to produce antibodies with a desired trait, e.g., enhanced amyloid beta 1-42 binding and/or reduced FcR binding).

[0191] In some embodiments, a substantial portion of an immunoglobulin variable domain can comprise at least one of the CDR regions and, optionally, their intervening framework regions from the variable regions as set out herein. The portion will also include at least about 50, 60, 70, 80, 85, 87, 88, 90, 92, 94, 95, 96, 97, 98% of either or both of FR1 and FR4. For example, the portion which may be contiguous or non-contiguous may include the C-terminal 50% of FR1 and the N-terminal 50% of FR4. Additional residues at the N-terminal or C-terminal end of the substantial part of the variable domain may be those not normally associated with naturally occurring variable domain regions. For example, construction of specific antigen-binding fragments made by recombinant DNA techniques may result in the introduction of N- or C-terminal residues encoded by linkers introduced to facilitate cloning or other manipulation steps. Other manipulation steps include the introduction of linkers to join variable domains described herein to further protein sequences, including immunoglobulin heavy chains, other variable domains (e.g., in the production of diabodies) or protein labels as discussed in more detail below.

[0192] The invention also encompasses binding fragments containing a single variable domain derived from either VH or VL domain sequences, especially VH domains. In the case of either of the single-chain specific binding domains, these domains may be used to screen for complementary domains capable of forming a two-domain specific antigen-binding domain capable of binding amyloid beta 1-42. This may be achieved by phage display screening methods using the so-called hierarchical dual combinatorial approach (as disclosed in, e.g., WO 92/01047) in which an individual colony containing either an H or L chain clone is used to infect a complete library of clones encoding the other chain (L or H) and the resulting two-chain specific antigen-binding domain is selected in accordance with phage display techniques such as those described in that reference. This technique is also disclosed in Marks et al., supra. Antibodies can be conjugated by chemical methods with radionuclides, drugs, macromolecules, or other agents, or can be made as fusion proteins comprising one or more CDRs described herein.

[0193] An antibody fusion protein contains a VH-VL pair where one of these chains (usually VH) and another protein are synthesized as a single polypeptide chain. These types of products differ from antibodies in that they generally have an additional functional element: e.g., the active moiety of a small molecule or the principal molecular structural feature of the conjugated or fused macromolecule.

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[0194] In addition to the changes to the amino acid sequence noted above, the antibodies can be glycosylated, pegylated, or linked to albumin or a nonproteinaceous polymer. For instance, the presently disclosed antibodies may be linked to one of a variety of nonproteinaceous polymers, e.g., polyethylene glycol, polypropylene glycol, or polyoxyalkylenes, in the manner set forth in U.S. Pat. Nos. 4,640,835; 4,496,689; 4,301,144; 4,670,417; 4,791,192; or 4,179,337. The antibodies are chemically modified by covalent conjugation to a polymer to increase their circulating half-life, for example. Exemplary polymers, and methods to attach them to peptides, are also shown in U.S. Pat. Nos. 4,766,106; 4,179,337; 4,495,285; and 4,609,546.

[0195] In other embodiments, the antibody may be modified to have an altered glycosylation pattern (e.g., altered from the original or native glycosylation pattern). As used in this context, "altered" means having one or more carbohydrate moieties deleted, and/or having one or more glycosylation sites added to the original antibody. Addition of glycosylation sites to the presently disclosed antibodies may be accomplished by altering the amino acid sequence to contain glycosylation site consensus sequences; such techniques are well known in the art. Another means of increasing the number of carbohydrate moieties on the antibodies is by chemical or enzymatic coupling of glycosides to the amino acid residues of the antibody. These methods are described in, e.g., WO 87/05330, and Aplin and Wriston ((1981) CRC Crit. Rev. Biochem. 22:259-306). Removal of any carbohydrate moieties present on the antibodies may be accomplished chemically or enzymatically as described in the art (Hakimuddin et al. (1987) Arch. Biochem. Biophys. 259:52; Edge et al. (1981) Anal. Biochem. 118:131; and Thotakura et al. (1987) Meth. Enzymol. 138:350). See, e.g., U.S. Pat. No. 5,869,046 for a modification that increases in vivo half life by providing a salvage receptor binding epitope.

[0196] In addition, combinatorial libraries of antibodies have been generated in bacteriophage lambda expression systems which may be screened as bacteriophage plaques or as colonies of lysogens (Huse et al. (1989) Science 246: 1275; Caton and Koprowski (1990) Proc. Natl. Acad. Sci. (U.S.A.) 87: 6450; Mullinax et al (1990) Proc. Natl. Acad. Sci. (U.S.A.) 87: 8095; Persson et al. (1991) Proc. Natl. Acad. Sci. (U.S.A.) 88: 2432). Various embodiments of bacteriophage antibody display libraries and lambda phage expression libraries have been described (Kang et al. (1991) Proc. Natl. Acad. Sci. (U.S.A.) 88: 4363; Clackson et al. (1991) Nature 352: 624; McCafferty et al. (1990) Nature 348: 552; Burton et al. (1991) Proc. Natl. Acad. Sci. (U.S.A.) 88: 10134; Hoogenboom et al. (1991) Nucleic Acids Res. 19: 4133; Chang et al. (1991) J. Immunol. 147: 3610; Breitling et al. (1991) Gene 104: 147; Marks et al. (1991) J. Mol. Biol. 222: 581; Barbas et al. (1992) Proc. Natl. Acad. Sci. (U.S.A.) 89: 4457; Hawkins and Winter (1992) J. Immunol. 22: 867; Marks et al. (1992) Biotechnology 10: 779; Marks et al. (1992) J. Biol. Chem. 267: 16007; Lowman et al (1991) Biochemistry 30: 10832; Lerner et al. (1992) Science 258: 1313, incorporated herein by reference). Typically, a bacteriophage antibody display library is screened with an antigen (e.g., polypeptide, carbohydrate, glycoprotein, nucleic acid) that is

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immobilized (e.g., by covalent linkage to a chromatography resin to enrich for reactive phage by affinity chromatography) and/or labeled (e.g., to screen plaque or colony lifts).

[0197] One particularly advantageous approach has been the use of so-called single-chain fragment variable (scFv) libraries (Marks et al. (1992) Biotechnology 10: 779; Winter G and Milstein C (1991) Nature 349: 293; Clackson et al. (1991) op.cit.; Marks et al. (1991) J. Mol. Biol. 222: 581; Chaudhary et al. (1990) Proc. Natl. Acad. Sci. (USA) 87: 1066; Chiswell et al. (1992) TIBTECH 10: 80; McCafferty et al. (1990) op.cit.; and Huston et al. (1988) Proc. Natl. Acad. Sci. (USA) 85: 5879). Various embodiments of scFv libraries displayed on bacteriophage coat proteins have been described.

[0198] Beginning in 1988, single-chain analogues of Fv fragments and their fusion proteins have been reliably generated by antibody engineering methods. The first step generally involves obtaining the genes encoding V_H and V_L regions with desired binding properties; these V genes may be isolated from a specific hybridoma cell line, selected from a combinatorial V-gene library, or made by V gene synthesis. The single-chain Fv is formed by connecting the component V genes with an oligonucleotide that encodes an appropriately designed linker peptide, such as $(Gly\text{-}Gly\text{-}Gly\text{-}Gly\text{-}Ser)_3$ or equivalent linker peptide(s). The linker bridges the C-terminus of the first V region and N-terminus of the second, ordered as either V_H -linker-V_L or V_L -linker-V_H. In principle, the scFv binding site can faithfully replicate both the affinity and specificity of its parent antibody combining site.

[0199] Thus, scFv fragments are comprised of V_H and V_L regions linked into a single polypeptide chain by a flexible linker peptide. After the scFv genes are assembled, they are cloned into a phagemid and expressed at the tip of the M13 phage (or similar filamentous bacteriophage) as fusion proteins with the bacteriophage pIII (gene 3) coat protein. Enriching for phage expressing an antibody of interest is accomplished by panning the recombinant phage displaying a population scFv for binding to a predetermined epitope (e.g., target antigen, receptor).

[0200] The linked polynucleotide of a library member provides the basis for replication of the library member after a screening or selection procedure, and also provides the basis for the determination, by nucleotide sequencing, of the identity of the displayed peptide sequence or V_H and V_L amino acid sequence. The displayed peptide(s) or single-chain antibody (e.g., scFv) and/or its V_H and V_L regions or their CDRs can be cloned and expressed in a suitable expression system. Often polynucleotides encoding the isolated V_H and V_L regions will be ligated to polynucleotides encoding constant regions (C_H and C_L) to form polynucleotides encoding complete antibodies (e.g., chimeric or fully-human), antibody fragments, and the like. Often polynucleotides encoding the isolated CDRs will be grafted into polynucleotides encoding a suitable variable region framework (and optionally constant regions) to form polynucleotides encoding complete antibodies (e.g., humanized or fully-human), antibody fragments, and the like. Antibodies can be used to isolate preparative quantities of the antigen by immunoaffinity

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chromatography. Various other uses of such antibodies are to diagnose and/or stage disease (e.g., neoplasia), and for therapeutic application to treat disease, such as for example: neoplasia, HIV infections and the like.

[0201] Various methods have been reported for increasing the combinatorial diversity of a scFv library to broaden the repertoire of binding species. The use of PCR (polymerase chain reaction) has permitted the variable regions to be rapidly cloned either from a specific hybridoma source or as a gene library from non-immunized cells, affording combinatorial diversity in the assortment of V_H and V_L cassettes which can be combined. Furthermore, the V_H and V_L cassettes can themselves be diversified, such as by random, pseudorandom, or directed mutagenesis. Typically, V_H and V_L cassettes are diversified in or near the complementarity-determining regions (CDRs), often the third CDR, CDR3. Enzymatic inverse PCR mutagenesis has been shown to be a simple and reliable method for constructing relatively large libraries of scFv site-directed mutants (Stemmer et al. (1993) Biotechniques 14: 256), as has error-prone PCR and chemical mutagenesis (Deng et al. (1994) J. Biol. Chem. 269: 9533). Riechmann et al. [Biochemistry 32: 8848; (1993)] showed semirational design of an antibody scFv fragment using site-directed randomization by degenerate oligonucleotide PCR and subsequent phage display of the resultant scFv mutants.

[0202] Selection of antibodies specific for amyloid beta 1-42 is based on binding affinity to amyloid beta 1-42 and may be determined by various well-known immunoassays including, enzyme-linked immunosorbent, chemiluminescent, immunofluorescent, immunohistochemical, radioimmunoassay, and immunoprecipitation assays and the like which may be performed in vitro, in vivo or in situ. The standard techniques known in the art for immunoassays are described in "Methods in Immunodiagnosis", 2nd Edition, Rose and Bigazzi, eds. John Wiley & Sons, 1980; Campbell et al., "Methods and Immunology", W.A. Benjamin, Inc., 1964; and Oellerich, M. (1984) J. Clin. Chem. Clin. Biochem. **22**:895-904.

[0203] Antibodies described herein may also be tagged with a detectable or functional label. Detectable labels include radiolabels such as ^{131}I or ^{99}Tc, which may be attached to antibodies described herein using conventional chemistry known in the art. Labels also include enzyme labels such as horseradish peroxidase or alkaline phosphatase. Labels further include chemical moieties such as biotin, which may be detected via binding to a specific cognate detectable moiety, e.g., labeled avidin.

[0204] The binding characteristics of an antibody disclosed herein may be measured by any suitable methods, including the following methods: Biacore analysis, Enzyme Linked Immunosorbent Assay (ELISA), x-ray crystallography, sequence analysis and scanning mutagenesis as described in the Examples below, and other methods that are well known in the art.

[0205] The antibodies of the present invention may be formulated, e.g., as a pharmaceutical composition.

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[0206] The anti-amyloid beta 1-42 antibodies disclosed herein are also useful for isolating, purifying, and/or detecting amyloid beta 1-42 in supernatant, cellular lysate, or on the cell surface or in tissue. Antibodies disclosed herein can be used diagnostically to monitor amyloid beta 1-42 protein levels as part of a clinical testing procedure. The present disclosure also provides novel isolated and purified polynucleotides and polypeptides related to novel antibodies directed against human amyloid beta 1-42. The genes, polynucleotides, proteins, and polypeptides disclosed herein include, but are not limited to, a human antibody to amyloid beta 1-42 and fragments, variants or derivatives thereof.

Anti-Amyloid Beta 1-42 Antibody Polynucleotides and Polypeptides

[0207] The disclosure provides purified and isolated polynucleotides encoding the anti-amyloid beta 1-42 antibodies and fragments thereof. The isolated nucleic acids of the present invention encoding the anti amyloid beta 1-42 antibodies can be made using (a) recombinant methods, (b) synthetic techniques, (c) purification techniques, or combinations thereof, as well-known in the art. DNA encoding the monoclonal antibodies is readily isolated and sequenced using methods known in the art (e.g., by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of murine antibodies). Where a hybridoma is produced, such cells can serve as a source of such DNA. Alternatively, using display techniques such as phage or ribosomal display libraries, the selection of the binder and the nucleic acid is simplified. After phage selection, the antibody coding regions from the phage can be isolated and used to generate whole antibodies, including human antibodies, or any other desired antigen binding fragment, and expressed in any desired host, including mammalian cells, insect cells, plant cells, yeast, and bacteria, e.g., as described below. Techniques to recombinantly produce Fab, Fab' and F(ab')2 fragments can also be employed using methods known in the art such as those disclosed in International publication No. WO 92/22324; Mullinax et al., 1992, BioTechniques 12(6):864-869; Sawai et al., 1995, AJR134:26-34; and Better et al., 1988, Science 240:1041-1043 (said references entirely incorporated by reference).

[0208] Once isolated, the DNA may be placed into expression vectors, which are then transfected into host cells such as E. coli cells, simian COS cells, Chinese Hamster Ovary (CHO) cells, or myeloma cells that do not otherwise produce antibody protein, to obtain the synthesis of monoclonal antibodies in the recombinant host cells. Review articles on recombinant expression in bacteria of DNA encoding the antibody include Skerra et al., Curr. Opinion in Immunol., 5:256-262 (1993) and Pluckthun, Immunol. Revs. 130:151-188 (1992).

[0209] Known methods of DNA or RNA amplification include, but are not limited to, polymerase chain reaction (PCR) and related amplification processes. PCR and other *in vitro* amplification methods can also be useful, for example, to clone nucleic acid sequences that code for proteins to be expressed, to make nucleic acids to use as probes for detecting the presence of the desired mRNA in samples, for nucleic acid sequencing, or for other purposes. Examples of

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techniques sufficient to direct persons of skill through in vitro amplification methods are found in Berger, supra, Sambrook, supra, and Mullis, et al., U.S. Pat. No. 4,683,202 (1987); and Innis, et al., PCR Protocols A Guide to Methods and Applications, Eds., Academic Press Inc., San Diego, Calif. (1990). Commercially available kits for genomic PCR amplification are known in the art. See, e.g., Advantage-GC Genomic PCR Kit (Clontech). Additionally, e.g., the T4 gene 32 protein (Boehringer Mannheim) can be used to improve yield of long PCR products.

[0210] The isolated nucleic acid compositions of this invention, such as RNA, cDNA, genomic DNA, or any combination thereof, can be obtained from biological sources using any number of cloning methodologies known to those of skill in the art. In some embodiments, oligonucleotide probes that selectively hybridize, under stringent conditions, to the polynucleotides of the present invention are used to identify the desired sequence in a cDNA or genomic DNA library. The isolation of RNA, and construction of cDNA and genomic libraries, is well known to those of ordinary skill in the art. (See, e.g., Sambrook, supra).

[0211] The nucleic acid molecules of the invention are nucleic acids encoding antibodies or antibody portions (e.g., VH, VL, CDR3) that bind to amyloid beta 1-42. "Isolated nucleic acid molecules" of the invention is intended to refer to a nucleic acid molecule in which the nucleotide sequences encoding the antibody or antibody portion are free of other nucleotide sequences encoding antibodies or antibody portions that bind antigens other than amyloid beta 1-42.

[0212] In one particular embodiment, mutations can be introduced randomly along all or part of an anti-amyloid beta 1-42 antibody coding sequence, such as by saturation mutagenesis or by recombination, and the resulting modified anti-amyloid beta 1-42 antibodies can be screened for binding activity.

[0213] In a more particular embodiment, the nucleic acid sequence encoding a single chain (scFv) recombinant antibody designated as AD38 (CECT accession number 7167) is set forth in SEQ ID NO: 9. The nucleic acid sequences encoding the heavy or light chain fragments from the antibody designated as AD38 (CECT accession number 7167) are set forth in SEQ ID NOs: 1 and 3, respectively.

[0214] In yet another particular embodiment, the nucleic acid sequence encoding a single chain (scFv) recombinant antibody designated as AD64 (CECT accession number 7168) is set forth in SEQ ID NO: 11. The nucleic acid sequences encoding the heavy or light chain fragments from the antibody designated as AD64 (CECT accession number 7168) are set forth in SEQ ID NOs: 5 and 7, respectively.

[0215] The polynucleotides can also include polynucleotides that hybridize under stringent conditions to any of the sequences set forth in SEQ ID NOs: 1, 3, 5, 7, 9 and 11, or complements thereof, and/or that encode polypeptides that retain substantial biological activity (i.e., active fragments) of the variable regions encoded by these sequences. The polynucleotides can also include continuous portions of the any of the sequences set forth in SEQ ID NOs: 1, 3, 5, 7, 9 and 11, comprising at least 21 consecutive nucleotides.

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[0216] The invention also provides for nucleotide sequences that are substantially homologous to the sequences encoding the antibodies to amyloid beta 1-42, as described herein. The percent identity between two nucleotide sequences can be determined using the GAP program in the GCG software package (available at http://www.gcg.com), using a NWSgapdna.CMP matrix and a gap weight of 40, 50, 60, 70, or 80 and a length weight of 1, 2, 3, 4, 5, or 6. The percent identity between two nucleotide or amino acid sequences can also determined using the algorithm of E. Meyers and W. Miller (Comput. AppL. Biosci., 4:11-17 (1988)) which has been incorporated into the ALIGN program (version 2.0), using a PAM 1 20 weight residue table, a gap length penalty of 12 and a gap penalty of 4. In addition, the percent identity between two amino acid sequences can be determined using the Needleman and Wunsch (J. Mol. Biol. 48:444-453 (1970)) algorithm which has been incorporated into the GAP program in the GCG software package (available at http://www.gcg.com), using either a Blossum 62 matrix or a PAM2 5 0 matrix, and a gap weight of 16, 14, 12, 10, 8, 6, or 4 and a length weight of 1, 2, 3, 4, 5, or 6. The nucleic acid and protein sequences of the present invention can further be used as a "query sequence" to perform a search against public databases to, for example, identify related sequences. Such searches can be performed using the NBLAST and XBLAST programs (version 2.0) of Altschul, et al. (1990) J. Mol. Biol. 215:403-10. BLAST nucleotide searches can be performed with the NBLAST program, score=100, wordlength=12 to obtain nucleotide sequences homologous to the nucleic acid molecules of the invention. BLAST protein searches can be performed with the XBLAST program, score=50, wordlength=3 to obtain amino acid sequences homologous to the protein molecules of the invention. To obtain gapped alignments for comparison purposes, Gapped BLAST can be utilized as described in Altschul et al., (1997) Nucleic Acids Res. 25(17):3389. When utilizing BLAST and Gapped BLAST programs, the default parameters of the respective programs (e.g., XBLAST and NBLAST) can be used. See http://www.ncbi.nlm.nih.gov.

[0217] In a further particular embodiment, the heavy chain variable domain CDR1, CDR2 or CDR3 of antibody AD38 as defined in Figure 8, the light chain variable domain CDR1, CDR2 or CDR3 of antibody AD38 as defined in Figure 9, the heavy chain variable domain CDR1, CDR2 or CDR3 of antibody AD64 as defined in Figure 10, and the light chain variable domain CDR1, CDR2 or CDR3 of antibody AD64 as defined in Figure 11, are understood as part of any of the amino acid sequences defined in sequences SEQ ID NO: 2, SEQ ID NO: 4, SEQ ID NO: 6 and SEQ ID NO: 8. It is to be understood that the CDR regions of any of the above-noted antibodies may be genetically engineered into any other protein or antibody molecule or scaffold protein, as described herein, for increasing their stability, e.g. their half-life, or for targeting to the brain or for decreasing their immunogenicity if necessary.

[0218] Polypeptides disclosed herein also include continuous portions of any of the sequences set forth in SEQ ID NOs: 2, 4, 6, 8, 10 and 12 comprising at least 4 consecutive amino acids and that retain substantial biological or binding activity (i.e., active fragments) of these sequences.

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Preferably, polypeptides of the present application include continuous portions of any of the sequences set forth in SEQ ID NOs: 2, 4, 6, 8, 10 and 12 comprising 5-7 amino acids. More preferred polypeptides of the present application include any continuous portion of the any of sequences set forth in SEQ ID NOs: 2, 4, 6, 8, 10 and 12 that retains substantial biological or binding activity of the antibodies designated as AD38 and AD64. Polynucleotides disclosed herein also include, in addition to those polynucleotides described above, polynucleotides that encode any of the amino acid sequences set forth in SEQ ID NOs: 2, 4, 6, 8, 10 and 12, or a continuous portion thereof, and that differ from the polynucleotides described in SEQ ID NOs: 1, 3, 5, 7, 9 and 11 only due to the well-known degeneracy of the genetic code.

[0219] The isolated polynucleotides disclosed herein may be used as hybridization probes and primers to identify and isolate nucleic acids having sequences identical to or similar to those encoding the disclosed polynucleotides. Hybridization methods for identifying and isolating nucleic acids include polymerase chain reaction (PCR), Southern hybridization, in situ hybridization and Northern hybridization, and are well known to those skilled in the art.

[0220] Hybridization reactions can be performed under conditions of different stringency. The stringency of a hybridization reaction includes the difficulty with which any two nucleic acid molecules will hybridize to one another. Preferably, each hybridizing polynucleotide hybridizes to its corresponding polynucleotide under reduced stringency conditions, more preferably stringent conditions, and most preferably highly stringent conditions. The term "standard hybridization conditions" refers to salt and temperature conditions substantially equivalent to 5 x SSC and 65°C for both hybridization and wash. However, one skilled in the art will appreciate that such "standard hybridization conditions" are dependent on particular conditions including the concentration of sodium and magnesium in the buffer, nucleotide sequence length and concentration, percent mismatch, percent formamide, and the like. Also important in the determination of "standard hybridization conditions" is whether the two sequences hybridizing are RNA-RNA, DNA-DNA or RNA-DNA. Such standard hybridization conditions are easily determined by one skilled in the art according to well known formulae, wherein hybridization is typically 10-20°C below the predicted or determined T_m with washes of higher stringency, if desired.

[0221] Procedures using such conditions of high stringency are as follows. Prehybridization of filters containing DNA is carried out for 15 minutes to overnight at 65°C in buffer composed of 6X SSC, 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.02% PVP, 0.02% Ficoll, 0.02% BSA, and 500 µg/ml denatured salmon sperm DNA. Filters are hybridized for 48 h at 65°C in prehybridization mixture containing 100 µg/ml denatured salmon sperm DNA and 5-20 X 10^6 cpm of ^{32}P-labeled probe. Washing of filters is done at 37°C for 1 h in a solution containing 2X SSC, 0.01% PVP, 0.01% Ficoll, and 0.01% BSA. This is followed by a wash in 0.1X SSC at 50°C for 45 min before autoradiography. Other conditions of high stringency that may be used are well known in the art.

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[0222] The isolated polynucleotides disclosed herein may be used as hybridization probes and primers to identify and isolate DNA having sequences encoding allelic variants of the disclosed polynucleotides. Allelic variants are naturally occurring alternative forms of the disclosed polynucleotides that encode polypeptides that are identical to or have significant similarity to the polypeptides encoded by the disclosed polynucleotides. Preferably, allelic variants have at least 90% sequence identity (more preferably, at least 95% identity; most preferably, at least 99% identity) with the disclosed polynucleotides.

[0223] The isolated polynucleotides disclosed herein may also be used as hybridization probes and primers to identify and isolate DNAs having sequences encoding polypeptides homologous to the disclosed polynucleotides. These homologs are polynucleotides and polypeptides isolated from a different species than that of the disclosed polypeptides and polynucleotides, or within the same species, but with significant sequence similarity to the disclosed polynucleotides and polypeptides. Preferably, polynucleotide homologs have at least 50%, 70%, 75%, 80%, 85%, 87%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99%, sequence with the disclosed polynucleotides

[0224] The isolated polynucleotides disclosed herein may also be used as hybridization probes and primers to identify cells and tissues that express the antibodies and the conditions under which they are expressed.

[0225] The isolated polynucleotides disclosed herein may be operably linked to an expression control sequence for recombinant production of the polypeptides described herein. A polynucleotide can be operably linked to a nucleotide sequence encoding a constant region, e.g., a constant region of one of the various antibody isotypes. For example, a polynucleotide that encodes a light chain disclosed herein (e.g., any one of those set forth in SEQ ID NOs: 4 or 8) may be operably linked to a nucleotide sequence that encodes the constant region (or derivatives thereof) of either a kappa light chain or lambda light chain, such that the expression of the linked nucleotides will result in a full kappa or lambda light chain with a variable region that specifically binds to amyloid beta 1-42 and interferes with the formation of a functional amyloid beta 1-42 signaling complex, and neutralizes or inhibits the formation of amyloid beta 1-42 or one or more amyloid beta 1-42 associated activities. Similarly, a polynucleotide that encodes a heavy chain region disclosed herein (e.g., any of those set forth in SEQ ID NOs:2 or 6) may be operably linked to a nucleotide sequence that encodes the constant region of a heavy chain isotype (or derivatives thereof), e.g., IgM, IgD, IgE, IgG and IgA. General methods of expressing recombinant proteins are well known in the art. Such recombinant proteins may be expressed in soluble form for use in treatment of disorders resulting from amyloid beta mediated signaling (e.g., Alzheimer's disease).

[0226] The recombinant expression vectors disclosed herein may carry additional sequences, such as sequences that regulate replication of the vector in host cells (e.g., origins of replication) and selectable marker genes. The selectable marker gene facilitates selection of host cells into

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which the vector has been introduced (see, e.g., U.S. Pat. Nos. 4,399,216, 4,634,665 and 5,179,017). For example, typically the selectable marker gene confers resistance to a drug, such as G418, hygromycin or methotrexate, on a host cell into which the vector has been introduced. Preferred selectable marker genes include the dihydrofolate reductase (DHFR) gene (for use in dhfr$^-$ host cells with methotrexate selection/amplification) and the neo gene (for G418 selection).

[0227] A number of cell lines are suitable host cells for recombinant expression. Mammalian host cell lines include, for example, COS cells, CHO cells, 293T cells, A431 cells, 3T3 cells, CV-1 cells, HeLa cells, L cells, BHK21 cells, HL-60 cells, U937 cells, HaK cells, Jurkat cells, as well as cell strains derived from in vitro culture of primary tissue and primary explants.

[0228] Alternatively, it may be possible to recombinantly produce polypeptides in lower eukaryotes such as yeast (e.g., Saccharomyces, Pichia, Kluyveromyces strains, and Candida strains) or in prokaryotes (e.g., *Escherichia coli*, *Bacillus subtilis*, and *Salmonella typhimurium*). Polypeptides made in yeast or bacteria can be modified, e.g., glycosylation of appropriate sites

[0229] Polypeptides can also be produced in animal cells, e.g., insect or mammalian cells. For example, a sequence encoding the polypeptide can be inserted into an insect expression vector, such as a baculovirus vector, and used in an insect cell expression system (e.g., the MAXBAC.RTM. kit, Invitrogen, Carlsbad, Calif.).

[0230] The polypeptides disclosed herein may then be purified from culture medium or cell extracts using known purification processes, such as gel filtration and ion exchange chromatography. Purification may also include affinity chromatography with agents known to bind the polypeptides disclosed herein.

[0231] Alternatively, the polypeptides disclosed herein may also be recombinantly expressed in a form that facilitates purification. For example, the polypeptides may be expressed as fusions with proteins such as maltose-binding protein (MBP), glutathione-S-transferase (GST), or thioredoxin (TRX) or as fusions to hexa-histidine, penta-histidine, or small epitope tags, e.g., the FLAG epitope.

[0232] The polypeptides disclosed herein also encompass molecules that are structurally different from the disclosed polypeptides (e.g., which have a slightly altered sequence), but which have substantially the same biochemical properties as the disclosed polypeptides (e.g., are changed only in functionally nonessential amino acid residues). Such molecules include naturally occurring allelic variants and deliberately engineered variants containing alterations, substitutions, replacements, insertions, or deletions. Techniques for such alterations, substitutions, replacements, insertions, or deletions are well known to those skilled in the art.

Other Specific Amyloid Beta 1-42 Binding Members or Agents

[0233] Also provided are binding members or agents, other than binding agents that are antibodies and fragments thereof, that bind to amyloid beta 1-42, particularly binding agents that compete with the antibodies AD38 and AD64 and other antibodies described herein for binding

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to amyloid beta 1-42. For example, the binding agents can bind to the same epitope or an overlapping epitope as AD38 and AD64 on amyloid beta 1-42. The binding agents preferably inhibit formation of a neurotoxic form of amyloid beta 1-42 or neutralize amyloid beta 1-42 activity. Such binding agents can be used in the methods described herein, e.g., the methods of treating and preventing disorders, in particular Alzheimer's disease. All embodiments described herein can be adapted for use with amyloid beta 1-42 binding agents.

[0234] Binding agents can be identified by a number of means, including modifying a variable domain described herein or grafting one or more CDRs of a variable domain described herein onto another scaffold. Binding agents can also be identified from diverse libraries, e.g., by screening. One method for screening protein libraries uses phage display. Particular regions of a protein are varied and proteins that interact with amyloid beta 1-42 are identified, e.g., by retention on a solid support or by other physical association. To identify particular binding agents that bind to the same epitope or an overlapping epitope as AD38 or AD64 on amyloid beta 1-42, binding agents can be eluted by adding AD38 or AD64 (or related antibody), or binding agents can be evaluated in competition experiments with AD38 or AD64 (or related antibody). It is also possible to deplete the library of agents that bind to other epitopes by contacting the library to a complex that contains amyloid beta 1-42 and antibody AD38 or AD64 (or related antibody). The depleted library can then be contacted to amyloid beta 1-42 to obtain a binding agent that binds to amyloid beta 1-42 but not to amyloid beta 1-42 when it is bound by AD38 or AD64. It is also possible to use peptides from amyloid beta 1-42 that contain the AD38 or AD64 epitope as a target.

[0235] Phage display is described, for example, in U.S. Pat. No. 5,223,409; Smith (1985) Science 228:1315-1317; WO 92/18619; WO 91/17271; WO 92/20791; WO 92/15679; WO 93/01288; WO 92/01047; WO 92/09690; WO 90/02809; WO 94/05781; Fuchs et al. (1991) Bio/Technology 9:1370-1372; Hay et al. (1992) Hum Antibod Hybridomas 3:81-85; Huse et al. (1989) Science 246:1275-1281; Griffiths et al. (1993) EMBO J. 12:725-734; Hawkins et al. (1992) J Mol Biol 226:889-896; Clackson et al. (1991) Nature 352:624-628; Gram et al. (1992) PNAS 89:3576-3580; Garrard et al. (1991) Bio/Technology 9:1373-1377; Rebar et al. (1996) Methods Enzymol. 267:129-49; and Barbas et al. (1991) PNAS 88:7978-7982. Yeast surface display is described, e.g., in Boder and Wittrup (1997) Nat. Biotechnol. 15:553-557. Another form of display is ribosome display. See, e.g., Mattheakis et al. (1994) Proc. Natl. Acad. Sci. USA 91:9022 and Hanes et al. (2000) Nat Biotechnol. 18:1287-92; Hanes et al. (2000) Methods Enzymol. 328:404-30. and Schaffitzel et al. (1999) J Immunol Methods. 231(1-2):119-35.

[0236] Binding agents that bind to amyloid beta 1-42 can have structural features of one scaffold proteins, e.g., a folded domain. An exemplary scaffold domain, based on an antibody, is a "minibody" scaffold has been designed by deleting three beta strands from a heavy chain variable domain of a monoclonal antibody (Tramontano et al., 1994, J. Mol. Recognit. 7:9; and Martin et al., 1994, The EMBO Journal 13, pp. 5303-5309). This domain includes 61 residues

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and can be used to present two hypervariable loops, e.g., one or more hypervariable loops of a variable domain described herein or a variant described herein. In another approach, the binding agent includes a scaffold domain that is a V-like domain (Coia et al. WO 99/45110). V-like domains refer to a domain that has similar structural features to the variable heavy (VH) or variable light (VL) domains of antibodies. Another scaffold domain is derived from tendamistatin, a 74 residue, six-strand beta sheet sandwich held together by two disulfide bonds (McConnell and Hoess, 1995, J. Mol. Biol. 250:460). This parent protein includes three loops. The loops can be modified (e.g., using CDRs or hypervariable loops described herein) or varied, e.g., to select domains that bind to amyloid beta 1-42. WO 00/60070 describes a β-sandwich structure derived from the naturally occurring extracellular domain of CTLA-4 is used as a scaffold.

[0237] Still another scaffold domain for an amyloid beta 1-42 binding agent is a domain based on the fibronectin type III domain or related fibronectin-like proteins. The overall fold of the fibronectin type III (Fn3) domain is closely related to that of the smallest functional antibody fragment, the variable region of the antibody heavy chain. Fn3 is a β-sandwich similar to that of the antibody VH domain, except that Fn3 has seven β-strands instead of nine. There are three loops at the end of Fn3; the positions of BC, DE and FG loops approximately correspond to those of CDR1, 2 and 3 of the VH domain of an antibody. Fn3 is advantageous because it does not have disulfide bonds. Therefore, Fn3 is stable under reducing conditions, unlike antibodies and their fragments (see WO 98/56915; WO 01/64942; WO 00/34784). An Fn3 domain can be modified (e.g., using CDRs or hypervariable loops described herein) or varied, e.g., to select domains that bind to amyloid beta 1-42.

[0238] Still other exemplary scaffold domains include: T-cell receptors; MHC proteins; extracellular domains (e.g., fibronectin Type III repeats, EGF repeats); protease inhibitors (e.g., Kunitz domains, ecotin, BPTI, and so forth); TPR repeats; trifoil structures; zinc finger domains; DNA-binding proteins; particularly monomeric DNA binding proteins; RNA binding proteins; enzymes, e.g., proteases (particularly inactivated proteases), RNase; chaperones, e.g., thioredoxin, and heat shock proteins; and intracellular signaling domains (such as SH2 and SH3 domains). U.S. 20040009530 describes examples of some alternative scaffolds.

[0239] Examples of small scaffold domains include: Kunitz domains (58 amino acids, 3 disulfide bonds), Cucurbida maxima trypsin inhibitor domains (31 amino acids, 3 disulfide bonds), domains related to guanylin (14 amino acids, 2 disulfide bonds), domains related to heat-stable enterotoxin IA from gram negative bacteria (18 amino acids, 3 disulfide bonds), EGF domains (50 amino acids, 3 disulfide bonds), kringle domains (60 amino acids, 3 disulfide bonds), fungal carbohydrate-binding domains (35 amino acids, 2 disulfide bonds), endothelin domains (18 amino acids, 2 disulfide bonds), and Streptococcal G IgG-binding domain (35 amino acids, no disulfide bonds). Examples of small intracellular scaffold domains include SH2, SH3, and EVH domains. Generally, any modular domain, intracellular or extracellular, can be used.

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[0240] Exemplary criteria for evaluating a scaffold domain can include: (1) amino acid sequence, (2) sequences of several homologous domains, (3) 3-dimensional structure, and/or (4) stability data over a range of pH, temperature, salinity, organic solvent, oxidant concentration. In one embodiment, the scaffold domain is a small, stable protein domains, e.g., a protein of less than 100, 70, 50, 40 or 30 amino acids. The domain may include one or more disulfide bonds or may chelate a metal, e.g., zinc.

[0241] Still other binding agents are based on peptides, e.g., proteins with an amino acid sequence that are less than 30, 25, 24, 20, 18, 15 or 12 amino acids. Peptides can be incorporated in a larger protein, but typically a region that can independently bind to amyloid beta 1-42, e.g., to an epitope described herein. Peptides can be identified by phage display as described previously.

[0242] An amyloid beta 1-42 binding agent may include non-peptide linkages and other chemical modification. For example, part or all of the binding agent may be synthesized as a peptidomimetic, e.g., a peptoid (see, e.g., Simon et al. (1992) Proc. Natl. Acad. Sci. USA 89:9367-71 and Horwell (1995) Trends Biotechnol. 13:132-4). A binding agent may include one or more (e.g., all) non-hydrolyzable bonds. Many non-hydrolyzable peptide bonds are known in the art, along with procedures for synthesis of peptides containing such bonds. Exemplary non-hydrolyzable bonds include --[CH_2NH]-- reduced amide peptide bonds, --[$COCH_2$]-- ketomethylene peptide bonds, --[CH(CN)NH]-- (cyanomethylene)amino peptide bonds, --[$CH_2CH(OH)$]-- hydroxyethylene peptide bonds, --[CH_2O]-- peptide bonds, and --[CH_2S]-- thiomethylene peptide bonds (see e.g., U.S. Pat. No. 6,172,043).

[0243] While the present invention contemplates the identification and development of novel antibodies through variegation of codons corresponding to the hypervariable region of an antibody's variable domain, another aspect of the invention provides for the development of binding proteins which are not antibodies or even variable domains of antibodies.

[0244] In order to obtain a novel binding protein against a chosen target, such as in the instance of the present invention, amyloid beta 1-42, DNA molecules, each encoding a protein comprising one of a family of similar potential binding domains and a structural signal calling for the display of the protein on the outer surface of a chosen bacterial cell, bacterial spore or phage (genetic package) are introduced into a genetic package. The protein is expressed and the potential binding domain is displayed on the outer surface of the package. The cells or viruses bearing the binding domains which recognize the target molecule are then isolated and amplified. The successful binding domains are then characterized. One or more of these successful binding domains is then used as a model for the design of a new family of potential binding domains, and the process is repeated until a novel binding domain having a desired affinity for the target molecule is obtained.

[0245] Most larger proteins fold into distinguishable globules called domains. Protein domains have been defined various ways, but all definitions fall into one of three classes: a) those that

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define a domain in terms of 3D atomic coordinates, b) those that define a domain as an isolable, stable fragment of a larger protein, and c) those that define a domain based on protein sequence homology plus a method from class a) or b). Frequently, different methods of defining domains applied to a single protein yield identical or very similar domain boundaries. The diversity of definitions for domains stems from the many ways that protein domains are perceived to be important, including the concept of domains in predicting the boundaries of stable fragments, and the relationship of domains to protein folding, function, stability, and evolution.

[0246] When a domain of a protein is primarily responsible for the protein's ability to specifically bind a chosen target, it is referred to herein as a "binding domain" (BD). A preliminary operation is to engineer the appearance of a stable protein domain, denoted as an "initial potential binding domain" (IPBD), on the surface of a genetic package.

[0247] For the purposes of this invention, the term "potential binding protein" refers to a protein encoded by one species of DNA molecule in a population of variegated DNA wherein the region of variation appears in one or more subsequences encoding one or more segments of the polypeptide having the potential of serving as a binding domain for the target substance. This is exemplified in U.S. Patent No. 6,979,538.

Pharmaceutical Compositions

[0248] Another aspect of the invention provides for pharmaceutical compositions for the treatment of Alzheimer's disease. The pharmaceutical composition comprises an agent, in the manner of the present invention, an anti-amyloid beta 1-42 antibody or fragment, derivative, or variant thereof, and a pharmaceutically acceptable carrier. While not wishing to be bound by theory, such an agent (antibody) may function in one or more ways such as:

a) preventing the production or generation of amyloid beta through cleavage of APP, or

b) preventing aggregation of amyloid beta fibrils and deposition in cerebral parenchymal tissues and blood vessels, or

c) preventing the formation of oligomeric forms of Abeta, Abeta 1-42, or

d) increasing the degradation of amyloid beta, or

e) increasing the clearance of amyloid beta from the brain, or

f) facilitating the metabolism of amyloid beta.

[0249] The pharmaceutical compositions may be formulated for oral delivery or parenteral delivery. Furthermore, the pharmaceutical compositions are formulated for delivery by a route selected from the group consisting of intravenous, intramuscular, oral, subcutaneous, intrathecal, intracranial and intraventricular. In a particular embodiment, the amyloid beta antibody in the composition binds to amyloid beta 1-42.

[0250] The compositions for parenteral administration will commonly comprise a solution of any of the agents described above, including an antibody or fragment thereof or a protein scaffold of

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the invention or a cocktail thereof dissolved in an acceptable carrier, preferably an aqueous carrier. A variety of aqueous carriers may be employed, e.g., water, buffered water, 0.4% saline, 0.3% glycine, and the like. These solutions are sterile and generally free of particulate matter. These solutions may be sterilized by conventional, well-known sterilization techniques. The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions such as pH adjusting and buffering agents, etc. The concentration of the agent of the invention in such pharmaceutical compositions may vary widely, i.e., from less than about 0.5%, usually at or at least about 1% to as much as 15 or 20% by weight, and will be selected primarily based on fluid volumes, viscosities, etc., according to the particular mode of administration selected.

[0251] Thus, a pharmaceutical composition of the invention for intramuscular injection could be prepared to contain 1 ml sterile buffered water, and a therapeutically effective amount of the antibody of the invention. Similarly, a pharmaceutical composition of the invention for intravenous infusion could be made up to contain 250 ml of sterile Ringer's solution, and a therapeutically effective amount of the antibody of the invention. Actual methods for preparing parenterally administrable compositions are well-known or will be apparent to those skilled in the art, and are described in more detail in, e.g., Remington's Pharmaceutical Science, 15th ed., Mack Publishing Company, Easton, PA. The therapeutically effective amount of the antibody can be determined by one skilled in the art. The age and weight of the patient, the stage of the disease, the mode of administration, the actual formulation and the possible coadministration of other therapeutic agents must all be taken into account when determining the optimal therapeutically effective amount of the antibody to be administered.

[0252] The agent (antibody) of the invention may be lyophilized for storage and reconstituted in a suitable carrier prior to use.

[0253] The pharmaceutical composition of the invention may be administered for prophylactic and/or therapeutic treatments. In therapeutic application, compositions are administered to a subject already suffering from Alzheimer's disease, in an amount sufficient to cure or at least partially arrest the disorder and its complications, or to stop the progression of the disease and one or more of its symptoms, for example, memory loss or anxiety or irritability. In prophylactic applications, compositions containing the present agents are administered to a subject not already in a disease state but one that may be predisposed to Alzheimer's disease to enhance the subject's resistance to such disorder. A "subject at risk for developing Alzheimer's disease" as described herein, refers to a subject who is predisposed to development of AD as described in the present application, whereby such risk factors are known to those skilled in the art.

[0254] It will be recognized by one of skill in the art that the optimal quantity and spacing of individual dosages of an agent of the invention will be determined by the nature and extent of the depressive disorder being treated, the form, route and site of administration, and the particular animal being treated, and that such optimums may be determined by conventional

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techniques. It will also be appreciated by one of skill in the art that the optimal course of treatment, i.e., the number of doses of an agent of the invention given per day for a defined number of days, may be ascertained by those skilled in the art using conventional course of treatment determination tests.

[0255] In practicing an exemplary method of treatment or use described herein, a therapeutically effective amount of antibody that binds to amyloid beta 1-42 can be administered to a subject, e.g., mammal (e.g., a human). An antibody may be administered in accordance with the methods described either alone or in combination with other therapies such as treatments employing cytokines, lymphokines hematopoietic factors, one or more other antibodies that may or may not bind amyloid beta or amyloid beta 1-42, or anti-inflammatory agents. Such additional factors and/or agents may be included in the pharmaceutical composition to produce a synergistic effect with an antibody described herein. When coadministered with one or more agents, the antibody may be administered either simultaneously with the second agent, or separately, e.g., sequentially. If administered separately, e.g., sequentially, the attending physician will decide on the appropriate sequence of administering the antibody in combination with other agents.

[0256] In one embodiment, the pharmaceutical composition includes the anti-amyloid beta 1-42 antibody as the sole biologic (e.g., the sole protein component) or as the sole biologically active ingredient. For example, the composition can include less than 25, 20, 15, 10, 5, 3, 2, 1, 0.4, or 0.1% other protein components on a w/w basis.

[0257] The pharmaceutical compositions may be in the form of a liposome in which the anti-amyloid beta 1-42 antibody is combined, in addition to other pharmaceutically acceptable carriers, with amphipathic agents such as lipids that exist in aggregated form as micelles, insoluble monolayers, liquid crystals, or lamellar layers while in aqueous solution. Suitable lipids for liposomal formulation include, without limitation, monoglycerides, diglycerides, sulfatides, lysolecithin, phospholipids, saponin, bile acids, and the like. Preparation of such liposomal formulations is within the level of skill in the art, as disclosed, for example, in U.S. Pat. Nos. 4,235,871; 4,501,728; 4,837,028; and 4,737,323, all of which are incorporated herein by reference.

[0258] The phrase "therapeutically effective amount" is used herein to mean an amount sufficient to prevent, and preferably reduce by at least about 25 percent, more preferably by at least 50 percent, most preferably by at least 90 percent, a clinically significant change in a feature of pathology such as for example, elevated blood pressure, fever, or white cell count, as may attend its presence and activity. As related to the present invention, the term may also mean an amount sufficient to ameliorate or reverse one or more symptoms associated with Alzheimer's disease. In particular, a "therapeutically effective amount" of the treatment may result in amelioration, reduction or elimination of at least one of the following symptoms: memory impairment, persistent sadness or anxiety, feelings of emptiness, hopelessness, pessimism,

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guilt, worthlessness, helplessness, a loss of interest or pleasure in hobbies and activities that were once enjoyed, decreased energy, or fatigue, difficulty concentrating, remembering, or making decisions, insomnia, early-morning awakening, or oversleeping, appetite and/or weight loss or overeating and weight gain, thoughts of death or suicide and suicide attempts, restlessness, irritability, and persistent physical symptoms that do not respond to treatment, such as headaches, digestive disorders, and chronic pain. When applied to an individual active ingredient, administered alone, the term refers to that ingredient alone. When applied to a combination, the term refers to combined amounts of the active ingredients that result in the therapeutic effect, whether administered in combination, serially or simultaneously.

Combination Therapy

[0259] It is envisioned that the antibodies of the present invention may be administered alone as a single composition or may be administered in combination with one or more other agents useful for treating Alzheimer's disease.

[0260] For example, amyloid beta formation could be inhibited by peptides homologous to Abeta (position 17-21) which have a similar degree of hydrophobicity, but also have a very low propensity to adopt a beta-sheet conformation due to the presence of proline residues (anti-beta-sheet peptides or beta-sheet inhibitors) (Soto C, Kindy MS, Baumann M, Frangione B.Biochem, (1996), Biophys Res Commun., Sep 24;226(3):672-80). An 11-residue peptide with these characteristics binds to Abeta, inhibits Abeta fibril formation and partially disaggregates preformed fibrils in vitro. Shorter anti-beta-sheet peptides and analogs containing D-amino acids are also able to inhibit Abeta fibrillogenesis. The latter are more resistant to proteolytic degradation and may serve as a starting point to design more efficient peptides derivatives to inhibit amyloidogenesis in vivo.

[0261] Alternatively, it has been determined that N-methyl amino acid containing congeners of the hydrophobic "core domain" of Abeta inhibit the fibrillogenesis of full-length Abeta. These peptides also disassemble preformed fibrils of full-length Abeta. A key feature of the inhibitor peptides is that they contain N-methyl amino acids in alternating positions of the sequence. The most potent of these inhibitors, termed Abeta 16-22m, has the sequence NH(2)-K(Me or L)V(Me or F)F(Me or A)E-CONH(2) (SEQ ID NO:13). These inhibitors appear to act by binding to growth sites of Abeta nuclei and/or fibrils and preventing the propagation of the network of hydrogen bonds that is essential for the formation of an extended beta-sheet fibril. (Gordon DJ, Sciarretta KL, Meredith SC., (2001), Biochemistry. Jul 27;40(28):8237-45). Furthermore, it has been shown that single N-methyl amino acid-containing peptides related to the central hydrophobic region β_{16-20} (Lys-Leu-Val-Phe-Phe (SEQ ID NO:14) of the β-amyloid protein are able to reduce the cytotoxicity of natural β_{1-42} in PC12 cell cultures. N-methyl phenylalanine analogs yield statistically significant increments in cell viability (Student's t-test < 0.01%) and are nontoxic in

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the same assay (M. Cruz, J.M. Tusell, D. Grillo-Bosch, F. Albericio, J. Serratosa, F. Rabanal and E. Giralt, (2004), The Journal of Peptide Research, 63(3): 324).

[0262] In addition, it has been shown that phenserine, a third generation acetylcholinesterase inhibitor (AChE-inhibitor), has the ability to reduce both beta amyloid precursor protein (APP) and beta amyloid peptide (amyloid-beta) formation in the brain (see http://www.axonyx.com/pipeline/index.html).

[0263] Other investigators have reported that injection of animals with low molecular weight heparins (enoxaprin and dalteparin) demonstrated a reduction in amyloid beta deposition. Moreover, these compounds were capable of arresting the progression of amyloid beta deposits and amyloid beta peptide fibril formation by impeding the structural changes necessary for fibril formation. Not only was amyloid beta progression retarded, but these molecules enhanced the clearance of established amyloid beta fibrils (Zhu, H. et al. (2001), Mol. Med. 7(8):517-522).

[0264] Furthermore, it has been shown that monoclonal antibodies are capable of inhibiting in vitro fibrillar aggregation of the Alzheimer beta-amyloid peptide (Solomon B, et al., (1996), Proc Natl Acad Sci U S A, 93(1):452-5). In one study, two monoclonal antibodies (mAbs), AMY-33 and 6F/3D, were raised against beta-amyloid fragments spanning amino acid residues 1-28 and 8-17 of the beta-amyloid peptide chain, respectively. In vitro aggregation of beta-amyloid peptide was induced by incubation for 3 hours at 37° C and monitored by ELISA, negative staining electron microscopy, and fluorimetric studies. The investigators found that the monoclonal antibodies prevented the aggregation of beta-amyloid peptide. Moreover, the inhibitory effect appeared to be related to the localization of the antibody-binding sites and the nature of the aggregating agents (Solomon B, et al., (1996), Proc Natl Acad Sci U S A, 93(1):452-5).

[0265] It may also be possible to prevent the effects of amyloid beta by administering an agent that effects a regulatory element necessary for its expression. Alternatively, the effects of amyloid beta in AD may be addressed by taking into account the effects of abeta in the inflammatory process. For example, studies by Suo Z. et al suggest that beta-amyloid (Abeta)-induced inflammatory reactions may partially drive the pathogenesis of Alzheimer's disease (AD) and in cerebral amyloid angiopathy (CAA). To evaluate the roles of Abeta in the inflammatory processes in vascular tissues, they tested the ability of Abeta to trigger inflammatory responses in cultured human vascular cells. They found that stimulation with Abeta dose-dependently increased the expression of CD40, and secretion of interferon-((IFN-() and interleukin-1ß (IL-1ß) in endothelial cells. Abeta also induced expression of IFN-(receptor (IFN-(R) both in endothelial and smooth muscle cells. Characterization of the Abeta-induced inflammatory responses in the vascular cells showed that the ligation of CD40 further increased cytokine production and/or the expression of IFN-(R. Moreover, IL-1 ß and IFN-(synergistically increased the Abeta-induced expression of CD40 and IFN-(R. They also found that Abeta induced expression of adhesion molecules, and that cytokine production and interaction of CD40-CD40 ligand (CD40L) further increased the Abeta-induced expression of adhesion molecules in these

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same cells. These results suggest that Abeta can function as an inflammatory stimulator to activate vascular cells and induces an auto-amplified inflammatory molecular cascade, through interactions among adhesion molecules, CD40-CD40L and cytokines. Additionally, Abeta 1-42, the more pathologic form of Abeta, induces much stronger effects in endothelial cells than in smooth muscle cells, while the reverse is true for Abeta
1-40. Collectively, these findings support the hypothesis that the Abeta-induced inflammatory responses in vascular cells may play a significant role in the pathogenesis of CAA and AD (Suo et al., (1998), Brain Res, 807(1-2): 110-7).

[0266] Studies have also focused on a means of enhancing the degradation and clearance of Abeta. Studies by Qiu et al. have shown increased degradation of amyloid beta protein by a metalloprotease secreted by microglia. Such a metalloprotease was identified in a microglial cell line, BV-2 (Qiu, WQ et al. (1997), J. Biol. Chem. 272(10):6641-6). Substrate analysis revealed that the enzyme responsible for the degradation of $Abeta_{1-40}$ and $Abeta_{1-42}$ was a non-matrix metalloprotease. Thus, it may be possible to develop novel therapeutics that act to enhance the activity of this enzyme or to identify other enzymes that act in a similar manner to degrade the amyloid beta proteins, or alternatively, to develop a means of directing specific enzymes with such amyloid beta degrading activity to the site where needed.

[0267] Another enzyme, neprilysin, has been shown to be a major Abeta degrading enzyme in vivo. Studies by Hama, et al. investigated whether or not manipulation of neprilysin activity in the brain would be an effective strategy for regulating Abeta levels. Neprilysin was expressed in primary cortical neurons using a Sindbis viral vector and the effect on Abeta metabolism was examined. The investigators showed that the corresponding recombinant protein, expressed in the cell bodies and processes, exhibited thiorphan-sensitive endopeptidase activity, whereas a mutant neprilysin with an amino acid substitution in the active site did not show any such activity. Expression of the wild-type neprilysin, but not the mutant, resulted in a dose-dependent decrease in both the Abeta 40 and 42 levels in the culture media (Hama E. et al. (2001), Journal of Biochemistry, Vol 130, Issue 6: 721-726). Moreover, neprilysin expression also resulted in reducing cell-associated Abeta, which could be more neurotoxic than extracellular Abeta. These results indicate that the manipulation of neprilysin activity in neurons, the major source of Abeta in the brain, would be a relevant strategy for controlling the Abeta levels and thus the Abeta-associated pathology in brain tissues. In the matter of the present invention, such a strategy could be used to treat AD associated with elevated levels of Abeta peptides.

[0268] Studies have also shown that endothelin converting enzyme (ECE) has been shown to play a fundamental role in amyloid metabolic pathways. A published study (Journal of Biological Chemistry: 276(27):24540-8, 2001) reported that both ECE-1a and ECE-1b activation reduced the intracellular generation of Abeta40 and Abeta42, and that ECE-1a may also be able to degrade preexisting protein. The development of strategies able to stimulate or over-express

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ECE could offer a novel therapy of treating AD characterized by elevated levels of Abeta 1-40 and Abeta 1-42.

[0269] Furthermore, it has been shown that a modest increase in astroglial TGF-beta 1 production in aged transgenic mice expressing the human beta-amyloid precursor protein (hAPP) results in a three-fold reduction in the number of parenchymal amyloid plaques, a 50% reduction in the overall Abeta load in the hippocampus and neocortex, and a decrease in the number of dystrophic neuritis (Wyss-Coray T, et al. (2001) Nat Med. 7(5):612-8). These investigators found that in mice expressing hAPP and TGF-ß1, Abeta accumulated substantially in cerebral blood vessels, but not in parenchymal plaques. Furthermore, in human cases of AD, Abeta immunoreactivity associated with parenchymal plaques was inversely correlated with Abeta in blood vessels and cortical TGF-ß1 mRNA levels. The reduction of parenchymal plaques in hAPP/TGF-beta1 mice was associated with a strong activation of microglia and an increase in inflammatory mediators. Recombinant TGF-ß1 stimulated Abeta clearance in microglial cell cultures. These results demonstrate that TGF-ß1 is an important modifier of amyloid beta deposition in vivo and indicate that TGF-ß1 might promote microglial processes that inhibit the accumulation of Abeta in the brain parenchyma. Accordingly, agents that modulate TGF-ß activity may prove useful to treat AD by virtue of their effect on Abeta deposition and/or clearance.

[0270] In studies related to the role of amyloid beta in Alzheimer's disease, several groups are exploring the possibility that Abeta-based vaccines can help the brain in Alzheimer's patients by increasing its clearance. Work by Lemere et al. demonstrated that vaccinating monkeys with synthetic amyloid beta peptide enhanced the clearance of amyloid beta protein from the brain and into the blood. In this study, five aged Caribbean vervet monkeys were given eight injections of the amyloid beta peptide over a period of nine-months. Five control monkeys did not receive the treatment. The investigators found that all of the immunized monkeys made antibodies to amyloid beta peptide. These antibodies were found in blood and, in lower amounts, in cerebrospinal fluid (CSF). More importantly, the investigators found that between 22 and 42 days after immunization, levels of amyloid beta protein dropped in the CSF and increased in the blood and no plaques were found in the immunized animals. These findings suggest that the antibodies bound to amyloid beta protein and increased its slow release from the CSF to the blood for clearance (http://www.ahaf.org/whatsnew/AD_Hope_Nov_2003.htm). One possible drawback to this approach is the finding that foreign beta-amyloid could have unwanted consequences, such as contributing to the death of brain cells or triggering harmful immune system responses. One possible concern is the induction of an inflammatory response in the brain. Thus, it may be necessary to administer other anti-inflammatory compounds at the time of vaccination. The present invention provides for such combination therapies.

[0271] One alternative is to find nontoxic molecules that will boost the clearance of beta-amyloid without inducing an inflammatory response. Agadjanyan et al. have found several

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molecules that mimic beta-amyloid (termed "mimotopes") in mice, and they are now looking for corresponding mimotopes in humans. Future studies by this group will concentrate on identification of beta-amyloid mimotopes in the blood from patients with Alzheimer's disease. These molecules will then be tested in a mouse model of Alzheimer's disease to determine whether vaccination with human mimotopes can reduce mouse brain levels of beta-amyloid and reverse memory deficits, without side effects (see http://www.alz.org/Research/Funded/2003/03USA_AgadjanyanIIRG.asp).

[0272] Any of the above agents, whether they are small organic molecules, enzymes, peptides or antibodies (other than those disclosed herein), which prevent the production or generation of amyloid beta through cleavage of APP, or aggregation of amyloid beta fibrils, or promote the degradation and/or clearance of amyloid beta peptides, may be useful for treatment of AD when combined with the antibodies of the invention.

Administration of the Pharmaceutical Compositions

[0273] According to the present invention, a therapeutic composition, e.g., an antibody that binds to amyloid beta 1-42, and which may act as an inhibitor of amyloid beta production or generation through cleavage of amyloid precursor protein (APP), or as an inhibitor of amyloid beta aggregation into fibrils, or as an enhancer of amyloid beta clearance from the brain, or as an enhancer of amyloid beta degradation, or as an agent that interferes with the neurotoxic effects of amyloid beta, and a pharmaceutically acceptable carrier of the invention, or an amyloid beta 1-42 binding agent or a protein or antibody prepared in a scaffold to enhance the half-life of the molecule, may be introduced orally or parenterally, e.g. intramuscularly, intravenously, subcutaneously, transmucosally, or nasally. Alternatively, administration is by intracranial, intrathecal or intraventricular administration. In addition, the therapeutic composition can be placed (e.g., injected) into the bloodstream after coupling the agent to a carrier that will allow the agent-carrier complex to cross the blood-brain barrier. Such methods are known to those skilled in the art.

[0274] In a preferred aspect, the agent of the present invention can cross cellular or nuclear membranes, which would allow for intravenous or oral administration. Strategies are available for such crossing, including but not limited to, increasing the hydrophobic nature of a molecule; introducing the molecule as a conjugate to a carrier, such as a ligand to a specific receptor, targeted to a receptor; and the like.

[0275] The present invention also provides for the use of the antibody molecules and fragments thereof as "targeting molecules". A "targeting molecule" as used herein shall mean a molecule which, when administered in vivo, localizes to desired location(s). In various embodiments, the targeting molecule can be a peptide or protein, an antibody or fragment thereof, a lectin, a carbohydrate, or a steroid. In one embodiment, the targeting molecule is a peptide ligand of a receptor on the target cell. In a more particular embodiment, the targeting molecule is an

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antibody, such as those described herein as AD38 (SEQ ID NO: 10), AD64 (SEQ ID NO: 12) and the amino acid sequences described in SEQ ID NOS: 2, 4, 6 and 8. Preferably, the targeting molecule is an antibody that binds to amyloid beta, preferably amyloid beta 1-42. The antibody may be useful for targeting other proteins or small organic molecules to the site of amyloid beta deposition in the brain.

[0276] Accordingly, in one embodiment, the use of agents that increase the transfer of molecules through the blood brain barrier is contemplated, such as that described in US 20060039859. One other strategy for drug delivery through the blood brain barrier (BBB) entails disruption of the BBB, either by osmotic means such as mannitol or leukotrienes, or biochemically by the use of vasoactive substances such as bradykinin. A BBB disrupting agent can be co-administered with the antibody therapeutic or imaging compositions of the invention when the compositions are administered by intravascular injection. Other strategies to go through the BBB may entail the use of endogenous transport systems, including carrier-mediated transporters such as glucose and amino acid carriers, receptor-mediated transcytosis for insulin or transferrin, and active efflux transporters such as p-glycoprotein. Active transport moieties may also be conjugated to the therapeutic or imaging compounds for use in the invention to facilitate transport across the epithelial wall of the blood vessel. Alternatively, drug delivery behind the BBB is by intrathecal delivery of therapeutics or imaging agents directly to the cranium, as through an Ommaya reservoir.

[0277] In one embodiment, to facilitate crosslinking, the antibody can be reduced to two heavy and light chain heterodimers, or the $F(ab')_2$ fragment can be reduced, and crosslinked to the agent via a reduced sulfhydryl.

[0278] In another embodiment, the therapeutic compound can be delivered in a vesicle, in particular a liposome (see Langer, (1990) Science, 249:1527-1533; Treat et al., (1989) in Liposomes in the Therapy of Infectious Disease and Cancer, Lopez-Berestein and Fidler (eds.), Liss: New York, pp. 353-365; Lopez-Berestein, ibid., pp. 317-327; see generally ibid.).

[0279] In yet another embodiment, the therapeutic compound can be delivered in a controlled release system. For example, the agent may be administered using intravenous infusion, an implantable osmotic pump, a transdermal patch, liposomes, or other modes of administration. In one embodiment, a pump may be used (see Langer (1990) supra; Sefton, (1987) CRC Crit. Ref. Biomed. Eng., 14:201; Buchwald et al., (1980) Surgery, 88:507; Saudek et al., (1989) N. Engl. J. Med., 321:574). In another embodiment, polymeric materials can be used (see Medical Applications of Controlled Release, Langer and Wise (eds.), CRC Press: Boca Raton, Florida (1974); Controlled Drug Bioavailability, Drug Product Design and Performance, Smolen and Ball (eds.), Wiley: New York (1984); Ranger and Peppas, (1983) J. Macromol. Sci. Rev. Macromol. Chem., 23:61; see also Levy et al., (1985) Science, 228:190; During et al., (1989) Ann. Neurol., 25:351; Howard et al., (1989) J. Neurosurg., 71:105). In yet another embodiment, a controlled release system can be placed in proximity of the therapeutic target, i.e., the brain, thus requiring

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only a fraction of the systemic dose (see, e.g., Goodson, in Medical Applications of Controlled Release, supra, vol. 2, pp. 115-138 (1984)).

[0280] When a therapeutically effective amount of an antibody that binds to amyloid beta 1-42 is administered orally, the binding agent will be in the form of a tablet, capsule, powder, solution or elixir. When administered in tablet form, a pharmaceutical composition may additionally contain a solid carrier such as a gelatin or an adjuvant. The tablet, capsule, and powder contain from about 5 to 95% binding agent, and preferably from about 25 to 90% binding agent. When administered in liquid form, a liquid carrier such as water, petroleum, oils of animal or plant origin such as peanut oil, mineral oil, soybean oil, or sesame oil, or synthetic oils may be added. The liquid form of the pharmaceutical composition may further contain physiological saline solution, dextrose or other saccharide solution, or glycols such as ethylene glycol, propylene glycol or polyethylene glycol. When administered in liquid form, the pharmaceutical composition contains from about 0.5 to 90% by weight of the binding agent, and preferably from about 1 to 50% the binding agent.

[0281] When a therapeutically effective amount of an antibody that binds to amyloid beta 1-42 is administered by intravenous, cutaneous, or subcutaneous injection, the binding agent will be in the form of a pyrogen-free, parenterally acceptable aqueous solution. The preparation of such parenterally acceptable protein solutions, having due regard to pH, isotonicity, stability, and the like, is within the skill in the art. A preferred pharmaceutical composition for intravenous, cutaneous, or subcutaneous injection should contain, in addition to binding agent an isotonic vehicle such as Sodium Chloride Injection, Ringer's Injection, Dextrose Injection, Dextrose and Sodium Chloride Injection, Lactated Ringer's Injection, or other vehicle as known in the art. A pharmaceutical composition may also contain stabilizers, preservatives, buffers, antioxidants, or other additive known to those of skill in the art.

[0282] The amount of an antibody (or other amyloid beta 1-42 binding agent) in the pharmaceutical composition can depend upon the nature and severity of the condition being treated, and on the nature of prior treatments that the patient has undergone. Ultimately, the attending physician will decide the amount of antibody with which to treat each individual patient. Initially, the attending physician will administer low doses of antibody and observe the patient's response. Larger doses of antibody may be administered until the optimal therapeutic effect is obtained for the patient, and at that point the dosage is not generally increased further. For example, doses in the range of 0.1-50 mg/kg, 0.5-50 mg/kg, 1-100 mg/kg, 0.5-25 mg/kg, 0.1-15 mg/kg, or 1-8 mg/kg of body weight can be administered. The physician may track the effectiveness of the pharmaceutical composition by monitoring the progression of the disease or the stabilization of the condition. For example, in the case of Alzheimer's disease, the physician may assess the overall mental health of the patient as related to signs of irritability or anxiety or may assess the effect of the composition on memory impairment. The pharmaceutical

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composition can be administered to normal patients or patients who do not show symptoms, e.g., in a prophylactic mode.

[0283] A composition that includes an amyloid beta 1-42 antibody or fragment thereof can be formulated for inhalation or other mode of pulmonary delivery. Accordingly, the compounds described herein can be administered by inhalation to pulmonary tissue. The term "pulmonary tissue" as used herein refers to any tissue of the respiratory tract and includes both the upper and lower respiratory tract, except where otherwise indicated. An amyloid beta 1-42 antibody or fragment thereof can be administered in combination with one or more of the existing modalities for treating pulmonary diseases.

[0284] In one example, the compound is formulated for a nebulizer. In one embodiment, the compound can be stored in a lyophilized form (e.g., at room temperature) and reconstituted in solution prior to inhalation.

[0285] It is also possible to formulate the compound for inhalation using a medical device, e.g., an inhaler (see, e.g., U.S. Pat. No. 6,102,035 (a powder inhaler) and U.S. Pat. No. 6,012,454 (a dry powder inhaler). The inhaler can include separate compartments for the active compound at a pH suitable for storage and another compartment for a neutralizing buffer, and a mechanism for combining the compound with a neutralizing buffer immediately prior to atomization. In one embodiment, the inhaler is a metered dose inhaler.

[0286] The three common systems used to deliver drugs locally to the pulmonary air passages include dry powder inhalers (DPIs), metered dose inhalers (MDIs) and nebulizers. MDIs, used in the most popular method of inhalation administration, may be used to deliver medicaments in a solubilized form or as a dispersion. Typically MDIs comprise a Freon or other relatively high vapor pressure propellant that forces aerosolized medication into the respiratory tract upon activation of the device. Unlike MDIs, DPIs generally rely entirely on the inspiratory efforts of the patient to introduce a medicament in a dry powder form to the lungs. Nebulizers form a medicament aerosol to be inhaled by imparting energy to a liquid solution. Direct pulmonary delivery of drugs during liquid ventilation or pulmonary lavage using a fluorochemical medium has also been explored. These and other methods can be used to deliver an amyloid beta 1-42 antibody or fragment thereof. In one embodiment, the amyloid beta 1-42 antibody or fragment thereof is associated with a polymer, e.g., a polymer that stabilizes or increases half-life of the compound.

[0287] For example, for administration by inhalation, an amyloid beta 1-42 antibody or fragment thereof is delivered in the form of an aerosol spray from a pressured container or dispenser that contains a suitable propellant or a nebulizer. The compound may be in the form of a dry particle or a liquid. Particles that include the compound can be prepared, e.g., by spray drying, by drying an aqueous solution of the amyloid beta 1-42 antibody or fragment thereof with a charge neutralizing agent and then creating particles from the dried powder, or by drying an aqueous solution in an organic modifier and then creating particles from the dried powder.

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[0288] The compound may be conveniently delivered in the form of an aerosol spray presentation from pressurized packs or a nebulizer, with the use of a suitable propellant, e.g., dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoroethane, carbon dioxide, or other suitable gas. In the case of a pressurized aerosol, the dosage unit may be determined by providing a valve to deliver a metered amount. Capsules and cartridges for use in an inhaler or insufflator may be formulated containing a powder mix of an amyloid beta 1-42 antibody or fragment thereof and a suitable powder base such as lactose or starch, if the particle is a formulated particle. In addition to the formulated or unformulated compound, other materials such as 100% DPPC or other surfactants can be mixed with the amyloid beta 1-42 42 antibody or fragment thereof to promote the delivery and dispersion of formulated or unformulated compound. Methods of preparing dry particles are described, for example, in PCT Publication WO 02/32406.

[0289] An amyloid beta 1-42 antibody or fragment thereof can be formulated for aerosol delivery, e.g., as dry aerosol particles, such that when administered it can be rapidly absorbed and can produce a rapid local or systemic therapeutic result. Administration can be tailored to provide detectable activity within 2 minutes, 5 minutes, 1 hour, or 3 hours of administration. In some embodiments, the peak activity can be achieved even more quickly, e.g., within 30 minutes or even within 10 minutes. An amyloid beta 1-42 antibody or fragment thereof can be formulated for longer biological half-life (e.g., by association with a polymer such as PEG) and can be used as an alternative to other modes of administration, e.g., such that the compound enters the circulation from the lungs and is distributed to other organs or to a particular target organ.

[0290] In one embodiment, the amyloid beta 1-42 antibody or fragment thereof is delivered in an amount such that at least 5% of the mass of the polypeptide is delivered to the lower respiratory tract or the deep lung. Deep lung has an extremely rich capillary network. The respiratory membrane separating the capillary lumen from the alveolar air space is very thin and extremely permeable. In addition, the liquid layer lining the alveolar surface is rich in lung surfactants. In other embodiments, at least 2%, 3%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, or 80% of the composition of an amyloid beta 1-42 antibody or fragment thereof is delivered to the lower respiratory tract or to the deep lung. Delivery to either or both of these tissues results in efficient absorption of the compound and high bioavailability. In one embodiment, the compound is provided in a metered dose using, e.g., an inhaler or nebulizer. For example, the compound is delivered in a dosage unit form of at least about 0.02, 0.1, 0.5, 1, 1.5, 2, 5, 10, 20, 40, or 50 mg/puff or more. The percent bioavailability can be calculated as follows:

Percent Bioavailability = (AUC noninvasive/AUC i.v. or s.c.) X (dose i.v. or s.c./dose noninvasive) X 100.

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[0291] Although not necessary, delivery enhancers such as surfactants can be used to further enhance pulmonary delivery. A "surfactant" as used herein refers to a compound having hydrophilic and lipophilic moieties that promote absorption of a drug by interacting with an interface between two immiscible phases. Surfactants are useful with dry particles for several reasons, e.g., reduction of particle agglomeration, reduction of macrophage phagocytosis, etc. When coupled with lung surfactant, a more efficient absorption of the compound can be achieved because surfactants, such as DPPC, will greatly facilitate diffusion of the compound. Surfactants are well known in the art and include, but are not limited to, phosphoglycerides, e.g., phosphatidylcholines, L-α-phosphatidylcholine dipalmitoyl (DPPC) and diphosphatidyl glycerol (DPPG); hexadecanol; fatty acids; polyethylene glycol (PEG); polyoxyethylene-9-; auryl ether; palmitic acid; oleic acid; sorbitan trioleate (SPAN.TM. 85); glycocholate; surfactin; poloxomer; sorbitan fatty acid ester; sorbitan trioleate; tyloxapol; and phospholipids.

Stabilization and Retention

[0292] In one embodiment, an amyloid beta 1-42 antibody or fragment thereof is physically associated with a moiety that improves its stabilization and/or retention in circulation, e.g., in blood, serum, lymph, or other tissues, e.g., by at least 1.5, 2, 5, 10, or 50 fold. For example, an amyloid beta 1-42 antibody or fragment thereof can be associated with a polymer, e.g., a substantially nonantigenic polymer, such as polyalkylene oxides or polyethylene oxides. Suitable polymers will vary substantially by weight. Polymers having molecular number average weights ranging from about 200 to about 35,000 (or about 1,000 to about 15,000, or about 2,000 to about 12,500) can be used. For example, an amyloid beta 1-42 antibody or fragment thereof can be conjugated to a water-soluble polymer, e.g., hydrophilic polyvinyl polymers, e.g. polyvinylalcohol and polyvinylpyrrolidone. A nonlimiting list of such polymers includes polyalkylene oxide homopolymers such as polyethylene glycol (PEG) or polypropylene glycols, polyoxyethylenated polyols, copolymers thereof.and block copolymers thereof, provided that the water solubility of the block copolymers is maintained.

[0293] The molecular weight of the polymer can range up to about 500,000 Da, and preferably is at least about 20,000 Da, or at least about 30,000 Da, or at least about 40,000 Da. The molecular weight chosen can depend upon the effective size of the conjugate to be achieved, the nature (e.g., structure, such as linear or branched) of the polymer, and the degree of derivatization. A covalent bond can be used to attach an amyloid beta 1-42 antibody or fragment thereof to a polymer, for example, cross linking to the N-terminal amino group of the antibody and epsilon amino groups found on lysine residues of the antibody, as well as other amino, imino, carboxyl, sulfhydryl, hydroxyl or other hydrophilic groups. Functionalized PEG polymers that can be attached to an amyloid beta 1-42 antibody or fragment thereof are available, e.g., from Shearwater Polymers, Inc. (Huntsville, Ala.). The reaction conditions for coupling PEG and other polymers may vary depending on the amyloid beta 1-42 antibody or fragment thereof, the

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desired degree of PEGylation, and the polymer utilized. Some factors involved in the choice of PEG derivatives include: the desired point of attachment (such as lysine or cysteine R-groups), hydrolytic stability and reactivity of the derivatives, stability, toxicity and antigenicity of the linkage, suitability for analysis, etc. Specific instructions for the use of any particular derivative are available from the manufacturer.

[0294] The conjugates of an amyloid beta 1-42 antibody or fragment thereof and a polymer can be separated from the unreacted starting materials, e.g., by gel filtration or ion exchange chromatography, e.g., HPLC. Heterologous species of the conjugates are purified from one another in the same fashion. Resolution of different species (e.g. containing one or two PEG residues) is also possible due to the difference in the ionic properties of the unreacted amino acids (see, e.g., WO 96/34015).

Therapeutic and Prophylactic Uses of Anti- amyloid beta 1-42 Antibodies

[0295] In yet another aspect, this disclosure features a method for neutralizing and/or inhibiting one or more disease-associated activities of amyloid beta 1-42 in vivo by administering an antibody that binds to amyloid beta 1-42, e.g., an antibody described herein in an amount sufficient to bind to and inhibit its activity and perhaps neutralize the neurotoxicity associated with beta amyloid 1-42. Such antibodies can also be administered to subjects having Alzheimer's disease or who are at risk of developing Alzheimer's disease or a related condition such as amyloidosis or other conditions in which the presence or accumulation of amyloid beta 1-42 may play a role in acquiring a disease or increasing the rate of progression of the disease.

Blood-Brain Barrier

[0296] The antibodies of the present invention, are contemplated for use in treating Alzheimer's disease. As such, the antibodies of the invention that exert their physiological effect *in vivo* in the brain may be more useful if they gain access to cells in the brain. Non-limiting examples of brain cells are neurons, glial cells (astrocytes, oligodendrocytes, microglia), cerebrovascular cells (muscle cells, endothelial cells), and cells that comprise the meninges. The blood brain barrier ("BBB") typically restricts access to brain cells by acting as a physical and functional blockade that separates the brain parenchyma from the systemic circulation (see, e.g., Pardridge, et al., J. Neurovirol. 5(6), 556-69 (1999); Rubin, et al., Rev. Neurosci. 22, 11-28 (1999)). Circulating molecules are normally able to gain access to brain cells via one of two processes: lipid-mediated transport through the BBB by free diffusion, or active (or catalyzed) transport.

[0297] The agents of the invention may be formulated to improve distribution *in vivo*, for example as powdered or liquid tablet or solution for oral administration or as a nasal spray, nose drops, a gel or ointment, through a tube or catheter, by syringe, by packtail, by pledget, or by submucosal infusion. For example, the blood-brain barrier (BBB) excludes many highly

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hydrophilic agents. To ensure that the more hydrophilic therapeutic agents of the invention cross the BBB, they may be formulated, for example, in liposomes. For methods of manufacturing liposomes, see, e.g., U.S. Pat. Nos. 4,522,811; 5,374,548; and 5,399,331. The liposomes may comprise one or more moieties which are selectively transported into specific cells or organs ("targeting moieties" or "targeting groups" or "transporting vectors"), thus providing targeted drug delivery (see, e.g., V.V. Ranade J. Clin. Pharmacol. 29, 685 (1989)). Likewise, the agents may be linked to targeting groups that facilitate penetration of the blood brain barrier.

[0298] To facilitate transport of agents of the invention across the BBB, they may be coupled to a BBB transport vector (for review of BBB transport vectors and mechanisms, see, Bickel, et al., Adv. Drug Delivery Reviews 46, 247-79 (2001)). Exemplary transport vectors include cationized albumin or the OX26 monoclonal antibody to the transferrin receptor; these proteins undergo absorptive-mediated and receptor-mediated transcytosis through the BBB, respectively. Natural cell metabolites that may be used as targeting groups, include putrescine, spermidine, spermine, or DHA. Other exemplary targeting moieties include folate or biotin (see, e.g., U.S. Pat. No. 5,416,016); mannosides (Umezawa, et al., Biochem. Biophys. Res. Commun. 153, 1038 (1988)); antibodies (P.G. Bloeman, et al., FEBS Lett. 357, 140 (1995); M. Owais, et al., Antimicrob. Agents Chemother. 39, 180 (1995)); surfactant protein A receptor (Briscoe, et al., Am. J. Physiol. 1233, 134 (1995)); gp120 (Schreier, et al., J. Biol. Chem. 269, 9090 (1994)); see also, K. Keinanen and M.L. Laukkanen, FEBS Lett. 346, 123 (1994); J.J. Killion and I.J. Fidler, Immunomethods 4, 273 (1994).

[0299] Examples of other BBB transport vectors that target receptor-mediated transport systems into the brain include factors such as insulin, insulin-like growth factors ("IGF-I," and "IGF-II"), angiotensin II, atrial and brain natriuretic peptide ("ANP," and "BNP"), interleukin I ("IL-1") and transferrin. Monoclonal antibodies to the receptors that bind these factors may also be used as BBB transport vectors. BBB transport vectors targeting mechanisms for absorptive-mediated transcytosis include cationic moieties such as cationized LDL, albumin or horseradish peroxidase coupled with polylysine, cationized albumin or cationized immunoglobulins. Small basic oligopeptides such as the dynorphin analogue E-2078 and the ACTH analogue ebiratide may also cross the brain via absorptive-mediated transcytosis and are potential transport vectors.

[0300] Other examples of BBB transport vectors include hexose moieties, e.g., glucose and monocarboxylic acids, e.g., lactic acid and neutral amino acids, e.g., phenylalanine and amines, e.g., choline and basic amino acids, e.g., arginine, nucleosides, e.g., adenosine and purine bases, e.g., adenine, and thyroid hormone, e.g., triiodothyridine. Antibodies to the extracellular domain of nutrient transporters may also be used as transport vectors. Other possible vectors include angiotensin II and ANP, which may be involved in regulating BBB permeability.

[0301] In some cases, the bond linking the therapeutic agent to the transport vector may be cleaved following transport into the brain in order to liberate the biologically active agent.

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Exemplary linkers include disulfide bonds, ester-based linkages, thioether linkages, amide bonds, acid-labile linkages, and Schiff base linkages. Avidin/biotin linkers, in which avidin is covalently coupled to the BBB drug transport vector, may also be used. Avidin itself may be a drug transport vector.

[0302] Transcytosis, including receptor-mediated transport of compositions across the blood brain barrier, may also be suitable for the agents of the invention. Transferrin receptor-mediated delivery is disclosed in U.S. Pat. Nos. 5,672,683; 5,383,988; 5,527,527; 5,977,307; and 6,015,555. Transferrin-mediated transport is also known. P.M. Friden, et al., Pharmacol. Exp. Ther. 278, 1491-98 (1996); H.J. Lee, J. Pharmacol. Exp. Ther. 292, 1048-52 (2000). EGF receptor-mediated delivery is disclosed in Y. Deguchi, et al., Bioconjug. Chem. 10, 32-37 (1999), and transcytosis is described in A. Cerletti, et al., J. Drug Target. 8, 435-46 (2000). Insulin fragments have also been used as carriers for delivery across the blood brain barrier. M. Fukuta, et al., Pharm. Res. 11. 1681-88 (1994). Delivery of agents via a conjugate of neutral avidin and cationized human albumin has also been described. Y.S. Kang, et al., Pharm. Res. 1, 1257-64 (1994).

[0303] Other modifications for enhancing penetration of the antibodies of the invention across the blood brain barrier may be accomplished using methods and derivatives known in the art. For example, U.S. Pat. No. 6,024,977 discloses covalent polar lipid conjugates for targeting to the brain and central nervous system. U.S. Pat. No. 5,017,566 discloses cyclodextrin derivatives comprising inclusion complexes of lipoidal forms of dihydropyridine redox targeting moieties. U.S. Pat. No. 5,023,252 discloses the use of pharmaceutical compositions comprising a neurologically active drug and a compound for facilitating transport of the drug across the blood-brain barrier including a macrocyclic ester, diester, amide, diamide, amidine, diamidine, thioester, dithioester, thioamide, ketone or lactone. U.S. Pat. No. 5,024,998 discloses parenteral solutions of aqueous-insoluble drugs with cyclodextrin derivatives. U.S. Pat. No. 5,039,794 discloses the use of a metastatic tumor-derived egress factor for facilitating the transport of compounds across the blood-brain barrier. U.S. Pat. No. 5,112,863 discloses the use of N-acyl amino acid derivatives as antipsychotic drugs for delivery across the blood-brain barrier. U.S. Pat. No. 5,124,146 discloses a method for delivery of therapeutic agents across the blood-brain barrier at sites of increase permeability associated with brain lesions. U.S. Pat. No. 5,153,179 discloses acylated glycerol and derivatives for use in a medicament for improved penetration of cell membranes. U.S. Pat. No. 5,177,064 discloses the use of lipoidal phosphonate derivatives of nucleoside antiviral agents for delivery across the blood-brain barrier. U.S. Pat. No. 5,254,342 discloses receptor-mediated transcytosis of the blood-brain barrier using the transferrin receptor in combination with pharmaceutical compounds that enhance or accelerate this process. U.S. Pat. No. 5,258,402 discloses treatment of epilepsy with imidate derivatives of anticonvulsive sulfamate. U.S. Pat. No. 5,270,312 discloses substituted piperazines as central nervous system agents. U.S. Pat. No. 5,284,876 discloses fatty acid conjugates of dopamine drugs. U.S. Pat.

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No. 5,389,623 discloses the use of lipid dihydropyridine derivatives of anti-inflammatory steroids or steroid sex hormones for delivery across the blood-brain barrier. U.S. Pat. No. 5,405,834 discloses prodrug derivatives of thyrotropin releasing hormone. U.S. Pat. No. 5,413,996 discloses acyloxyalkyl phosphonate conjugates of neurologically-active drugs for anionic sequestration of such drugs in brain tissue. U.S. Pat. No. 5,434,137 discloses methods for the selective opening of abnormal brain tissue capillaries using bradykinin infused into the carotid artery. U.S. Pat. No. 5,442,043 discloses a peptide conjugate between a peptide having a biological activity and incapable of crossing the blood-brain barrier and a peptide which exhibits no biological activity and is capable of passing the blood-brain barrier by receptor-mediated endocytosis. U.S. Pat. No. 5,466,683 discloses water soluble analogues of an anticonvulsant for the treatment of epilepsy. U.S. Pat. No. 5,525,727 discloses compositions for differential uptake and retention in brain tissue comprising a conjugate of a narcotic analgesic and agonists and antagonists thereof with a lipid form of dihydropyridine that forms a redox salt upon uptake across the blood-brain barrier that prevents partitioning back to the systemic circulation.

[0304] Still further examples of modifications that enhance penetration of the blood brain barrier are described in International (PCT) Application Publication No. WO 85/02342, which discloses a drug composition comprising a glycerolipid or derivative thereof. PCT Publication No. WO 089/11299 discloses a chemical conjugate of an antibody with an enzyme which is delivered specifically to a brain lesion site for activating a separately-administered neurologically-active prodrug. PCT Publication No. WO 91/04014 discloses methods for delivering therapeutic and diagnostic agents across the blood-brain barrier by encapsulating the drugs in liposomes targeted to brain tissue using transport-specific receptor ligands or antibodies. PCT Publication No. WO 91/04745 discloses transport across the blood-brain barrier using cell adhesion molecules and fragments thereof to increase the permeability of tight junctions in vascular endothelium. PCT Publication No. WO 91/14438 discloses the use of a modified, chimeric monoclonal antibody for facilitating transport of substances across the blood-brain barrier. PCT Publication No. WO 94/01131 discloses lipidized proteins, including antibodies. PCT Publication No. WO 94/03424 discloses the use of amino acid derivatives as drug conjugates for facilitating transport across the blood-brain barrier. PCT Publication No. WO 94/06450 discloses conjugates of neurologically-active drugs with a dihydropyridine-type redox targeting moiety and comprising an amino acid linkage and an aliphatic residue. PCT Publication No. WO 94/02178 discloses antibody-targeted liposomes for delivery across the blood-brain barrier. PCT Publication No. WO 95/07092 discloses the use of drug-growth factor conjugates for delivering drugs across the blood-brain barrier. PCT Publication No. WO 96/00537 discloses polymeric microspheres as injectable drug-delivery vehicles for delivering bioactive agents to sites within the central nervous system. PCT Publication No. WO 96/04001 discloses omega-3-fatty acid conjugates of neurologically-active drugs for brain tissue delivery. PCT Publication No. WO 96/22303 discloses fatty acid and glycerolipid conjugates of neurologically-active drugs for brain

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tissue delivery.

[0305] Methods for Diagnosing, Prognosing and Monitoring Amyloid Beta Associated Disorders

Non-Invasive Alzheimer's Disease (AD) Diagnostics

[0306] Certain non-invasive AD diagnostic probes are known, and hold some promise for enabling in vivo evaluation of the presence and/or extent of brain amyloid. Known non-invasive AD diagnostic probes include: a) Congo Red derivatized small organic molecules (Dezutter et al., 1999b; Klunk et al., 2002; Skovronsky et al., 2000); b) anti-Abeta. monoclonal antibodies that bind specific amino acid residues of Abeta 1-42/43 (Majocha et al., 1992; Walker et al., 1994); c) Abeta 1-40 peptide derivatized with putrescine for increased permeability across the BBB, with appended chelation cores holding gadolinium (Gd-DTPA) or monocrystalline iron oxide nanoparticles (MION) (see, e.g. Weissleder et al., 2000); and d) iodine-123/125 and carbon-11 labeled thioflavin-based organic compounds that have been developed for in vivo labeling of A.beta. plaques (Klunk et al., 2001; Kung et al., 2002).

[0307] However, these known imaging agents bear significant limitations. The Congo Red derivatized compounds are neutral, small molecular weight compounds which can permeate the blood-brain barrier, and can provide localization of an Abeta targeted probe, but do not provide quantification capabilities.

[0308] Thus, known non-invasive imaging tools as they apply to *in vivo* diagnosis of AD are currently quite limited for a variety of reasons. A clear need remains for tools and methods enabling premortem diagnosis of AD, elucidation of the pathogenesis of AD, and efficient monitoring of patients undergoing anti-amyloid therapeutic treatment. In particular, a need exists for non-invasive imaging techniques for visualizing AD-associated changes in the brain.

[0309] The proteins that bind to amyloid beta 1-42, e.g., antibodies, described herein have *in vitro* and *in vivo* diagnostic utilities for Alzheimer's disease. An exemplary method includes: (i) administering the amyloid beta 1-42 antibody to a subject; and (ii) detecting the amyloid beta 1-42 antibody in the subject. The detecting can include determining the location of the amyloid beta 1-42 antibody in the subject. Another exemplary method includes contacting an amyloid beta 1-42 antibody to a sample, e.g., a sample from a subject.

[0310] In another aspect, the present invention provides a diagnostic method for detecting the presence of amyloid beta 1-42, in vitro (e.g., a biological sample, such as tissue, biopsy) or in vivo (e.g., in vivo imaging in a subject). The method includes: (i) contacting a sample with amyloid beta 1-42 antibody; and (ii) detecting formation of a complex between the amyloid beta 1-42 antibody and the sample. The method can also include contacting a reference sample (e.g., a control sample) with the ligand, and determining the extent of formation of the complex between the ligand and the sample relative to the same for the reference sample. A change, e.g., a statistically significant change, in the formation of the complex in the sample or subject

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relative to the control sample or subject can be indicative of the presence of amyloid beta 1-42 in the sample.

[0311] The amyloid beta 1-42 antibody can be directly or indirectly labeled with a detectable substance to facilitate detection of the bound or unbound protein. Suitable detectable substances include various enzymes, prosthetic groups, fluorescent materials, luminescent materials, and radioactive materials.

[0312] Complex formation between the amyloid beta 1-42 antibody and amyloid beta 1-42 can be detected by measuring or visualizing either the ligand bound to the amyloid beta 1-42 or unbound ligand. Conventional detection assays can be used, e.g., an enzyme-linked immunosorbent assays (ELISA), a radioimmunoassay (RIA) or tissue immunohistochemistry. Further to labeling the amyloid beta 1-42 antibody, the presence of amyloid beta 1-42 can be assayed in a sample by a competition immunoassay utilizing standards labeled with a detectable substance and an unlabeled amyloid beta 1-42 antibody.

[0313] The foregoing antibodies can be used in methods known in the art relating to the localization and activity of the amyloid beta 1-42 polypeptide, e.g., for Western blotting, imaging amyloid beta in situ, measuring levels thereof in appropriate physiological samples, etc. using any of the detection techniques mentioned herein or known in the art. The standard techniques known in the art for immunoassays are described in "Methods in Immunodiagnosis", 2nd Edition, Rose and Bigazzi, eds. John Wiley & Sons, 1980; Campbell et al., "Methods and Immunology", W.A. Benjamin, Inc., 1964; and Oellerich, M. (1984) J. Clin. Chem. Clin. Biochem. 22:895-904.

[0314] One aspect of the invention provides a method of using an antibody against amyloid beta, for example amyloid beta 1-42, to diagnose Alzheimer's disease (AD) in a subject. As amyloid beta levels correlate with the presence of AD in a subject, amyloid beta 1-42 serves as a general biomarker for AD, and may be predictive of the future onset of such disease. Alternatively, it may also serve as a marker for monitoring efficacy of therapy for such disorder, as described herein. Thus, the antibody compositions and methods provided herein are particularly deemed useful for the diagnosis of AD. In a particular embodiment, AD is the result of build-up of amyloid beta in the brains of individuals suffering from such disease. More particularly, the amyloid beta is amyloid beta 1-42.

[0315] The diagnostic method of the invention provides contacting a biological sample such as a biopsy sample, tissue, cell or fluid (e.g., whole blood, plasma, serum, urine, or CSF) isolated from a subject with an antibody which binds amyloid beta (Ghiso et al. FEBS Letters 408 (1997) pages 105-108). The antibody is allowed to bind to the antigen to form an antibody-antigen complex. The conditions and time required to form the antibody-antigen complex may vary and are dependent on the biological sample being tested and the method of detection being used. Once non-specific interactions are removed by, for example, washing the sample, the antibody-antigen complex is detected using any one of the immunoassays described above as well a

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number of well-known immunoassays used to detect and/or quantitate antigens (see, for example, Harlow and Lane, Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory, New York (1988) 555-612). Such well-known immunoassays include antibody capture assays, antigen capture assays, and two-antibody sandwich assays. In an antibody capture assay, the antigen is attached to solid support, and labeled antibody is allowed to bind. After washing, the assay is quantitated by measuring the amount of antibody retained on the solid support. In an antigen capture assay, the antibody is attached to a solid support, and labeled antigen is allowed to bind. The unbound proteins are removed by washing, and the assay is quantitated by measuring the amount of antigen that is bound. In a two-antibody sandwich assay, one antibody is bound to a solid support, and the antigen is allowed to bind to this first antibody. The assay is quantitated by measuring the amount of a labeled second antibody that binds to the antigen.

[0316] These immunoassays typically rely on labeled antigens, antibodies, or secondary reagents for detection. These proteins may be labeled with radioactive compounds, enzymes, biotin, or fluorochromes. Of these, radioactive labeling may be used for almost all types of assays. Enzyme-conjugated labels are particularly useful when radioactivity must be avoided or when quick results are needed. Biotin-coupled reagents usually are detected with labeled streptavidin. Streptavidin binds tightly and quickly to biotin and may be labeled with radioisotopes or enzymes. Fluorochromes, although requiring expensive equipment for their use, provide a very sensitive method of detection. Those of ordinary skill in the art will know of other suitable labels which may be employed in accordance with the present invention. The binding of these labels to antibodies or fragments thereof may be accomplished using standard techniques such as those described by Kennedy, et al. ((1976) Clin. Chim. Acta 70:1-31), and Schuurs, et al. ((1977) Clin. Chim Acta 81:1-40).

[0317] In accordance with the diagnostic method of the invention, the presence or absence of the antibody-antigen complex is correlated with the presence or absence in the biological sample of the antigen, or a peptide fragment thereof. A biological sample containing elevated levels of said antigen (amyloid beta 1-42) is indicative of AD in a subject from which the biological sample was obtained. Accordingly, the diagnostic method of the invention may be used as part of a routine screen in subjects suspected of having AD or for subjects who may be predisposed to having AD. Moreover, the diagnostic method of the invention may be used alone or in combination with other well-known diagnostic methods to confirm the presence of AD.

[0318] The diagnostic method of the invention further provides that an antibody of the invention may be used to monitor the levels of amyloid beta antigen in patient samples at various intervals of drug treatment to identify whether and to which degree the drug treatment is effective in reducing or inhibiting the symptoms associated with such disorder, such reduction being an indication that the therapy may ultimately result in amelioration and/or cure of the disorder. Furthermore, antigen levels may be monitored using an antibody of the invention in studies evaluating efficacy of drug candidates in model systems and in clinical trials. The antigens

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provide for surrogate biomarkers in biological fluids to non-invasively assess the global status of AD. For example, using an antibody of this invention, antigen levels may be monitored in biological samples of individuals treated with known or unknown therapeutic agents or toxins. This may be accomplished with cell lines in vitro or in model systems and clinical trials. Persistently increased total levels of amyloid beta antigen in biological samples during or immediately after treatment with a drug candidate indicates that the drug candidate has little or no effect on AD. Likewise, the reduction in total levels of amyloid beta antigen indicates that the drug candidate is effective in reducing or inhibiting the symptoms of AD. Furthermore, the continued reduction of amyloid beta in the subject may ultimately result in full remission of the individual suffering from such disorder. This may provide valuable information at all stages of pre-clinical drug development, clinical drug trials as well as subsequent monitoring of patients undergoing drug treatment.

[0319] The present invention also provides methods for monitoring the progression of AD by detecting the upregulation of amyloid beta 1-42. Monitoring methods involve determining the test amounts of amyloid beta 1-42in biological samples taken from a subject at a first and second time, and comparing the amounts. A change in amount of amyloid beta 1-42 between the first and second time indicates a change in the course of AD, with a decrease in amount indicating stabilization of AD progression, and an increase in amount indicating progression of AD. Such monitoring assays are also useful for evaluating the efficacy of a particular therapeutic intervention (e.g., disease attenuation and/or reversal) in patients being treated for AD.

[0320] Fluorophore- and chromophore-labeled protein ligands can be prepared. Since antibodies and other proteins absorb light having wavelengths up to about 310 nm, the fluorescent moieties should be selected to have substantial absorption at wavelengths above 310 nm, and preferably above 400 nm. A variety of suitable fluorescers and chromophores are described by Stryer (Science (1968) 162:526) and by Brand et al. (Annual Rev. Biochem. (1972) 41:843 868). The protein ligands can be labeled with fluorescent chromophore groups by conventional procedures such as those disclosed in U.S. Pat. Nos. 3,940,475, 4,289,747, and 4,376,110. One group of fluorescers having a number of the desirable properties described above is the xanthene dyes, which include the fluoresceins and rhodamines. Another group of fluorescent compounds are the naphthylamines. Once labeled with a fluorophore or chromophore, the protein ligand can be used to detect the presence or localization of the amyloid beta 1-42 in a sample, e.g., using fluorescent microscopy (such as confocal or deconvolution microscopy).

[0321] Immunohistochemistry can be performed using the protein ligands described herein. For example, in the case of an antibody, the antibody can be synthesized with a label (such as a purification or epitope tag), or can be detectably labeled, e.g., by conjugating a label or label-binding group. For example, a chelator can be attached to the antibody. The antibody is then contacted to a histological preparation, e.g., a fixed section of tissue that is on a microscope

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slide. After an incubation for binding, the preparation is washed to remove unbound antibody. The preparation is then analyzed, e.g., using microscopy, to identify if the antibody bound to the preparation.

[0322] The antibody (or other polypeptide or peptide) can be unlabeled at the time of binding. After binding and washing, the antibody is labeled in order to render it detectable.

[0323] The amyloid beta 1-42 antibody can also be immobilized on a protein array. The protein array can be used as a diagnostic tool, e.g., to screen medical samples (such as isolated cells, blood, sera, biopsies, and the like). The protein array can also include other ligands, e.g., that bind to amyloid beta 1-42 or to other target molecules.

[0324] Methods of producing polypeptide arrays are described, e.g., in De Wildt et al. (2000) Nat. Biotechnol. 18:989-994; Lueking et al. (1999) Anal. Biochem. 270:103-111; Ge (2000) Nucleic Acids Res. 28, e3, I-VII; MacBeath and Schreiber (2000) Science 289:1760-1763; WO 01/40803 and WO 99/51773A1. Polypeptides for the array can be spotted at high speed, e.g., using commercially available robotic apparati, e.g., from Genetic MicroSystems or BioRobotics. The array substrate can be, for example, nitrocellulose, plastic, glass, e.g., surface-modified glass. The array can also include a porous matrix, e.g., acrylamide, agarose, or another polymer.

[0325] For example, the array can be an array of antibodies, e.g., as described in De Wildt, supra. Cells that produce the protein ligands can be grown on a filter in an arrayed format. Polypeptide production is induced, and the expressed polypeptides are immobilized to the filter at the location of the cell.

[0326] A protein array can be contacted with a labeled target to determine the extent of binding of the target to each immobilized polypeptide from the diversity strand library. If the target is unlabeled, a sandwich method can be used, e.g., using a labeled probed, to detect binding of the unlabeled target.

[0327] Information about the extent of binding at each address of the array can be stored as a profile, e.g., in a computer database. The protein array can be produced in replicates and used to compare binding profiles, e.g., of a target and a non-target. Thus, protein arrays can be used to identify individual members of the diversity strand library that have desired binding properties with respect to one or more molecules.

[0328] The amyloid beta 1-42 antibody can be used to label cells, e.g., cells in a sample (e.g., a patient sample). The ligand is also attached (or attachable) to a fluorescent compound. The cells can then be sorted using fluorescent activated cell sorted (e.g., using a sorter available from Becton Dickinson Immunocytometry Systems, San Jose Calif.; see also U.S. Pat. Nos. 5,627,037; 5,030,002; and 5,137,809). As cells pass through the sorter, a laser beam excites the fluorescent compound while a detector counts cells that pass through and determines whether a fluorescent compound is attached to the cell by detecting fluorescence. The amount of label bound to each cell can be quantified and analyzed to characterize the sample.

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[0329] The sorter can also deflect the cell and separate cells bound by the ligand from those cells not bound by the ligand. The separated cells can be cultured and/or characterized.

[0330] In still another embodiment, the invention provides a method for detecting the presence of a amyloid beta 1-42 within a subject *in vivo*. The method includes (i) administering to a subject (e.g., a patient having an amyloid beta 1-42 associated disorder such as AD) an anti-amyloid beta 1-42 antibody, conjugated to a detectable marker; (ii) exposing the subject to a means for detecting the detectable marker. For example, the subject is imaged, e.g., by NMR or other tomographic means.

[0331] Examples of labels useful for diagnostic imaging include radiolabels such as ^{131}I, ^{111}In, ^{123}I, ^{99m}Tc, ^{32}P, ^{33}P, ^{125}I, ^{3}H, ^{14}C, and ^{188}Rh, fluorescent labels such as fluorescein and rhodamine, nuclear magnetic resonance active labels, positron emitting isotopes detectable by a positron emission tomography ("PET") scanner, chemiluminescers such as luciferin, and enzymatic markers such as peroxidase or phosphatase. Short range radiation emitters, such as isotopes detectable by short range detector probes can also be employed. The protein ligand can be labeled with such reagents using known techniques. For example, see Wensel and Meares (1983) Radioimmunoimaging and Radioimmunotherapy, Elsevier, N.Y. for techniques relating to the radiolabeling of antibodies and Colcher et al. (1986) Meth. Enzymol. 121: 802 816.

[0332] A radiolabeled ligand can also be used for in vitro diagnostic tests. The specific activity of a isotopically-labeled ligand depends upon the half life, the isotopic purity of the radioactive label, and how the label is incorporated into the antibody.

[0333] Procedures for labeling polypeptides with the radioactive isotopes (such as ^{14}C, ^{3}H, ^{35}S, ^{99m}Tc, ^{125}I, ^{32}P, ^{33}P, and ^{131}I) are generally known. See, e.g., U.S. Pat. No. 4,302,438; Goding, J. W. (Monoclonal antibodies: principles and practice: production and application of monoclonal antibodies in cell biology, biochemistry, and immunology 2nd ed. London; Orlando: Academic Press, 1986. pp 124 126) and the references cited therein; and A. R. Bradwell et al., "Developments in Antibody Imaging", Monoclonal Antibodies for Cancer Detection and Therapy, R. W. Baldwin et al., (eds.), pp 65 85 (Academic Press 1985).

[0334] Amyloid beta 1-42 antibodies described herein can be conjugated to Magnetic Resonance Imaging (MRI) contrast agents. Some MRI techniques are summarized in EP-A-0 502 814. These methods may be used in conjunction with contrast agents. Examples of such contrast agents include a number of magnetic agents paramagnetic agents (which primarily alter T1) and ferromagnetic or superparamagnetic (which primarily alter T2 response). Chelates (e.g., EDTA, DTPA and NTA chelates) can be used to attach (and reduce toxicity) of some paramagnetic substances (e.g., Fe^{3+}, Mn^{2+}, Gd^{3+}). Other agents can be in the form of particles, e.g., less than 10 mm to about 10 nm in diameter and having ferromagnetic, antiferromagnetic, or superparamagnetic properties.

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[0335] The amyloid beta 1-42 antibodies can also be labeled with an indicating group containing the NMR active ^{19}F atom, or a plurality of such atoms as described by Pykett ((1982) Scientific American 246:78-88) to locate and image amyloid beta 1-42 distribution.

Cellular Transfection and Gene Therapy

[0336] The present invention encompasses the use of nucleic acids encoding the antibodies of the invention for transfection of cells *in vitro* and *in vivo*. These nucleic acids can be inserted into any of a number of well-known vectors for transfection of target cells and organisms. The nucleic acids are transfected into cells ex vivo and in vivo, through the interaction of the vector and the target cell. The compositions are administered to a subject in an amount sufficient to elicit a therapeutic response.

[0337] In another aspect, the invention provides a method of treating a target site, i.e., a target cell or tissue, in a human or other animal comprising transfecting a cell with a nucleic acid encoding an antibody of the invention, wherein the nucleic acid comprises an inducible promoter operably linked to the nucleic acid encoding the fusion polypeptide. For gene therapy procedures in the treatment or prevention of human disease, see for example, Van Brunt (1998) Biotechnology 6:1149-1154.

[0338] Non-limiting examples of techniques which can be used to introduce an expression vector encoding the antibodies into a host cell include: Adenovirus-Polylysine DNA Complexes: Naked DNA can be introduced into cells by complexing the DNA to a cation, such as polylysine, which is then coupled to the exterior of an adenovirus virion (e.g., through an antibody bridge, wherein the antibody is specific for the adenovirus molecule and the polylysine is covalently coupled to the antibody) (see Curiel et al. (1992) Human Gene Therapy 3:147-154). Entry of the DNA into cells exploits the viral entry function, including natural disruption of endosomes to allow release of the DNA intracellularly. A particularly advantageous feature of this approach is the flexibility in the size and design of heterologous DNA that can be transferred to cells.

[0339] Receptor-Mediated DNA Uptake: Naked DNA can also be introduced into cells by complexing the DNA to a cation, such as polylysine, which is coupled to a ligand for a cell-surface receptor (see for example Wu et al. (1988) J. Biol. Chem. 263:14621; Wilson et al. (1992) J Biol. Chem. 267:963-967; and U.S. Pat. No. 5,166,320). Binding of the DNA-ligand complex to the receptor facilitates uptake of the DNA by receptor-mediated endocytosis. Receptors to which a DNA-ligand complex have targeted include the transferrin receptor and the asialoglycoprotein receptor. Additionally, a DNA-ligand complex can be linked to adenovirus capsids which naturally disrupt endosomes, thereby promoting release of the DNA material into the cytoplasm and avoiding degradation of the complex by intracellular lysosomes (see for example Curiel et al. (1991) Proc. Natl. Acad. Sci. USA 88:8850; and Cotten et al. (1992) Proc. Natl. Acad. Sci. USA 89:6094-6098; Wagner et al. (1992) Proc. Natl. Acad. Sci. USA 89:6099-6103). Receptor-mediated DNA uptake can be used to introduce DNA into cells either in vitro or

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in vivo and, additionally, has the added feature that DNA can be selectively targeted to a particular cell type by use of a ligand which binds to a receptor selectively expressed on a target cell of interest.

[0340] Liposome-Mediated transfection ("lipofection"): Naked DNA can be introduced into cells by mixing the DNA with a liposome suspension containing cationic lipids. The DNA/liposome complex is then incubated with cells. Liposome mediated transfection can be used to stably (or transiently) transfect cells in culture in vitro. Protocols can be found in Current Protocols in Molecular Biology, Ausubel, F. M. et al. (eds.) Greene Publishing Associates, (1989), Section 9.4 and other standard laboratory manuals. Additionally, gene delivery in vivo has been accomplished using liposomes. See for example Nicolau et al. (1987) Meth. Enz. 149:157-176; Wang and Huang (1987) Proc. Natl. Acad. Sci. USA 84:7851-7855; Brigham et al. (1989) Am. J Med. Sci. 298:278; and Gould-Fogerite et al. (1989) Gene 84:429-438.

[0341] Direct Injection: Naked DNA can be introduced into cells by directly injecting the DNA into the cells. For an in vitro culture of cells, DNA can be introduced by microinjection, although this not practical for large numbers of cells. Direct injection has also been used to introduce naked DNA into cells in vivo (see e.g., Acsadi et al. (1991) Nature 332:815-818; Wolff et al. (1990) Science 247:1465-1468). A delivery apparatus (e.g., a "gene gun") for injecting DNA into cells in vivo can be used. Such an apparatus is commercially available (e.g., from BioRad).

[0342] Retroviral Mediated Gene Transfer: Defective retroviruses are well characterized for use in gene transfer for gene therapy purposes (for a review see Miller, A. D. (1990) Blood 76:271). A recombinant retrovirus can be constructed having a nucleic acid encoding a gene of interest (e.g., an antibody homologue) inserted into the retroviral genome. Additionally, portions of the retroviral genome can be removed to render the retrovirus replication defective. The replication defective retrovirus is then packaged into virions which can be used to infect a target cell through the use of a helper virus by standard techniques. Protocols for producing recombinant retroviruses and for infecting cells *in vitro* or *in vivo* with such viruses can be found in Current Protocols in Molecular Biology, Ausubel, F. M. et al. (eds.) Greene Publishing Associates, (1989), Sections 9.10-9.14 and other standard laboratory manuals. Examples of suitable retroviruses include pLJ, pZIP, pWE and pEM which are well known to those skilled in the art.

[0343] Retroviruses have been used to introduce a variety of genes into many different cell types, including epithelial cells, endothelial cells, lymphocytes, myoblasts, hepatocytes, bone marrow cells, in vitro and/or in vivo (see for example Eglitis, et al. (1985) Science 230:1395-1398; Danos and Mulligan (1988) Proc. Natl. Acad. Sci. USA 85:6460-6464; Wilson et al. (1988) Proc. Natl. Acad. Sci. USA 85:3014-3018; Armentano et al. (1990) Proc. Natl. Acad. Sci. USA 87:6141-6145; Huber et al. (1991) Proc. Natl. Acad. Sci. USA 88:8039-8043; Ferry et al. (1991) Proc. Natl. Acad. Sci. USA 88:8377-8381; Chowdhury et al. (1991) Science 254:1802-1805; van Beusechem et al. (1992) Proc. Natl. Acad. Sci. USA 89:7640-7644; Kay et al. (1992) Human Gene Therapy 3:641-647; Dai et al. (1992) Proc. Natl. Acad. Sci. USA 89:10892-10895; Hwu et

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al. (1993) J Immunol. 150:4104-4115; U.S. Pat. No. 4,868,116; U.S. Pat. No. 4,980,286; and PCT Publication Nos. WO 89/07136; WO 89/02468; WO 89/05345; and WO 92/07573). While any retrovirus may be utilized, the lentivirus approach allows for delivery to a broad variety of cellular targets, both ex vivo (cell lines, primary cells including stem cells, fertilized oocytes, and blastocysts) and in vivo (e.g., brain, lung, liver). The lentivirus vector-mediated delivery of siRNAs allows for the controllable suppression of cellular genes both with a high degree of efficacy and without significant leakiness.

[0344] Adenoviral Mediated Gene Transfer: The genome of an adenovirus can be manipulated such that it encodes and expresses a gene product of interest (e.g., an antibody homologue) but is inactivated in terms of its ability to replicate in a normal lytic viral life cycle. See for example Berkner et al. (1988) BioTechniques 6:616; Rosenfeld et al. (1991) Science 252:431-434; and Rosenfeld et al. (1992) Cell 68:143-155. Suitable adenoviral vectors derived from the adenovirus strain Ad type 5 dl324 or other strains of adenovirus (e.g., Ad2, Ad3, Ad7 etc.) are well known to those skilled in the art. Recombinant adenoviruses are advantageous in that they do not require dividing cells to be effective gene delivery vehicles and can be used to infect a wide variety of cell types, including airway epithelium (Rosenfeld et al. (1992) cited supra), endothelial cells (Lemarchand et al. (1992) Proc. Natl. Acad. Sci. USA 89:6482-6486), hepatocytes (Herz and Gerard (1993) Proc. Natl. Acad. Sci. USA 90:2812-2816) and muscle cells (Quantin et al. (1992) Proc. Natl. Acad. Sci. USA 89:2581-2584). Additionally, introduced adenoviral DNA (and foreign DNA contained therein) is not integrated into the genome of a host cell but remains episomal, thereby avoiding potential problems that can occur as a result of insertional mutagenesis in situations where introduced DNA becomes integrated into the host genome (e.g., retroviral DNA). Moreover, the carrying capacity of the adenoviral genome for foreign DNA is large (up to 8 kilobases) relative to many other gene delivery vectors (Berkner et al. cited supra; Haj-Ahmand and Graham (1986) J Virol. 57:267). Most replication-defective adenoviral vectors currently in use are deleted for all or parts of the viral E1 and E3 genes but retain as much as 80% of the adenoviral genetic material.

[0345] Adeno-Associated Viral Mediated Gene Transfer: Adeno-associated virus (AAV) is a naturally occurring defective virus that requires another virus, such as an adenovirus or a herpes virus, as a helper virus for efficient replication and a productive life cycle. (For a review see Muzyczka et al. Curr. Topics in Micro. and Immunol. (1992) 158:97-129). It is also one of the few viruses that may integrate its DNA into non-dividing cells, and exhibits a high frequency of stable integration (see for example Flotte et al. (1992) Am. J Respir. Cell. Mol. Biol. 7:349-356; Samulski et al. (1989) J Virol. 63:3822-3828; and McLaughlin et al. (1989) J Virol. 62:1963-1973). Vectors containing as little as 300 base pairs of AAV can be packaged and can integrate. Space for exogenous DNA is limited to about 4.5 kb. An AAV vector such as that described in Tratschin et al. (1985) Mol. Cell. Biol. 5:3251-3260 can be used to introduce DNA into cells. A variety of nucleic acids have been introduced into different cell types using AAV vectors (see for

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example Hermonat et al. (1984) Proc. Natl. Acad. Sci. USA 81:6466-6470; Tratschin et al. (1985) Mol. Cell. Biol. 4:2072-2081; Wondisford et al. (1988) Mol. Endocrinol. 2:32-39; Tratschin et al. (1984) J Virol. 51:611-619; and Flotte et al. (1993) J Biol. Chem. 268:3781-3790).

[0346] The efficacy of a particular expression vector system and method of introducing nucleic acid into a cell can be assessed by standard approaches routinely used in the art. For example, DNA introduced into a cell can be detected by a filter hybridization technique (e.g., Southern blotting) and RNA produced by transcription of the introduced DNA can be detected, for example, by Northern blotting, RNase protection or reverse transcriptase-polymerase chain reaction (RT-PCR). Expression of the introduced gene product (e.g., the antibody homologue) in the cell can be detected by an appropriate assay for detecting proteins, for example by immunohistochemistry.

[0347] As will be appreciated by those skilled in the art, the choice of expression vector system will depend, at least in part, on the host cell targeted for introduction of the nucleic acid. For example, nucleic acid encoding a fusion polypeptide is preferably introduced into muscle cells.

[0348] The functional outcome of expression of the antibodies can be assessed by suitable assays that monitor the expression and/or function of the target protein, including standard immunohistochemistry or immunoelectron microscopy techniques.

Kits

[0349] Also within the scope described herein are kits comprising the protein ligand that binds to amyloid beta 1-42 and instructions for diagnostic use, e.g., the use of the amyloid beta 1-42 antibody (e.g., antibody or antigen-binding fragment thereof, or other polypeptide or peptide) to detect amyloid beta 1-42, *in vitro*, e.g., in a sample, e.g., a biopsy or cells from a patient having an amyloid beta 1-42associated disorder, or in vivo, e.g., by imaging a subject. The kit can further contain a least one additional reagent, such as a label or additional diagnostic agent. For *in vivo* use the ligand can be formulated as a pharmaceutical composition.

[0350] The foregoing antibodies can be used in methods known in the art relating to the localization and activity of the amyloid beta, e.g., for Western blotting, imaging amyloid beta in situ, measuring levels thereof in appropriate physiological samples, etc. using any of the detection techniques mentioned herein or known in the art. The standard techniques known in the art for immunoassays are described in "Methods in Immunodiagnosis", 2nd Edition, Rose and Bigazzi, eds. John Wiley & Sons, 1980; Campbell et al., "Methods and Immunology", W.A. Benjamin, Inc., 1964; and Oellerich, M. (1984) J. Clin. Chem. Clin. Biochem. 22:895-904.

[0351] An amyloid beta 1-42 antibody or fragment thereof, can be provided in a kit, e.g., as a component of a kit. For example, the kit includes (a) amyloid beta 1-42 antibody or fragment thereof, e.g., a composition that includes an amyloid beta 1-42 antibody or fragment thereof, and, optionally (b) informational material. The informational material can be descriptive, instructional, marketing, or other material that relates to the methods described herein and/or

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the use of an amyloid beta 1-42 antagonist, e.g., anti- amyloid beta 1-42 antibody or fragment thereof, for the methods described herein.

[0352] The informational material of the kits is not limited in its form. In one embodiment, the informational material can include information about production of the compound, molecular weight of the compound, concentration, date of expiration, batch, or production site information, and so forth. In one embodiment, the informational material relates to using the ligand to treat, prevent, diagnose, prognose, or monitor a disorder described herein.

[0353] In one embodiment, the informational material can include instructions to administer an amyloid beta 1-42 antagonist, e.g., anti- amyloid beta 1-42 antibody or fragment thereof, in a suitable manner to perform the methods described herein, e.g., in a suitable dose, dosage form, or mode of administration (e.g., a dose, dosage form, or mode of administration described herein). In another embodiment, the informational material can include instructions to administer an amyloid beta 1-42 antagonist, e.g., anti- amyloid beta 1-42 antibody or fragment thereof, to a suitable subject, e.g., a human, e.g., a human having, or at risk for, Alzheimer's disease, or an amyloid beta 1-42 mediated disorder.

[0354] For example, the material can include instructions to administer an amyloid beta 1-42 antagonist, e.g., amyloid beta 1-42 antibody or fragment thereof, to a patient, a patient with or at risk for AD.

[0355] The kit can include one or more containers for the composition containing an amyloid beta 1-42 antagonist, e.g., anti- amyloid beta 1-42 antibody or fragment thereof. In some embodiments, the kit contains separate containers, dividers or compartments for the composition and informational material. For example, the composition can be contained in a bottle, vial, or syringe, and the informational material can be contained in a plastic sleeve or packet. In other embodiments, the separate elements of the kit are contained within a single, undivided container. For example, the composition is contained in a bottle, vial, or syringe that has attached thereto the informational material in the form of a label. In some embodiments, the kit includes a plurality (e.g., a pack) of individual containers, each containing one or more unit dosage forms (e.g., a dosage form described herein) of an amyloid beta 1-42 antagonist, e.g., anti- amyloid beta 1-42 antibody or fragment thereof. For example, the kit includes a plurality of syringes, ampules, foil packets, atomizers, or inhalation devices, each containing a single unit dose of an amyloid beta 1-42 antagonist, e.g., anti- amyloid beta 1-42 antibody or fragment thereof, or multiple unit doses.

[0356] The kit optionally includes a device suitable for administration of the composition, e.g., a syringe, inhalant, pipette, forceps, measured spoon, dropper (e.g., eye dropper), swab (e.g., a cotton swab or wooden swab), or any such delivery device. In a preferred embodiment, the device is an implantable device that dispenses metered doses of the ligand.

Screening Assays

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[0357] A still further aspect of the invention relates to screening assays to identify agents which bind amyloid beta e.g. a specific binding member thereof, particularly antibodies or mimics thereof. While not wishing to be bound by theory, these agents may inhibit the production or generation of Abeta by cleavage of APP or the aggregation of amyloid beta fibrils or to enhance the degradation or clearance of Abeta. The invention encompasses both *in vivo* and *in vitro* assays to screen small molecules, compounds, recombinant proteins, peptides, enzymes, nucleic acids, antibodies, etc. which exhibit any of the above activities.

[0358] In a particular embodiment, the binding of the agent is determined through the use of competitive binding assays. The competitor is an antibody of the invention known to bind to amyloid beta protein, including amyloid beta 1-42, or fragments thereof. Competitive screening assays may be done by combining the amyloid beta protein and an antibody of the invention in a first sample. A second sample comprises a test agent, amyloid beta and an antibody of the invention. The binding of the antibody is determined for both samples, and a change, or difference in binding between the two samples indicates the presence of a test agent capable of binding to amyloid beta and potentially modulating its conformational structure and/or activity. That is, if the binding of the antibody is different in the second sample relative to the first sample, the test agent is capable of binding to amyloid beta protein. Similar designs that utilize antibodies of this invention for the identification of non-antibody compounds that bind to amyloid beta are obvious to those skilled in the art.

[0359] One variation provides that the agent is labeled. Either the agent, or the competitor, or both, is added first to amyloid beta protein for a time sufficient to allow binding. Incubations may be performed at any temperature which facilitates optimal activity, typically between 4°C and 40°C. Incubation periods are selected for optimum activity, but may also be optimized to facilitate rapid high-throughput screening. Typically between 0.1 and 1 hour will be sufficient. Excess reagent is generally removed or washed away. The second component is then added, and the presence or absence of the labeled component is followed, to indicate binding.

[0360] It is preferred that the competitor is added first, followed by the test agent. Displacement of the competing antibody of this invention is an indication that the test agent is binding to amyloid beta protein and thus is capable of binding to, and potentially modulating, the conformational structure and/or activity of amyloid beta protein. In this reaction either component may be labeled. Thus, for example, if the competitor is labeled, the presence of label in the wash solution indicates displacement by the agent. Alternatively, if the test agent is labeled, the presence of the label on the support indicates displacement.

[0361] Alternatively, the test agent is added first, with incubation and washing, followed by the competitor. The absence of binding by the competitor may indicate the test agent is bound to amyloid beta protein with a higher affinity. Thus, if the agent is labeled, the presence of the label on the support, coupled with a lack of competitor binding, may indicate the test agent is capable of binding to amyloid beta protein.

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[0362] The agents encompass numerous chemical classes, though typically they are organic molecules, preferably small organic compounds having a molecular weight of more than 100 and less than about 3,500 daltons. Agents comprise functional groups necessary for structural interaction with proteins, particularly hydrogen bonding, and typically include at least an amine, carbonyl, hydroxyl or carboxyl group, preferably at least two of the functional chemical groups. The agents often comprise cyclical carbon or heterocyclic structures and/or aromatic or polyaromatic structures substituted with one or more of the above functional groups. Agents may also be found among biomolecules including peptides, saccharides, fatty acids, steroids, purines, pyrimidines, derivatives, structural analogs or combinations thereof.

[0363] Agents are obtained from a wide variety of sources including libraries of synthetic or natural compounds.

[0364] Alternatively, the antibodies of this invention may be used for the design and synthesis of either peptide or non-peptide compounds (mimetics) that specifically bind to amyloid beta.

[0365] The assays provided use amyloid beta protein, amyloid beta 1-42, or fragments of any of these molecules. In addition, the assays described herein may use either isolated amyloid beta, amyloid beta 1-42, or fragments thereof or cells expressing these molecules or animal models that express these molecules.

[0366] A variety of other reagents may be included in the screening assays. These include reagents like salts, neutral proteins, e.g. albumin, detergents, etc. which may be used to facilitate optimal protein-protein binding and/or reduce non-specific or background interactions. Also, reagents that otherwise improve the efficiency of the assay, such as protease inhibitors, nuclease inhibitors, anti-microbial agents, and the like may be used. The mixture of components may be added in any order that provides for the requisite binding.

[0367] The methods of the invention are used to identify compounds that bind amyloid beta and which may inhibit the production or generation of Abeta through cleavage of APP, such as a gamma or beta secretase inhibitor, or the aggregation of amyloid beta fibrils, or which enhance the degradation or clearance of Abeta, and are therefore useful in the treatment of disorders, diseases or conditions attributed to elevated levels of amyloid beta, in particular, amyloid 1-42 or fragments thereof.

[0368] In the same manner that a small organic molecule or enzyme or mimotope having the desired activity and characteristics noted above, *e.g.* a molecule that blocks the production or generation of Abeta, or blocks the aggregation of amyloid beta, or enhances its degradation or clearance, may be administered to treat AD, so may an antibody specific for amyloid beta, Abeta 1-40, Abeta 1-42 or fragments thereof be administered to treat AD. Accordingly, a further aspect of the invention provides methods for treating AD by administering to a subject with such disorder an antibody which binds to and therefore blocks the functionally significant region(s) of amyloid beta, Abeta 1-42 or fragments thereof. As one of skill in the art may appreciate, the pharmaceutical compositions comprising the antibody and a pharmaceutically acceptable

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carrier, as well as the route of administration of such pharmaceutical compositions, would be similar to those provided above for the other agents of this invention. The optimal quantity and spacing of individual dosages of an agent of the invention will be determined by the nature of the agent, the nature and extent of the disorder being treated, the form, route and site of administration, and the particular animal being treated. Such optimums may be determined by conventional techniques of monitoring clinical depression.

EXAMPLES

[0369] The following Examples and Figures are provided as illustrative examples and not for the purpose of limiting the present invention.

EXAMPLE 1.- Obtaining genetic immunoglobulin repertoires from peripheral blood of healthy elderly donors

Choice of suitable donors for constructing combinatorial human antibody libraries

[0370] A group of 4 healthy elderly individuals with ages comprised between 90-102 years was selected for obtaining human antibody or recombinant antibody fragment libraries. Said non-immunized healthy donors (Vj2, Vj3, Vj4 and Vj5) were chosen as optimal candidates for obtaining antibodies against the beta-amyloid 1-42 peptide for having full cognitive capacity and good health with no symptoms of Alzheimer's or another neuropathy without having received any medication for the prevention or treatment of neurodegenerative diseases.

Determination of specific antibodies in the serum of the chosen donors

[0371] Detection of natural antibodies in the serum of the donors was examined by means of a specific immunodetection assay that allows detecting reactivity against the beta-amyloid 1-42 peptide.

[0372] A specific enzymatic immunodetection assay (ELISA) was carried out to identify natural anti beta-amyloid 1-42 peptide antibodies in human serum, which included optimization of the working dilutions of commercial antibodies as well as the adjustment of the detection of antibodies against the beta-amyloid 1-42 peptide. A commercial mouse anti beta-amyloid 1-42 antibody (Pharmingen) was evaluated by means of plate immunoassay, using human beta-amyloid 1-42 peptide (Pharmingen) at 5 µg/ml as a coating. The working concentration of the secondary antibody to be used was then determined. To that end, several assays were conducted using the secondary antibodies: anti-hFab-HRP (Pierce), anti IgG+IgM+IgA-HRP (Pierce); anti IgG (H+L)-HRP (Pierce), anti IgM (Pharmingen), anti MIg1-biothin (Pharmingen). It was concluded that only two of the tested secondary antibodies present suitable characteristics for the inventors' purpose: anti IgG+IgM+IgA-HRP (Pierce) and anti IgG (H+L) HRP (Pierce) antibody.

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[0373] Once the working concentrations of the commercial anti-beta 1-42 antibody and of the secondary antibody were optimized, the existence of anti beta-amyloid 1-42 peptide antibodies was then detected in the donors' serums. To that end, an ELISA using the beta-amyloid 1-42 peptide (Pharmingen) at a concentration of 5 µg/ml as the coating was performed and several serum dilutions were incubated. The secondary antibody used was anti IgG+IgM+IgA-HRP (Pierce). The results are shown in Figures 1A-1D. In view of these results, reactivity against the beta-amyloid 1-42 peptide (1 µg/ml) of the Vj2 and Vj5 serums was checked by means of dilutions. Anti IgG (H+L)-HRP antibody (Pierce) was used as the secondary antibody. In Figures 2A and 2B, it can be observed how these two serums titrate against the beta-amyloid 1-42 peptide, the serum from donor Vj2 exhibiting the greatest titer of anti beta-amyloid antibodies. Based on the results obtained in the antibody detection assays and on the clinical history, donor Vj2 was chosen as the optimal candidate for isolating the genetic repertoire of immunoglobulins and the construction of libraries for selecting antibodies against Alzheimer's disease.

Obtaining the immunoglobulin repertoire

Purification of peripheral blood B cells and obtaining the RNA mold

[0374] Peripheral blood lymphocytes (between 0.1 and 2.10^8 cells) of the selected donor (Vj2) were obtained by means of Ficoll-Hypaque density gradient centrifugation. The genetic material (mRNA) was extracted using commercial product Tri-Reagent following the manufacturer's protocol. Integrity and purity of the extracted material was analyzed by means of agarose/high resolution formaldehyde gels and analytically using the bio-sizing method for its quantification. After checking the integrity of each sample, the resulting material was considered satisfactory in terms of quality and integrity and was stored at -80 °C for its subsequent processing into aliquots suitable for being used as a mold for amplification of all the genetic families of the immunoglobulin genes.

[0375] The obtained samples were used for constructing the following libraries:

- Antibody fragment (Fab) library
- Single chain antibody fragment (scFv) library

Obtaining the immunoglobulin gene repertoire by means of PCR

[0376] Single chain complementary DNA (cDNA) was synthesized from mRNA by means of reverse transcriptase reaction, using deoxythimine octomers (oligo-dt) (Pharmacia) as primers. The material was aliquoted and used as a mold to amplify the fragments corresponding to the heavy chains (HC) of the IgG1 immunoglobulins and the kappa light chains (LC) by PCR. PCR amplification was done using standard protocols and in an independent manner. The different IgG1 fragments were obtained independently, using various single chain oligonucleotides, representative of all families of variable genes (VH) as primers, together with the Cγ1 specific primer of the first gamma-1 chain constant domain (CH1). The kappa light chains (LC) were

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amplified in a similar manner, using primers representative of each VK family, together with kappa constant region (CK) specific primers. In addition to containing sequences specific for the amplification of variable or constant genes, all the primers include specific restriction targets not present in the genes for the purpose of facilitating their cloning. The pairing temperature (Tp) used for each pair of primers was optimized in prior experiments for the purpose of achieving amplification of about 95% of the genes of each VH and VK family.

[0377] The PCR products (HC or LC) for each amplified family were isolated from purified, quantified and proportionally combined 2% agarose gels in order to obtain the final repertoire (Figures 3A and 3B). This material was used as a mold in a second PCR amplification using external oligonucleotides for the purpose of extending the products, facilitating the subsequent digestion with restriction enzymes and increasing their cloning efficiency. The results are illustrated in Figure 3C. Once amplified, the products of each repertoire (HC-G1 and LC-kappa) were independently purified. The VH repertoires were digested with restriction enzymes Xho I and SpeI and were purified from agarose gels by Quiackick. The LC-kappa repertoire was digested with Sac I and XbaI and purified in the same manner.

[0378] Additionally, the same starting material was used to obtain a single chain antibody fragment (scFv) repertoire by means of using specific primers. The approach used allows obtaining from the same donor two antibody libraries: on in Fab (antibody fragments) format and another one in scFv fragment. Said single chain fragments consist of VH segments fused by means of a flexible linker (G_4S_2) to the light chain variable region. All amplifications were previously optimized until obtaining a yield of 90-100%. The results are illustrated in Figures 4A and 4B. Once the amplifications were carried out, the positive bands representative of each family were extracted and purified from preparative gels, and the material was subsequently fused by means of PCR overlap for its combined amplification using for that purpose primers RSC-F and RSC-B (Figure 4C). The obtained bands were finally digested and purified.

EXAMPLE 2.- Construction of high-complexity combinatorial antibody libraries

Fab fragment library

[0379] The Fab fragment library was constructed by means of cloning and assembly, using the gamma-1 repertoires combined with all the kappa light chains in Fab format, using the phagemid vector pComb3 XX. This vector allows cloning the CLs at restriction sites SacI-Xba I and the HCs at restriction sites XhoI–SpeI. The libraries were constructed sequentially. First the corresponding light chain repertoire was cloned and then the heavy chain repertoire was cloned.

[0380] Preparation of vector pComb3 XX SacI/XbaI was optimized in pilot experiments until obtaining a high CL ligation level (>$1x10^8$ transforming colonies/µg of DNA) and a low religation efficiency (<1%) in the absence of inserts, before cloning the kappa LC repertoire. Then, all the ligation product was used to transform highly competent E. coli bacteria (Efficiency > 5.10^9

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transforming colonies/µg of DNA) by means of electroporation. After bacterial growth, the entire bacterial culture was centrifuged at low speed and duly treated to extract the phagemid DNA. The DNA was purified, quantified and subjected to digestion with restriction enzymes XhoI and SpeI. The resulting vector was quantified and used for cloning the HC-IgG1 repertoires. After precipitation, the ligation products were transformed independently by means of electroporation in E. coli and the final assembly was carried out by means of infection with M13 helper phage to construct a kappa-IgG1 repertoire antibody expression library of $1x\ 10^7$ antibodies.

Single chain fragment (scFv) library

[0381] The single chain antibody fragment (scFv) library of the same donor (Vj2) was constructed by means of cloning single fragments of the variable regions of the immunoglobulins corresponding to the Gamma and Kappa repertoires overlapped by means of a flexible linker G_4S_2. After enzymatic digestion, the repertoires were cloned into vector pComB3XX. The obtained size was 5×10^6 independent clones.

Checking the diversity of antibody libraries by HCDR3 segment sequencing

[0382] The genetic material of 15-20 antibody-producing clones was extracted from each library. Random sequences of the HCDR3 segment indicated that the libraries consist of a wide diversity, both in the size of HC CDR3 (6-21 amino acids long), and in their sequence. As an example, Figure 5 shows the HCDR3 sequences of several clones chosen randomly from the antibody fragment (Fab) library and from the single chain antibody fragment (scFv) library.

[0383] Human antibody libraries were obtained including two formats (Fab and scFv). Said libraries represent a broad collection of human antibodies and constitute a highly useful tool in the search for specific antibodies directed against the beta-amyloid 1-42 peptide.

EXAMPLE 3.- Antigen selection from the single chain fragment library

[0384] The antigen selection against the beta-amyloid 1-42 peptide of the single chain fragment (scFv) library was carried out by means of an immunoassay against the beta-amyloid 1-42 peptide immobilized in solid phase (ELISA), using BSA as a negative control (c-). In each cycle, the scFv fragment library was amplified up to 10^{13} clones/ml and selected in the previously determined conditions, increasing the astringency of the washings in each selection round. The peptide concentrations to be used in the selection against the peptide amyloid 1-42 were previously optimized in solid phase. A significant degree of selection was obtained in the antigen selection process, as illustrated in Figure 6.

EXAMPLE 4.- Identification of high-affinity specific target human antibodies

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[0385] After antigen selection from the single chain fragment library, the identification of antibody clones specific against the beta amyloid peptide was carried out by means of transfection of the genetic material of the antibodies obtained in bacteria suitable for the expression of soluble scFv molecules.

[0386] In the antigenic selection process, clones were chosen randomly from the total number of clones obtained in the last selection cycle and were arranged in master culture dishes. Antibody production in bacterial clones was optimized by means of pilot assays to obtain maximum expression. After determining the optimal conditions 235 clones chosen randomly from the master were analyzed. Each bacterial clone was grown in optimal culture conditions for antibody production and these were finally extracted from the supernatant for their characterization.

EXAMPLE 5.- Characterization of the obtained human antibodies against the beta-amyloid 1-42 peptide

[0387] After the identification of specific human antibodies, the genetic, structural and functional characterization of said antibodies were performed.

[0388] The antibodies obtained from the selection of the single chain antibody fragment library were analyzed by means of immunoassay specific against the beta-amyloid 1-42 peptide and BSA. A total of 5 recombinant antibodies showing specific reactivity against the beta-amyloid 1-42 peptide were obtained and the remaining antibodies showed reactivity against beta amyloid 1-42 and BSA. Two of said recombinant antibodies, AD38 and AD64, showed better beta-amyloid 1-42 peptide-binding profiles. These antibodies were purified to mid-scale (0.5-1 µg/ml) and evaluated at different concentrations in immunoassay against peptide 1-42. The obtained results are summarized in Figures 7A and 7B, in which it is seen that both antibodies specifically recognize the target binding in a dose-dependent manner.

[0389] Genetic characterization was carried out by means of the automatic determination of the nucleotide sequences. Reading and edition was conducted using the SeqEd program (Applied Biosystems) and subsequent analysis was conducted using GCG (University of Wisconsin Genetic Computer Group-Madison, WI) and Analize (MacMolly ®Tetra Sof Gen GmbH (version 3.10)). Identity of the VH, DH, JH and VK, JK genes was determined by using FASTA (GenBank), Blast and IgBlast (NCBI) together with MacMolly (Aling and Complign) and DNAPLOT (Version 2.0.1) locally applied to the human antibody database VBASE (Tomlinson et al., MRC Centre for Protein Engineering, UK) (Tomlinson, 1992). The index of replacement and silent (R:S) mutations observed in the HC and LC, after comparison with the corresponding genomic genes (germline), was calculated manually. The theoretical or inherent R:S indexes or each germinal gene were calculated individually for each codon and region using the InhSusCalc v1.0 program.

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[0390] Once the nucleotide sequences of the scFv antibody fragments of AD38 (SEQ ID NO: 9) and AD64 (SEQ ID NO: 11) were known, said recombinant antibodies were used as the only antibodies. Their heavy chains are derived from the VHIII gene family and the light chains are derived from the VKIII and VKII gene families. Table 1 summarizes the genetic derivation and sequences of the CDR3 (Complementary-Determining Region-3) segments of recombinant antibodies AD38 and AD64; and Table 2 shows the R:S indexes. Numerous somatic mutations in comparison to their corresponding genomic genes (germline) were observed in both antibodies, indicating the existence of antigen maturation processes against the beta-amyloid 1-42 peptide.

[0391] Both the functional analysis and the genetic characteristics found after sequencing indicate that antibodies AD38 and AD64 are specific, exhibit antigenic maturation characteristics and reflect part of a competent immune response against the beta amyloid peptide. This makes them and antibodies derived from their structures excellent candidates as diagnostic, therapeutic or imaging agents.

EXAMPLE 6

Proposed Treatment of Subjects

[0392] A single-dose phase I trial can be performed to determine safety of the anti-amyloid beta 1-42 antibody. A therapeutic agent can be administered in increasing dosages to different patients starting from about 0.01mg per patient to a level of presumed efficacy, and increasing by a factor of three until a level of about 10 times the proposed effective dosage is reached.

[0393] A phase II trial can be performed to determine therapeutic efficacy of the anti-amyloid beta 1-42 antibody. Patients with early to mid Alzheimer's Disease defined using Alzheimer's disease and Related Disorders Association (ADRDA) criteria for probable AD will be selected. Suitable patients score in the 12-26 range on the Mini-Mental State Exam (MMSE). Other selection criteria are that patients are likely to survive the duration of the study and lack complicating issues such as use of concomitant medications that may interfere. Baseline evaluations of patient function can be made using classic psychometric measures, such as the MMSE, and the ADAS, which is a comprehensive scale for evaluating patients with Alzheimer's Disease status and function. These psychometric scales provide a measure of progression of the Alzheimer's condition. Suitable qualitative life scales can also be used to monitor treatment. Disease progression can also be monitored by MRI. Blood profiles of patients can also be monitored including assays of immunogen-specific antibodies and T-cells responses.

[0394] Following baseline measures, patients can begin receiving treatment. They will be randomized and treated with either therapeutic agent or placebo in a blinded fashion. Patients will be monitored at least every six months. Efficacy will be determined by a significant reduction in progression of a treatment group relative to a placebo group.

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[0395] A second phase II trial can be performed to evaluate conversion of patients from non-Alzheimer's Disease early memory loss, sometimes referred to as age-associated memory impairment (AAMI), to probable Alzheimer's disease as defined as by ADRDA criteria. Patients with high risk for conversion to Alzheimer's Disease may be selected from a non-clinical population by screening reference populations for early signs of memory loss or other difficulties associated with pre-Alzheimer's symptomatology, a family history of Alzheimer's Disease, genetic risk factors, age, sex, and other features found to predict high-risk for Alzheimer's Disease. Baseline scores on suitable metrics including the MMSE and the ADAS together with other metrics designed to evaluate a more normal population will be collected. These patient populations will be divided into suitable groups with placebo comparison against dosing alternatives with the agent. These patient populations will be followed at intervals of about six months, and the endpoint for each patient is whether or not he or she converts to probable Alzheimer's Disease as defined by ADRDA criteria at the end of the observation.

[0396] **Table 1**

Antibody	**VH germline**	**DH germline**	**JH germline**	**HCDR3**
AD38	COS-6/DA-8	D6-6/DN4	JH4a	ELRGWSIEY
AD64	DP-38/9-1	D2	JH3a	DDYDF
Antibody	**Vk germline**		**JL germline**	**LCDR3**
AD38	Vg/38K		JK5	QHRRDWPPGAT
AD64	DPK15/A19		JK4	MQALHNPLT

[0397] **Table 2**

Heavy chain (HC)							
Antibody	**FR1**	**CDR1**	**FR2**	**CDR2**	**FR3**	**FRs R:S**	**CDRs R:S**
AD38	5:2	1:0	2:1	8:4	8:3	15:6	9:4
AD64	6:3	1:1	0:0	10:2	7:6	13:9	11:3
Light chain (LC)							
Antibody	**FR1**	**CDR1**	**FR2**	**CDR2**	**FR3**	**FRs R:S**	**CDRs R:S**
AD38	5:2	4:0	3:2	3:0	4:1	12:5	7:0
AD64	4:0	3:0	1:1	2:1	2:1	7:2	5:1

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[0398] Biological Material Deposit

An *Escherichia coli* XL 1-B culture containing the AD38 encoding plasmid vector has been deposited pursuant to, and in satisfaction of, the requirements of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure with the Spanish Type Culture Collection (CECT) (Colección Española de Cultivos Tipo), Burjassot, Valencia (Spain), on March 28, 2006, under the access number CECT 7167.

Also, an *Escherichia coli* XL 1-B culture containing the AD64 encoding plasmid vector has been deposited pursuant to, and in satisfaction of, the requirements of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure with the Spanish Type Culture Collection (CECT) (Colección Española de Cultivos Tipo), Burjassot, Valencia (Spain), on March 28, 2006, under the access number CECT 7168.

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Applicant's or agent's file reference P1480PC00	International application No. PCT/EP2006/003363

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Address of depositary institution *(including postal code and country)*
Universidad de Valencia
Campus de Burjassot
Edificio de Investigaciópn
46100 Burjassot (Valencia)
Spain

Date of deposit	Accession Number
March 28, 2006	CECT 7167

C. ADDITIONAL INDICATIONS *(leave blank if not applicable)* This information is continued on an additional sheet ☐

Escherichia coli XL 1-B culture containing the AD38 encoding plasmid vector

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Date of deposit	Accession Number
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Escherichia coli XL 1-B culture containing the AD64 encoding plasmid vector

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WHAT IS CLAIMED IS:

1. An isolated specific binding member which recognizes an epitope on amyloid beta 1-42, wherein said binding member is obtained from healthy elderly subjects having full cognitive capacity and who exhibit no symptoms of Alzheimer's disease or other neuropathies.

2. The isolated specific binding member of claim 1, wherein the subjects from which the binding member is obtained range in age from 60 years of age and above, 70 years of age and above, 80 years of age and above or 90 years of age and above.

3. An isolated specific binding member of claim 1, wherein the binding member is an antibody having amyloid beta 1-42 binding capacity, and wherein the antibody comprises an amino acid sequence of SEQ ID NOs: 2, or 4, or an amino acid sequence that is substantially homologous to SEQ ID NOs: 2 or 4, and functional fragments, derivatives or variants thereof.

4. An antibody, functional fragment or derivative thereof with beta-amyloid 1-42 peptide-binding capacity comprising one or more of the amino acid sequences selected from the group consisting of SEQ ID NO: 2, SEQ ID NO: 4 and sequences substantially homologous to either one of SEQ ID NO: 2 or SEQ ID NO: 4.

5. An antibody, functional fragment thereof or derivative with beta-amyloid 1-42 peptide-binding capacity, according to claim 4, comprising at least one heavy chain and one light chain, wherein the heavy chain comprises the amino acid sequence of SEQ ID NO: 2 and the light chain comprises the amino acid sequence of SEQ ID NO: 4.

6. An antibody, functional fragment or derivative thereof with beta-amyloid 1-42 peptide-binding capacity comprising one or more of the amino acid sequences selected from the group consisting of SEQ ID NO: 6, SEQ ID NO: 8 and sequences substantially homologous to either one of SEQ ID NO: 6 or SEQ ID NO: 8.

7. An antibody, functional fragment thereof or derivative with beta-amyloid 1-42 peptide-binding capacity, according to the claim 6, comprising at least one heavy chain and one light chain, wherein the heavy chain comprises the amino acid sequence of SEQ ID NO: 6 and the light chain comprises the amino acid sequence of SEQ ID NO: 8.

8. An isolated specific binding member which recognizes amyloid beta 1-42, wherein said binding member is obtained by means of affinity maturation strategies from the antibodies of any one of claims 1-7.

9. A specific binding member or functional antibody fragment or derivative thereof according to any of claims 1 to 8, wherein said fragment is a scFv.

10. A specific binding member, or antibody, functional fragment thereof or derivative thereof, of any one of claims 1-8, which is detectably labeled.

11. The specific binding member, or antibody, functional fragment thereof or derivative thereof, of claim 10, wherein the detectable label is selected from an enzyme, a radioisotope, a fluorophore and a heavy metal.

12. A specific binding member, or antibody, functional fragment thereof or derivative thereof, of any one of claims 1-9 which is attached to a drug.

13. A specific binding member, or antibody, functional fragment thereof or derivative thereof, comprising a functional or binding portion of any one of the sequences set forth in claims 3 to 7 or combinations thereof with beta-amyloid 1-42 peptide-binding capacity.

14. An isolated nucleic acid molecule selected from the group consisting of:

a. a nucleic acid molecule encoding a specific binding member, or antibody, functional fragment thereof or derivative thereof,, according to any one of claims 1 to 7;

b. a nucleic acid molecule the nucleotide sequence of which differs from the sequences defined in (a) due to degeneration of the genetic code; and

c. a nucleic acid molecule substantially homologous to the sequences defined in (a).

15. The isolated nucleic acid of claim 14 encoding a binding member which is an antibody molecule or a fragment, derivative or variant thereof, which binds amyloid beta 1-42.

16. The isolated nucleic acid molecule of claim 15, comprising any one of the nucleotide sequences set forth in SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 5 or SEQ ID NO: 7, or combinations thereof.

17. A vector comprising a nucleic acid molecule according to any of claims 14,15 or 16.

18. A host cell containing a vector according to claim 17.

19. A unicellular host transformed with:

(A) the DNA sequence of SEQ ID NO:1;

(B) the DNA sequence of SEQ ID NO:3;

(C) the DNA sequence of SEQ ID NO:1 and the DNA sequence of SEQ ID NO:3;

(D) a DNA sequence that hybridizes to any of the foregoing DNA sequences under standard hybridization conditions;

(E) a DNA sequence that codes on expression for an amino acid sequence encoded by any of the foregoing DNA sequences; or

(F) a fragment of any one of the foregoing DNA sequences which encodes a specific binding member, or antibody, functional fragment thereof or derivative thereof, as defined in any one of claims 1 to 7,

wherein said DNA sequence is operatively linked to an expression control sequence.

20. A unicellular host transformed with:

(A) the DNA sequence of SEQ ID NO:5;

(B) the DNA sequence of SEQ ID NO:7;

(C) the DNA sequence of SEQ ID NO:5 and the DNA sequence of SEQ ID NO:7;

(D) a DNA sequence that hybridizes to any of the foregoing DNA sequences under standard hybridization conditions;

(E) a DNA sequence that codes on expression for an amino acid sequence encoded by any of the foregoing DNA sequences; or

(F) a fragment of any one of the foregoing DNA sequences which encodes a specific binding member, or antibody, functional fragment thereof or derivative thereof, as defined in any one of claims 1 to 7,

wherein said DNA sequence is operatively linked to an expression control sequence.

21. The unicellular host of either one of claims 19 or 20 wherein the unicellular host is selected from the group consisting of E. coli, Pseudomonas, Bacillus, Streptomyces, yeasts, CHO, YB/20, NSO, SP2/0, R1.1, B-W, L-M, COS 1, COS 7, BSC1, BSC40, and BMT10 cells, plant cells, insect cells, and human cells in tissue culture.

22. The use of a specific binding member, or antibody, fragment thereof or derivative thereof according to any of claims 1 to 8, 9, 12 or 13 for preparing a pharmaceutical composition for the treatment of Alzheimer's disease.

23. A pharmaceutical composition comprising a specific binding member, or antibody, fragment thereof or derivative thereof according to any of claims 1 to 8, 9, 12 or 13, or an isolated nucleic acid molecule of claims 14-16, for use in the treatment of Alzheimer's disease.

24. A pharmaceutical composition according to claim 22, for use in treating or preventing the progression of Alzheimer's disease, or for the amelioration of symptoms associated with Alzheimer's disease, or for diagnosing or screening a subject for the presence of Alzheimer's disease or for determining a subject's risk for developing Alzheimer's disease, said composition comprising a binding member that recognizes amyloid beta 1-42 and a pharmaceutically acceptable carrier.

25. The use of a specific binding member, or antibody, fragment thereof or derivative thereof according to any of claims 1 to 8, 9, 12 or 13, for the manufacture of a medicament for the treatment of Alzheimer's disease.

26. The pharmaceutical composition of claim 22 or the use of claim 25, wherein the binding member is an antibody comprising one or more of the amino acid sequences set forth in SEQ ID NOs: 2, 4, 6, or 8 or amino acid sequences having substantial homology to one or more of the sequences set forth in SEQ ID NOs: 2, 4, 6 or 8.

27. The pharmaceutical composition of claim 24 or the use of claim 25, wherein the binding member is a scFv.

28. The pharmaceutical composition of claim 24 or the use of claim 25, wherein said composition or medicament further comprises an additional agent useful for treating Alzheimer's disease, selected from the group consisting of small organic molecules, other amyloid beta antibodies, and combinations thereof.

29. A process of obtaining antibodies or functional fragments thereof with beta-amyloid 1-42 peptide-binding capacity from the genetic repertoire of immunoglobulins isolated from healthy donors, comprising the steps of:

A) purifying B cells from a sample, said sample being obtained from a subject fulfilling the following criteria;
   (i) being 60 years of age or older;
   (ii) having full cognitive capacity and good health;
   (iii) having no symptoms of Alzheimer's disease or another neuropathy;
   (iv) not having previously received any medication for the prevention or treatment of neurodegenerative diseases; and
   (v) possessing detectable anti beta-amyloid 1-42 peptide antibodies in the blood serum;
B) obtaining the immunoglobulin gene repertoire for said anti beta-amyloid 1-42 peptide antibodies from said B cells;
C) using said repertoire to express said antibodies.

30. The process of claim 29, wherin step B) comprises the steps of:
   (i) obtaining mRNA from the said B cells;
   (ii) obtaining cDNA from the mRNA of step (d); and
   (iii) using a primer extension reaction to amplify from said cDNA the fragments corresponding to the heavy chains (HC) and the kappa light chains (LC) of said anti beta-amyloid 1-42 peptide antibodies.

31. An antibody, functional fragment thereof or derivative with beta-amyloid 1-42 peptide-binding capacity obtained by a process according to claims 29 and 30.

32. The binding member or antibody, fragment or derivative thereof, according to any of claims 1 to 8, 9, 12 or 13 for use as a medicament.

33. A pharmaceutical composition comprising a binding member or an antibody, fragment or derivative thereof, according to any of claims 1 to 8, 9, 12 or 13 and a pharmaceutically acceptable excipient.

34. The use of a binding member or an antibody, fragment or derivative thereof, according to any of claims 1 to 8, 9, 12 or 13 for the *in vitro* detection of the beta-amyloid 1-42 peptide.

35. The use of a binding member or an antibody, fragment or derivative thereof, according to any of claims 1 to 8, 9, 12 or 13 for the *in vivo* detection of the beta-amyloid 1-42 peptide.

36. The use of a binding member or an antibody, fragment or derivative thereof, according to any of claims 1 to 8, 9, 12 or 13 for the preparation of a composition for the *in vivo* detection of the beta-amyloid 1-42 peptide.

37. The use of a binding member or an antibody, fragment or derivative thereof, according to any of claims 1 to 8, 9, 12 or 13, for the identification of a compound able to compete with said antibody, fragment or derivative.

38. A specific binding member or antibody or fragment, derivative or variant thereof according to any one of claims 1 to 8, 9, 12 or 13 for use in a method of treatment or diagnosis of the human or animal body.

39. A method of preparing a specific binding member capable of binding an epitope on amyloid beta 1-42, which method comprises:

a) providing a starting repertoire of nucleic acids encoding a VH domain which lacks a CDR3 encoding region;

b) combining said repertoire with a donor nucleic acid encoding an amino acid sequence substantially as set out in SEQ ID NOs: 2, 4, 6 or 8 such that said donor nucleic acid is inserted into the missing CDR3 region, so as to provide a product repertoire of nucleic acids encoding a VH domain;

c) expressing the nucleic acids of said product repertoire; and

d) selecting a specific binding member which recognizes amyloid beta 1-42; and

e) recovering said binding member or the nucleic acid encoding it.

40. A method of treating or preventing the progression of Alzheimer's disease in a subject, or for ameliorating the symptoms associated with Alzheimer's disease, or for diagnosing or screening a subject for the presence of Alzheimer's disease or for determining a subject's risk for developing Alzheimer's disease, which method comprises administering to said subject an effective amount of a specific binding member or antibody or fragment, derivative or variant thereof, as defined in any one of claims 1 to 8, 9, 12 or 13.

41. A method of treating or preventing the progression of Alzheimer's disease in a subject, or for ameliorating the symptoms associated with Alzheimer's disease, or for diagnosing or screening a subject for the presence of Alzheimer's disease or for determining a subject's risk for developing Alzheimer's

disease, which method comprises administering to said subject an effective amount of the pharmaceutical composition of any one of claims 23, 24 or 26 to 28.

42. A kit for the diagnosis of Alzheimer's disease, said kit comprising a specific binding member or antibody of any one of claims 1 to 8, 9, 12 or 13, optionally with reagents and/or instructions for use.

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FIG. 1A

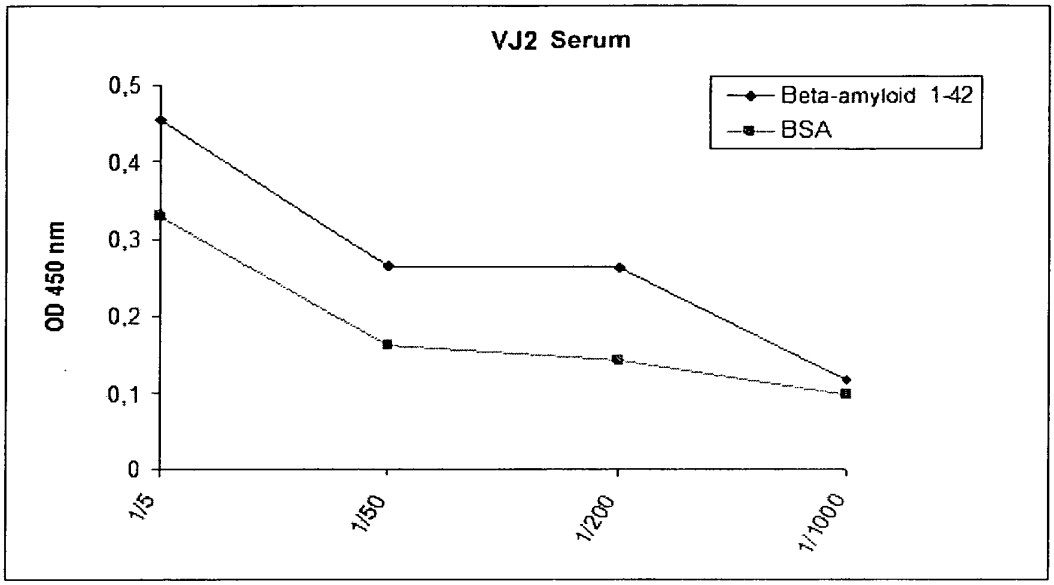

VJ2 Serum
Beta-amyloid 1-42
BSA
OD 450 nm
0,5
0,4
0,3
0,2
0,1
0
1/5
1/50
1/200
1/1000

FIG. 1B

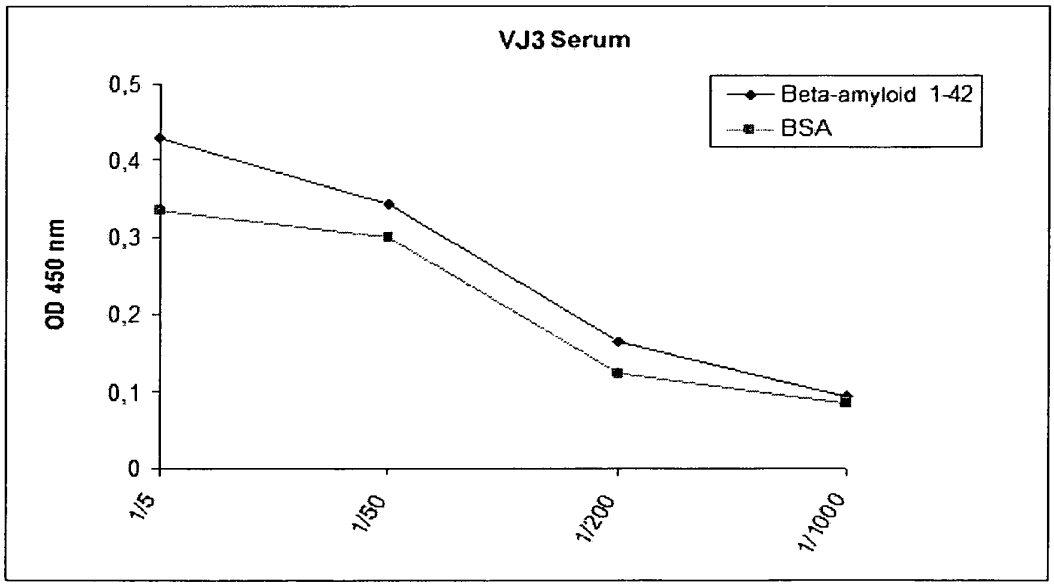

VJ3 Serum
Beta-amyloid 1-42
BSA
OD 450 nm
0,5
0,4
0,3
0,2
0,1
0
1/5
1/50
1/200
1/1000

FIG. 1C

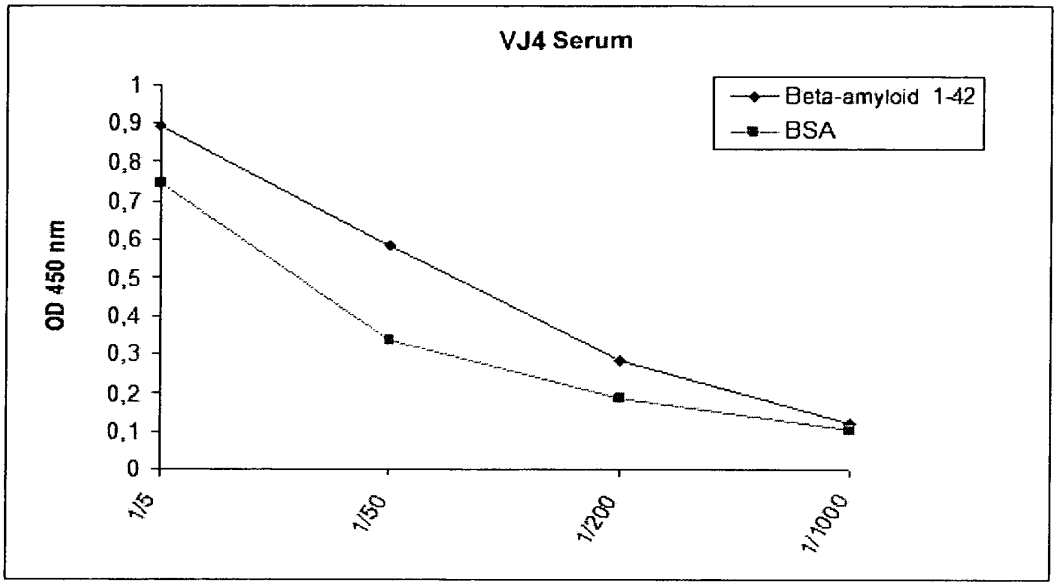

VJ4 Serum
Beta-amyloid 1-42
BSA
OD 450 nm
1
0,9
0,8
0,7
0,6
0,5
0,4
0,3
0,2
0,1
0
1/5
1/50
1/200
1/1000

FIG. 1D

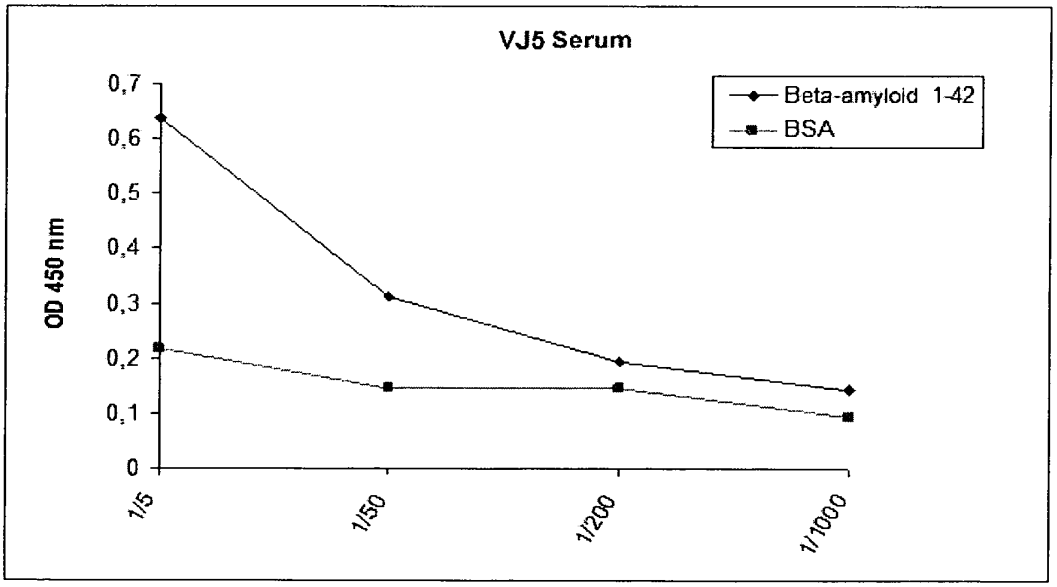

VJ5 Serum
Beta-amyloid 1-42
BSA
OD 450 nm
0,7
0,6
0,5
0,4
0,3
0,2
0,1
0
1/5
1/50
1/200
1/1000

FIG. 2A

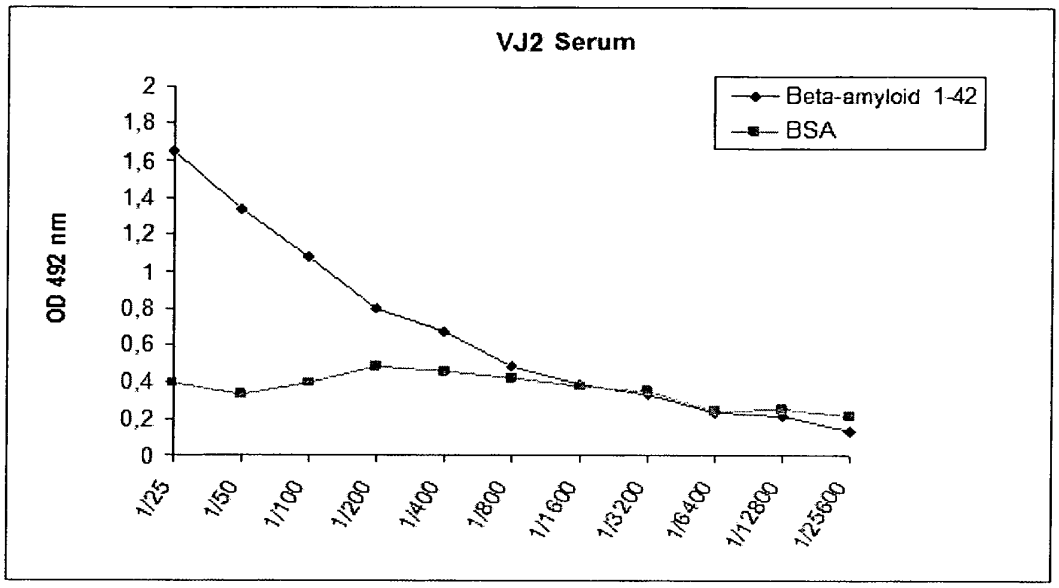

VJ2 Serum
Beta-amyloid 1-42
BSA
OD 492 nm
2
1,8
1,6
1,4
1,2
1
0,8
0,6
0,4
0,2
0
1/25
1/50
1/100
1/200
1/400
1/800
1/1600
1/3200
1/6400
1/12800
1/25600

FIG. 2B

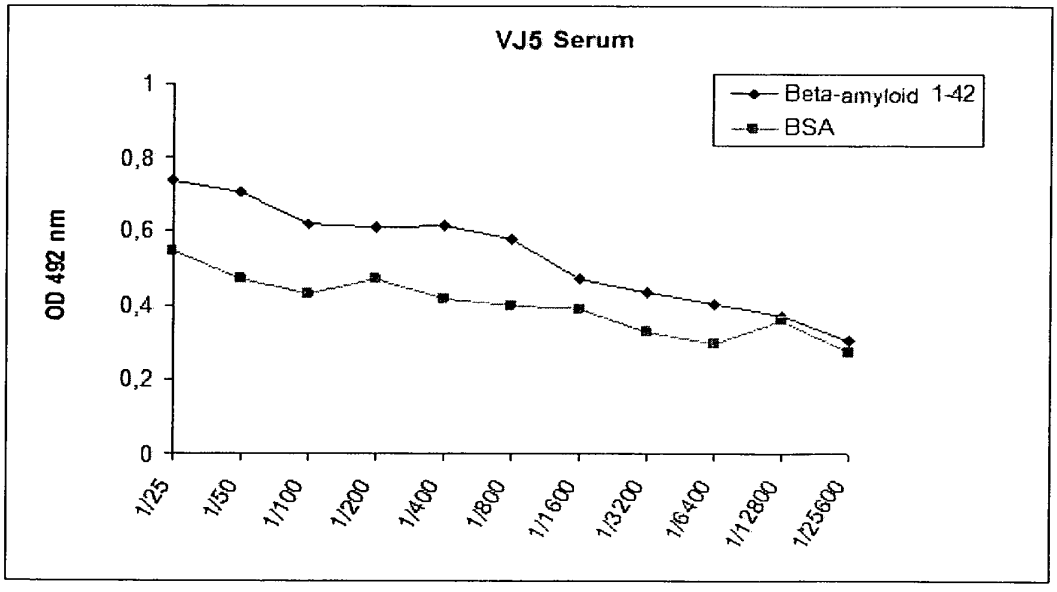

VJ5 Serum
Beta-amyloid 1-42
BSA
OD 492 nm
1
0,8
0,6
0,4
0,2
0
1/25
1/50
1/100
1/200
1/400
1/800
1/1600
1/3200
1/6400
1/12800
1/25600

FIG. 3A

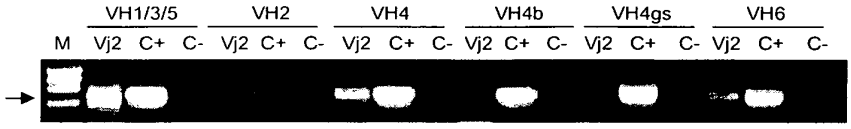


FIG. 3B

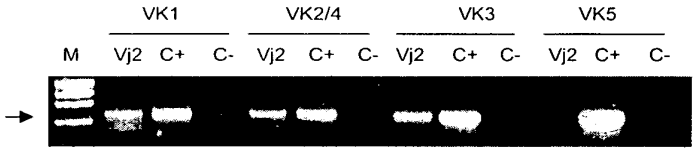


FIG. 3C

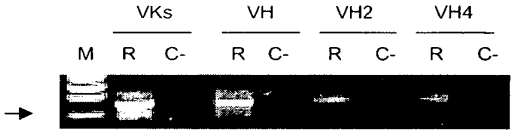


FIG. 4A

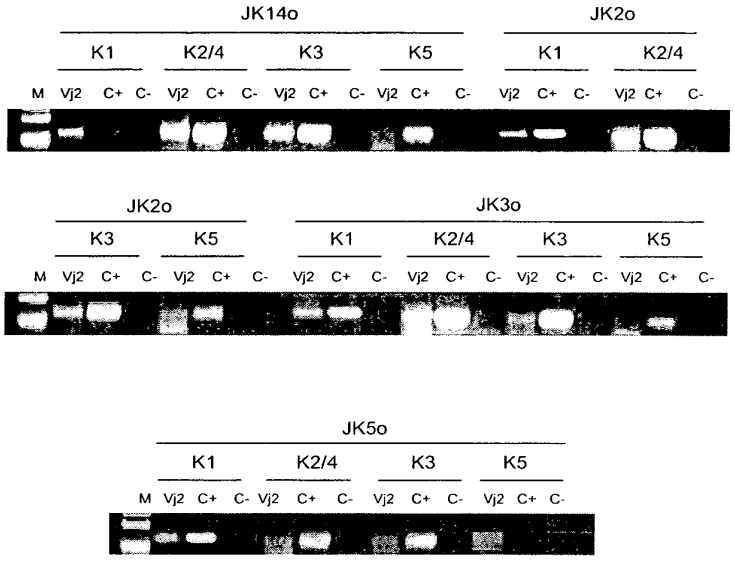


FIG. 4B

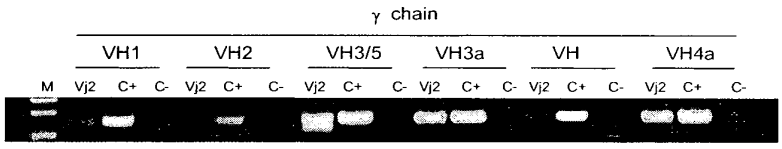


FIG. 4C

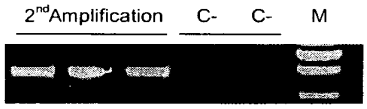


FIG. 5

YYF	/ CLQHDSFPRT(10)	/ FGQG	CAR	/ LSEYYSRAHGMDV(13)	/ WGQG
YYC	/ QQYGSSLT(8)	/ FGGG	CAR	/ DRGLGWLRFPL(10)	/ WGQG
YYC	/ QQSYSYPLT(9)	/ FGQG	CAR	/ DGYDFWSGYRMDV(13)	/ WGQG
YYC	/ QQRNNWPLT(9)	/ FGGG	CAR	/ GGTGEDWYFDL(11)	/ WGQG
YYC	/ QQYNSYSWT(9)	/ FGQG	CAR	/ DGAPLDYKNSGYYMDV(16)	/ WGEG
YYC	/ QQYGTSWT(8)	/ FGQG	CAT	/ DPLTVPTGPLTTGTMET(17)	/ WGEG
YYC	/ QQIYSSPPEST(11)	/ FGQG	CAR	/ LRYLKEGFDY(10)	/ WGQG
YYC	/ QQRSNWPPSLT(11)	/ FGGG	CAT	/ GDVFDV(6)	/ WGQG
YYC	/ QQYGSSEWT(9)	/ FGQG	CNR	/ DPPPSIGVESPAIGDDY(17)	/ WGQG
YYC	/ QQRNSWPWT(9)	/ FGQG	CAR	/ ESPTAGDYSEDY(12)	/ WCQD
			CAR	/ HQQLYYYYIDV(11)	/ WGKG
			CAL	/ TSGWYPRSASEI(12)	/ WDGQ
			CAR	/ VSRYDYKSSLKY(12)	/ WGQG
			CAR	/ DPYGANGPSNDT(12)	/ WGAG

FIG. 6

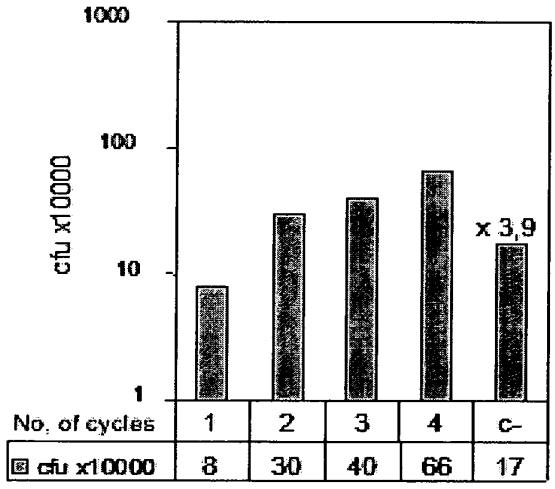


FIG. 7A

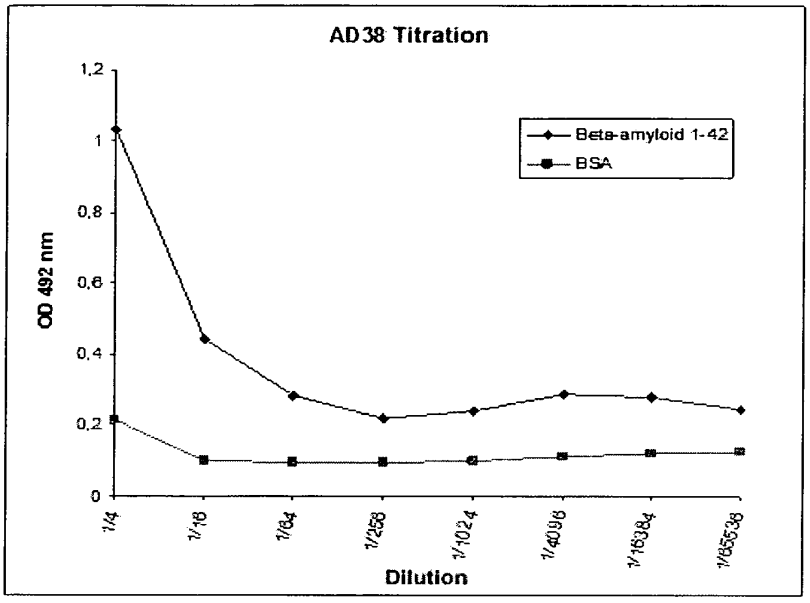

AD38 Titration
Beta-amyloid 1-42
BSA
OD 492 nm
1,2
1
0,8
0,6
0,4
0,2
0
1/4
1/16
1/64
1/256
1/1024
1/4096
1/16384
1/65536
Dilution

FIG. 7B

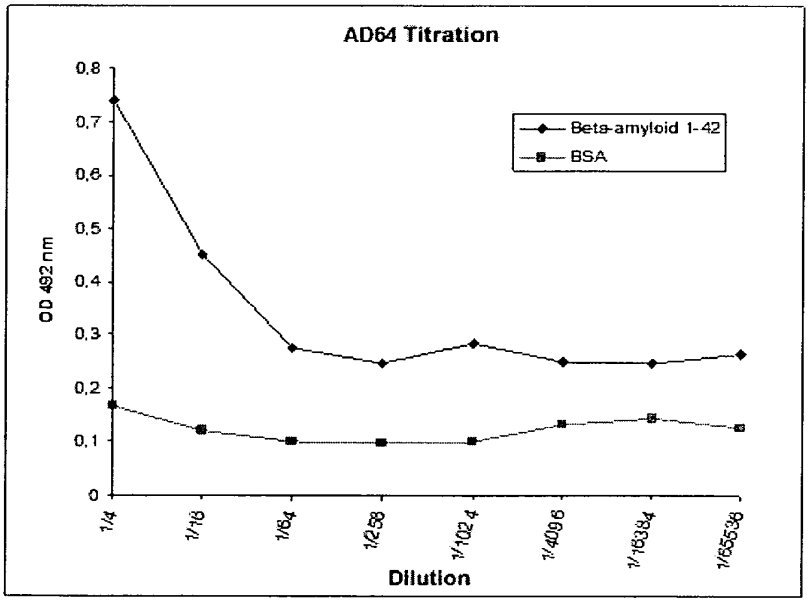

AD64 Titration
Beta-amyloid 1-42
BSA
OD 492 nm
0,8
0,7
0,6
0,5
0,4
0,3
0,2
0,1
0
1/4
1/16
1/64
1/256
1/1024
1/4096
1/16384
1/65536
Dilution

FIG. 8

HEAVY CHAIN AD38 (HC38)

```
HC/38
GAG GTG CAG CTG TTG GAG TCT GGG GGA GGC CTA GTT CAG CCG GGG GGG TCC CTG AGA CTC TCC
 E  V  Q  L  L  E  S  G  G  G  L  V  Q  P  G  G  S  L  R  L  S

                                  CDR1
HC/38
TGT ACA GCC TCT GGA TTC GTC TTC AGT GAC TAC TGG ATG CAC TGG GTC CGC CAA GCT CCA GGG
 C  T  A  S  G  F  V  F  S  D  Y  W  M  H  W  V  R  Q  A  P  G

                                                    CDR2
HC/38
AAG GGG CTG GTT TGG ATT TCA CGT CTT AAG AGT GAT GGA AGT AGT AGA AGT TAT GCG GAC TTT
 K  G  L  V  W  I  S  R  L  K  S  D  G  S  S  R  S  Y  A  D  F

HC/38
GTG AAG GGC CGA TTC ACC GTC TCC AGA GAC AAC GTC AAG AAT ACA CTG TTT TTG CAA ATC AAC
 V  K  G  R  F  T  V  S  R  D  N  V  K  N  T  L  F  L  Q  I  N

                                                          CDR3
HC/38
AGT CTG AGA GCC GAG GAC ACG GCT GTC TAT TAC TGT GCA AGA GAG TTA CGT GGC TGG TCC ATT
 S  L  R  A  E  D  T  A  V  Y  Y  C  A  R  E  L  R  G  W  S  I

HC/38
GAA TAT TGG GGC CAC GGA ACT CAG GTC ACC GTC TCC TCA GCT TCC ACC AAG
 E  Y  W  G  H  G  T  Q  V  T  V  S  S  A  S  T  K
```

FIG. 9

LIGHT CHAIN AD38 (LC38)

```
                GAG CTC GTG TTG ACG CAG TCT CCA GCC ACC CTG TCT TTG TCT CCA GGA GAA AGA GCC ACC CTC
LC/38             E   L   V   L   T   Q   S   P   A   T   L   S   L   S   P   G   E   R   A   T   L

                                        CDR1
                TTC TGC AGG GCC AGT CAG GAT ATT AGC ACC TCC TTA GCC TGG TAT CAA CAA AAA CCT GGC CAG
LC/38             F   C   R   A   S   Q   D   I   S   T   S   L   A   W   Y   Q   Q   K   P   G   Q

                                                CDR2
                GTT CCC AGG CTC CTC ATC TAT GGC ACA TCC TAT AGG GCC ACT GGC ATC TCA GCC AGG TTC AGT
LC/38             V   P   R   L   L   I   Y   G   T   S   Y   R   A   T   G   I   S   A   R   F   S

                GGC CGT GGG TCT GGG ACA GAC TTC ACT CTC ACC ATC AGC AGC CTA GAG CCT GAA GAC TTT GCA
LC/38             G   R   G   S   G   T   D   F   T   L   T   I   S   S   L   E   P   E   D   F   A

                                                CDR3
                TTG TAT TAC TGT CAA CAC CGG AGG GAC TGG CCT CCG GGG GCC ACC TTC GGC CAA GGG ACA CGA
LC/38             L   Y   Y   C   Q   H   R   R   D   W   P   P   G   A   T   F   G   Q   G   T   R

                CTG GAG ATT AAA
LC/38             L   E   I   K
```

FIG. 10

HEAVY CHAIN AD64 (HC64)

```
                                                           ________CDR1_______
HC/64   GAG GTG CAG CTG GTG CAG TCT GGG GGA GGC TTG GTA AAG CCG GGG GGG TCC CTC AGA CTC TCC
         E   V   Q   L   V   Q   S   G   G   G   L   V   K   P   G   G   S   L   R   L   S

                                            ________CDR1_______
HC/64   TGT GTC GGC TCT GGA TTC ACT TTC ACT AAT GCC TGG ATG AGC TGG GTC CGC CAG GCT CCA GGG
         C   V   G   S   G   F   T   F   T   N   A   W   M   S   W   V   R   Q   A   P   G

                                ________________________________CDR2_______________________
HC/64   AAG GGG CTG GAG TGG GTT GGC CGT ATG AAA AGC AAG ACT TAT GGC TGG ACA ACA GAA TTT GCT
         K   G   L   E   W   V   G   R   M   K   S   K   T   Y   G   W   T   T   E   F   A

        ___________________
HC/64   ACA GCC GTG GAA GGC AGA TTC ACC ATG TCA AGA GAT GAT TCA AAA AAC ACA CTC TAT TTG CAA
         T   A   V   E   G   R   F   T   M   S   R   D   D   S   K   N   T   L   Y   L   Q

                                                                        ________CDR3_______
HC/64   ATG AAC AGC CTG AAA ACC GAG GAC ACA GCC GTG TAT TAT TGC TCC ACA GAT GAC TAT GAT TTT
         M   N   S   L   K   T   E   D   T   A   V   Y   Y   C   S   T   D   D   Y   D   F

        TGG GGC CAA GGG ACA ATG GTC ACC GTC TCT TCA GCT TCC ACC AAG
         W   G   Q   G   T   M   V   T   V   S   S   A   S   T   K
```

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EX-1045
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FIG. 11

LIGHT CHAIN AD64 (LC AD64)

LC/64
GAG CTC GTG ATG ACT CAG TCT CCA GTC TCC CTG GCC GTC ACC CCT GGA GAG CCG GCC TCC ATC
E L V M T Q S P V S L A V T P G E P A S I

CDR1
TCC TGC AGG TCT AGT CAG AGC CTC CTG TTT AGT AAT GGA TAC AAC TAT TTG GAT TGG TAC CTT
S C R S S Q S L L F S N G F H Y L D W Y L

LC/64
CDR2
CAG AAG CCA GGG CAG TCT CCA CAG CTC CTG ATC TAT TTG GGT TCC AGT CGG GCC TCC GGG GTC
Q K P G Q S P Q L L I Y L G S S R A S G V

LC/64
CCT GAC AGG TTC AGT GGC AGT GGA TCA GGC TCA GAT TTC ACA CTG AAA ATC AGC AGA GTG GAG
P D R F S G S G S G S D F T L K I S R V E

LC/64
CDR3
GCT GAG GAT GTT GGG GTT TAT TAC TGC ATG CAA GCT CTA CAT AAT CCG CTC ACT TTC GGC GGA
A E D V G V Y Y C M Q A L H N P L T F G G

LC/64
GGG ACC AAG CTG GAG ATC AAA
G T K L E I K

List of Sequences

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<151> 2005-04-01

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<212> DNA
<213> Homo sapiens

<220>
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<222> (1)..(366)
<223> Recombinant antibody AD38 heavy chain variable domain

<400> 1

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Glu Val Gln Leu Leu Glu Ser Gly Gly Gly Leu Val Gln Pro Gly Gly
1               5                   10                  15

tcc ctg aga ctc tcc tgt aca gcc tct gga ttc gtc ttc agt gac tac       96
Ser Leu Arg Leu Ser Cys Thr Ala Ser Gly Phe Val Phe Ser Asp Tyr
            20                  25                  30

tgg atg cac tgg gtc cgc caa gct cca ggg aag ggg ctg gtt tgg att      144
Trp Met His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Val Trp Ile
        35                  40                  45

tca cgt ctt aag agt gat gga agt agt aga agt tat gcg gac ttt gtg      192
Ser Arg Leu Lys Ser Asp Gly Ser Ser Arg Ser Tyr Ala Asp Phe Val
    50                  55                  60

aag ggc cga ttc acc gtc tcc aga gac aac gtc aag aat aca ctg ttt      240
Lys Gly Arg Phe Thr Val Ser Arg Asp Asn Val Lys Asn Thr Leu Phe
65                  70                  75                  80

ttg caa atc aac agt ctg aga gcc gag gac acg gct gtc tat tac tgt      288
Leu Gln Ile Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys
                85                  90                  95

gca aga gag tta cgt ggc tgg tcc att gaa tat tgg ggc cac gga act      336
Ala Arg Glu Leu Arg Gly Trp Ser Ile Glu Tyr Trp Gly His Gly Thr
            100                 105                 110

cag gtc acc gtc tcc tca gct tcc acc aag                             366
Gln Val Thr Val Ser Ser Ala Ser Thr Lys
        115                 120
```

<210> 2
<211> 122
<212> PRT
<213> Homo sapiens

<400> 2

Glu Val Gln Leu Leu Glu Ser Gly Gly Gly Leu Val Gln Pro Gly Gly
1 5 10 15

Ser Leu Arg Leu Ser Cys Thr Ala Ser Gly Phe Val Phe Ser Asp Tyr
20 25 30

Trp Met His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Val Trp Ile
35 40 45

Ser Arg Leu Lys Ser Asp Gly Ser Ser Arg Ser Tyr Ala Asp Phe Val
50 55 60

Lys Gly Arg Phe Thr Val Ser Arg Asp Asn Val Lys Asn Thr Leu Phe
65 70 75 80

Leu Gln Ile Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys
85 90 95

Ala Arg Glu Leu Arg Gly Trp Ser Ile Glu Tyr Trp Gly His Gly Thr
100 105 110

Gln Val Thr Val Ser Ser Ala Ser Thr Lys
115 120

<210> 3
<211> 327
<212> DNA
<213> Homo sapiens

<220>
<221> CDS
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<223> Recombinant antibody AD38 light chain variable domain

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Glu Leu Val Leu Thr Gln Ser Pro Ala Thr Leu Ser Leu Ser Pro Gly
1 5 10 15

gaa aga gcc acc ctc ttc tgc agg gcc agt cag gat att agc acc tcc 96
Glu Arg Ala Thr Leu Phe Cys Arg Ala Ser Gln Asp Ile Ser Thr Ser
20 25 30

```
tta gcc tgg tat caa caa aaa cct ggc cag gtt ccc agg ctc ctc atc      144
Leu Ala Trp Tyr Gln Gln Lys Pro Gly Gln Val Pro Arg Leu Leu Ile
        35                  40                  45

tat ggc aca tcc tat agg gcc act ggc atc tca gcc agg ttc agt ggc      192
Tyr Gly Thr Ser Tyr Arg Ala Thr Gly Ile Ser Ala Arg Phe Ser Gly
    50                  55                  60

cgt ggg tct ggg aca gac ttc act ctc acc atc agc agc cta gag cct      240
Arg Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu Glu Pro
65                  70                  75                  80

gaa gac ttt gca ttg tat tac tgt caa cac cgg agg gac tgg cct ccg      288
Glu Asp Phe Ala Leu Tyr Tyr Cys Gln His Arg Arg Asp Trp Pro Pro
                85                  90                  95

ggg gcc acc ttc ggc caa ggg aca cga ctg gag att aaa                 327
Gly Ala Thr Phe Gly Gln Gly Thr Arg Leu Glu Ile Lys
            100                 105
```

<210> 4
<211> 109
<212> PRT
<213> Homo sapiens

<400> 4

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Glu Leu Val Leu Thr Gln Ser Pro Ala Thr Leu Ser Leu Ser Pro Gly
1               5                   10                  15

Glu Arg Ala Thr Leu Phe Cys Arg Ala Ser Gln Asp Ile Ser Thr Ser
            20                  25                  30

Leu Ala Trp Tyr Gln Gln Lys Pro Gly Gln Val Pro Arg Leu Leu Ile
        35                  40                  45

Tyr Gly Thr Ser Tyr Arg Ala Thr Gly Ile Ser Ala Arg Phe Ser Gly
    50                  55                  60

Arg Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu Glu Pro
65                  70                  75                  80

Glu Asp Phe Ala Leu Tyr Tyr Cys Gln His Arg Arg Asp Trp Pro Pro
                85                  90                  95

Gly Ala Thr Phe Gly Gln Gly Thr Arg Leu Glu Ile Lys
            100                 105
```

<210> 5
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<212> DNA
<213> Homo sapiens

<220>
<221> CDS
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<223> Recombinant antibody AD64 heavy chain variable domain

<400> 5

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Glu Val Gln Leu Val Gln Ser Gly Gly Gly Leu Val Lys Pro Gly Gly
1               5                   10                  15

tcc ctc aga ctc tcc tgt gtc ggc tct gga ttc act ttc act aat gcc        96
Ser Leu Arg Leu Ser Cys Val Gly Ser Gly Phe Thr Phe Thr Asn Ala
            20                  25                  30

tgg atg agc tgg gtc cgc cag gct cca ggg aag ggg ctg gag tgg gtt       144
Trp Met Ser Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val
        35                  40                  45

ggc cgt atg aaa agc aag act tat ggc tgg aca aca gaa ttt gct aca       192
Gly Arg Met Lys Ser Lys Thr Tyr Gly Trp Thr Thr Glu Phe Ala Thr
    50                  55                  60

gcc gtg gaa ggc aga ttc acc atg tca aga gat gat tca aaa aac aca       240
Ala Val Glu Gly Arg Phe Thr Met Ser Arg Asp Asp Ser Lys Asn Thr
65                  70                  75                  80

ctc tat ttg caa atg aac agc ctg aaa acc gag gac aca gcc gtg tat       288
Leu Tyr Leu Gln Met Asn Ser Leu Lys Thr Glu Asp Thr Ala Val Tyr
                85                  90                  95

tat tgc tcc aca gat gac tat gat ttt tgg ggc caa ggg aca atg gtc       336
Tyr Cys Ser Thr Asp Asp Tyr Asp Phe Trp Gly Gln Gly Thr Met Val
            100                 105                 110

acc gtc tct tca gct tcc acc aag                                      360
Thr Val Ser Ser Ala Ser Thr Lys
        115                 120
```

<210> 6
<211> 120
<212> PRT
<213> Homo sapiens

<400> 6

```
Glu Val Gln Leu Val Gln Ser Gly Gly Gly Leu Val Lys Pro Gly Gly
1               5                   10                  15

Ser Leu Arg Leu Ser Cys Val Gly Ser Gly Phe Thr Phe Thr Asn Ala
            20                  25                  30

Trp Met Ser Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val
        35                  40                  45

Gly Arg Met Lys Ser Lys Thr Tyr Gly Trp Thr Thr Glu Phe Ala Thr
    50                  55                  60
```

```
Ala Val Glu Gly Arg Phe Thr Met Ser Arg Asp Asp Ser Lys Asn Thr
65                  70                  75                  80

Leu Tyr Leu Gln Met Asn Ser Leu Lys Thr Glu Asp Thr Ala Val Tyr
                85                  90                  95

Tyr Cys Ser Thr Asp Asp Tyr Asp Phe Trp Gly Gln Gly Thr Met Val
            100                 105                 110

Thr Val Ser Ser Ala Ser Thr Lys
        115                 120
```

<210> 7
<211> 336
<212> DNA
<213> Homo sapiens

<220>
<221> CDS
<222> (1)..(336)
<223> Recombinant antibody AD64 light chain variable domain

<400> 7

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Glu Leu Val Met Thr Gln Ser Pro Val Ser Leu Ala Val Thr Pro Gly
1               5                   10                  15

gag ccg gcc tcc atc tcc tgc agg tct agt cag agc ctc ctg ttt agt       96
Glu Pro Ala Ser Ile Ser Cys Arg Ser Ser Gln Ser Leu Leu Phe Ser
            20                  25                  30

aat gga ttc cac tat ttg gat tgg tac ctt cag aag cca ggg cag tct      144
Asn Gly Phe His Tyr Leu Asp Trp Tyr Leu Gln Lys Pro Gly Gln Ser
        35                  40                  45

cca cag ctc ctg atc tat ttg ggt tcc agt cgg gcc tcc ggg gtc cct      192
Pro Gln Leu Leu Ile Tyr Leu Gly Ser Ser Arg Ala Ser Gly Val Pro
    50                  55                  60

gac agg ttc agt ggc agt gga tca ggc tca gat ttc aca ctg aaa atc      240
Asp Arg Phe Ser Gly Ser Gly Ser Gly Ser Asp Phe Thr Leu Lys Ile
65                  70                  75                  80

agc aga gtg gag gct gag gat gtt ggg gtt tat tac tgc atg caa gct      288
Ser Arg Val Glu Ala Glu Asp Val Gly Val Tyr Tyr Cys Met Gln Ala
                85                  90                  95

cta cat aat ccg ctc act ttc ggc gga ggg acc aag ctg gag atc aaa      336
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Asp Arg Phe Ser Gly Ser Gly Ser Gly Ser Asp Phe Thr Leu Lys Ile
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tat ggc aca tcc tat agg gcc act ggc atc tca gcc agg ttc agt ggc      192
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                85                  90                  95

ggg gcc acc ttc ggc caa ggg aca cga ctg gag att aaa ggt ggt tcc      336
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            100                 105                 110

tct aga tct tcc gag gtg cag ctg ttg gag tct ggg gga ggc cta gtt      384
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65                  70                  75                  80

Glu Asp Phe Ala Leu Tyr Tyr Cys Gln His Arg Arg Asp Trp Pro Pro
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    50                  55                  60

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Asp Arg Phe Ser Gly Ser Gly Ser Gly Ser Asp Phe Thr Leu Lys Ile
65                  70                  75                  80

agc aga gtg gag gct gag gat gtt ggg gtt tat tac tgc atg caa gct       288
Ser Arg Val Glu Ala Glu Asp Val Gly Val Tyr Tyr Cys Met Gln Ala
                85                  90                  95

cta cat aat ccg ctc act ttc ggc gga ggg acc aag ctg gag atc aaa       336
Leu His Asn Pro Leu Thr Phe Gly Gly Gly Thr Lys Leu Glu Ile Lys
            100                 105                 110

ggt ggt tcc tct aga tct tcc gag gtg cag ctg gtg cag tct ggg gga       384
Gly Gly Ser Ser Arg Ser Ser Glu Val Gln Leu Val Gln Ser Gly Gly
        115                 120                 125

ggc ttg gta aag ccg ggg ggg tcc ctc aga ctc tcc tgt gtc ggc tct       432
Gly Leu Val Lys Pro Gly Gly Ser Leu Arg Leu Ser Cys Val Gly Ser
    130                 135                 140

gga ttc act ttc act aat gcc tgg atg agc tgg gtc cgc cag gct cca       480
Gly Phe Thr Phe Thr Asn Ala Trp Met Ser Trp Val Arg Gln Ala Pro
145                 150                 155                 160

ggg aag ggg ctg gag tgg gtt ggc cgt atg aaa agc aag act tat ggc       528
Gly Lys Gly Leu Glu Trp Val Gly Arg Met Lys Ser Lys Thr Tyr Gly
                165                 170                 175

tgg aca aca gaa ttt gct aca gcc gtg gaa ggc aga ttc acc atg tca       576
Trp Thr Thr Glu Phe Ala Thr Ala Val Glu Gly Arg Phe Thr Met Ser
            180                 185                 190

aga gat gat tca aaa aac aca ctc tat ttg caa atg aac agc ctg aaa       624
Arg Asp Asp Ser Lys Asn Thr Leu Tyr Leu Gln Met Asn Ser Leu Lys
        195                 200                 205

acc gag gac aca gcc gtg tat tat tgc tcc aca gat gac tat gat ttt       672
Thr Glu Asp Thr Ala Val Tyr Tyr Cys Ser Thr Asp Asp Tyr Asp Phe
    210                 215                 220

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225                 230                 235
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Trp Thr Thr Glu Phe Ala Thr Ala Val Glu Gly Arg Phe Thr Met Ser
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Thr Glu Asp Thr Ala Val Tyr Tyr Cys Ser Thr Asp Asp Tyr Asp Phe
210 215 220

Trp Gly Gln Gly Thr Met Val Thr Val Ser Ser Ala Ser Thr Lys
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INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2006/003363

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K39/395 G01N33/53 A61P25/28

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

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 practical, search terms used)
EPO-Internal, Sequence Search, PAJ, WPI Data, EMBASE, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 1 172 378 A (DODEL, RICHARD, DR; DU, YANSHENG, DR) 16 January 2002 (2002-01-16) the whole document	1,2, 8-12, 22-25, 31-38, 40-42
X	CAI JIONG ET AL: "Cloning and expression of human single-chain Fv antibody against amyloid beta peptide involved in Alzheimer's disease" ZHONGGUO YIXUE KEXUEYUAN XUEBAO - ACTA ACADEMIAE MEDICINAE SINICAE, BEIJING, CN, vol. 25, no. 5, October 2003 (2003-10), pages 557-562, XP002994528 ISSN: 1000-503X abstract; figure 4	1,2,8,9, 31

-/--

[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

"E" earlier document 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
28 June 2006	17/07/2006

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, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Bernhardt, W

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

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2006/003363

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2005/028511 A (CENTOCOR, INC; MERCKEN, MARC; BENSON, JACQUELINE, M) 31 March 2005 (2005-03-31) pages 2-3; claims 1-38; examples 1,2,4	1,2, 8-12, 22-25, 31-38, 40-42
X	WO 2004/108895 A (NEURALAB LIMITED; WYETH; BASI, GURIQ; SALDANHA, JOSE, W; BARD, FREDERI) 16 December 2004 (2004-12-16) page 4; claims 43,69-75; figure 5a; sequence 8	6,13-15, 17-26, 31-33, 38,40-42
X	GEYLIS V ET AL: "P4-388 Human monoclonal antibodies against amyloid-beta (Abeta) engendered by EBV-immortalized lymphocytes from healthy adults" NEUROBIOLOGY OF AGING, TARRYTOWN, NY, US, vol. 25, July 2004 (2004-07), page S585, XP004626560 ISSN: 0197-4580 abstract	1-8, 13-16, 29-32,39
X,P	GEYLIS ET AL: "Immunotherapy of Alzheimer's disease (AD): From murine models to anti-amyloid beta (Abeta) human monoclonal antibodies" AUTOIMMUNITY REVIEWS, ELSEVIER, AMSTERDAM, NL, vol. 5, no. 1, 1 August 2005 (2005-08-01), pages 33-39, XP005203566 ISSN: 1568-9972 the whole document	1-42
A	KANG J ET AL: "THE PRECURSOR OF ALZHEIMER'S DISEASE AMYLOID A4 PROTEIN RESEMBLES A CELL- SURFACE RECEPTOR" NATURE, NATURE PUBLISHING GROUP, LONDON, GB, vol. 325, 19 February 1987 (1987-02-19), pages 733-736, XP000196840 ISSN: 0028-0836 abstract; figure 1	1-42

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INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2006/003363

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
	& DATABASE UniProt [Online] 13 August 1987 (1987-08-13), "Amyloid beta A4 protein precursor (APP) (ABPP) (Alzheimer disease amyloid protein) (Cerebral vascular amyloid peptide) (CVAP) (Protease nexin-II) (PN-II) (APPI) (PreA4) [Contains: Soluble APP-alpha (S-APP-alpha); Soluble APP-beta (S-APP-beta); C99; Beta-amyloid protein 42 (Beta-APP42); Beta-amyloid" retrieved from EBI accession no. UNIPROT:P05067 Database accession no. P05067 -----	
A	LITTLE M ET AL: "Of mice and men: hybridoma and recombinant antibodies" IMMUNOLOGY TODAY, ELSEVIER PUBLICATIONS, CAMBRIDGE, GB, vol. 21, no. 8, 1 August 2000 (2000-08-01), pages 364-370, XP004215163 ISSN: 0167-5699 abstract; figure 3 -----	1-42

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

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box II.1

Although claims 40 and 41 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

In addition, although claim 35 is directed to a diagnostic method practised on the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

Continuation of Box II.1

Rule 39.1(iv) PCT - Method for treatment of the human or animal body by therapy
Rule 39.1(iv) PCT - Diagnostic method practised on the human or animal body

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP2006/003363

Box II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

see FURTHER INFORMATION sheet PCT/ISA/210

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

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

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

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7873

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2006/003363

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 1172378 A	16-01-2002	NONE	
WO 2005028511 A	31-03-2005	AU 2004274390 A1	31-03-2005
		CA 2520853 A1	31-03-2005
		EP 1613657 A2	11-01-2006
WO 2004108895 A	16-12-2004	AU 2004245989 A1	16-12-2004
		CA 2525975 A1	16-12-2004
		EP 1636268 A2	22-03-2006

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(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
8 February 2007 (08.02.2007)

PCT

(10) International Publication Number
WO 2007/016357 A1

(51) International Patent Classification:
G01N 33/68 (2006.01) *A61K 31/00* (2006.01)
A61K 39/00 (2006.01)

(21) International Application Number:
PCT/US2006/029452

(22) International Filing Date: 27 July 2006 (27.07.2006)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/703,653 29 July 2005 (29.07.2005) US

(71) Applicant *(for all designated States except US)*: **REGENTS OF THE UNIVERSITY OF MINNESOTA** [US/US]; 450 McNamara Alumni Center, 200 Oak Street SE, Minneapolis, MN 55455 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: **ASHE, Karen, H.** [US/US]; 7 Oriole Lane, North Oaks, MN 55127 (US). **LESNE, Sylvain, E.** [FR/US]; Apartment A112, 18 13th Street No., Minneapolis, MN 55403 (US). **NEWMAN, Eric, A.** [US/US]; 1905 James Avenue So., Minneapolis, MN 55403 (US).

(74) Agent: **JOHNSON, Nancy, A.**; Mueting, Raasch & Gebhardt, P.A., P.O. Box 581415, Minneapolis, MN 55458-1415 (US).

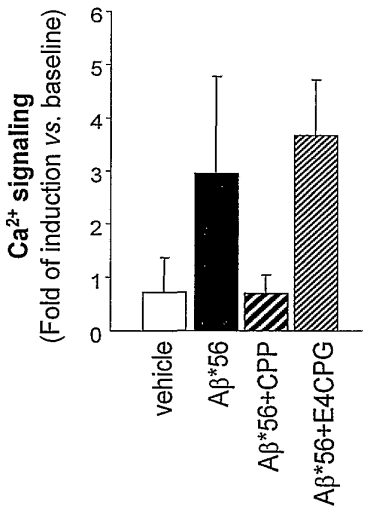
(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LV, LY, MA, MD, MG, MK, MN, MW, MX, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT, LU, LV, MC, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report*
— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: AMYLOID BETA RECEPTOR AND USES THEREOF



(57) Abstract: The present invention relates to the identification that soluble assemblies of amyloid β function as a NMDA receptor antagonist. The present invention also provides methods and compositions for the detection and treatment of neurodegenerative and cognitive disorders and screening methods to identify agents that modulate the antagonistic effect of soluble assemblies of amyloid β on NMDA receptor function.

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AMYLOID BETA RECEPTOR AND USES THEREOF

CONTINUING APPLICATION DATA

This application claims the benefit of U.S. Provisional Application Serial No. 60/703,653, filed July 29, 2005, which is incorporated by reference herein.

GOVERNMENT FUNDING

The present invention was made with government support under Grant No.NS33249, awarded by the National Institutes of Health. The Government may have certain rights in this invention.

BACKGROUND

The amyloid-β protein (Aβ) is implicated in the pathogenesis of Alzheimer's disease (AD). The Aβ peptides are the major amyloid protein deposited in AD brains and both natural and synthetic forms have devastating effects on the viability and function of neurons. See, for example, Yankner et al., Science 250, 279-82 (1990); Pike et al., Brain Res 563, 311-4 (1991); Pike et al., J Neurosci 13, 1676-87 (1993); Lambert et al., Proc Natl Acad Sci U S A 95, 6448-53 (1998); Walsh et al., Nature 416, 535-9 (2002); and Kayed et al., Science 300, 486-9 (2003). However, the mechanism by which the accumulation of Aβ proteins alters physiologic functioning in the brain and disrupts memory and cognitive function is unclear.

SUMMARY OF THE INVENTION

The present invention includes a method of detecting a neurodegenerative disease and/or cognitive disorder in a subject, the method including obtaining a sample from the subject; immunoprecipitating the sample with an antibody to a NMDA receptor; wherein the coprecipitation of amyloid β along with the NMDA receptor indicates the subject has a neurodegenerative disease and/or cognitive disorder. In some embodiments, the amyloid β is a soluble assembly of amyloid β protein. In some embodiments, the antibody to an NMDA receptor is an antibody that binds to a NMDA receptor subunit selected from NR1, NR2A, and/or NR2B.

The present invention also includes a method of detecting a presymptomatic neurodegenerative disease and/or cognitive disorder in a subject, the method including obtaining a sample from the subject; immunoprecipitating the sample with an antibody to a NMDA receptor; wherein the coprecipitation of amyloid β along with the NMDA receptor indicates the subject has a presymptomatic neurodegenerative disease and/or cognitive disorder disease. In some embodiments, the amyloid β is a soluble assembly of amyloid β protein. In some embodiments, the antibody to an NMDA receptor is an antibody that binds to a NMDA receptor subunit selected from NR1, NR2A, and/or NR2B.

The present invention includes a method of inhibiting NMDA receptor function, the method including contacting a NMDA receptor with a soluble assembly of amyloid β protein.

The present invention includes a method of screening for an agent that alters the antagonistic effect of a soluble assembly of amyloid β protein on NMDA receptor function, the method including: contacting a NMDA receptor with the agent and a soluble assembly of amyloid β protein; determining NMDA receptor function for the NMDA receptor contacted with the agent and the soluble assembly of amyloid β protein; comparing NMDA receptor function for the NMDA receptor contacted with the agent and the soluble assembly of amyloid β protein to NMDA receptor function for a NMDA receptor contacted with the soluble assembly of amyloid β protein and not contacted with the agent; wherein a difference in the level of NMDA receptor function in the NMDA receptor contacted with the agent and the soluble assembly of amyloid β protein compared to NMDA receptor function in the NMDA receptor contacted with the soluble assembly of amyloid β protein and not contacted with the agent indicates the agent alters the antagonistic effect of the soluble assembly of amyloid β protein on NMDA receptor function. In some embodiments, the agent inhibits the antagonistic effect of the soluble assembly of amyloid β protein on NMDA receptor function. In some embodiments, NMDA receptor function is determined by whole cell patch clamp recording. In some embodiments, NMDA receptor function is determined by Ca^{+2} fluorescence imaging. The present invention also includes agents identified by such methods.

The present invention includes a method of screening for an agent for the treatment of neurodegenerative disease and/or cognitive disorder, the method

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including: contacting a NMDA receptor with the agent and a soluble assembly of amyloid β protein; determining NMDA receptor function for the NMDA receptor contacted with the agent and the soluble assembly of amyloid β protein; comparing NMDA receptor function for the NMDA receptor contacted with the agent and the soluble assembly of amyloid β protein to NMDA receptor function for an NMDA receptor contacted with the soluble assembly of amyloid β protein and not contacted with the agent; wherein an altered level of NMDA receptor function in the NMDA receptor contacted with the agent and the soluble assembly of amyloid β protein compared to the NMDA receptor function of the NMDA receptor contacted with the soluble assembly of amyloid β protein and not contacted with the agent indicates the agent as an agent for the treatment of neurodegenerative disease and/or cognitive disorder. In some embodiments, the agent inhibits the antagonistic effect of the soluble assembly of amyloid β protein on NMDA receptor function. In some embodiments, NMDA receptor function is determined by whole cell patch clamp recording. In some embodiments, NMDA receptor function is determined by Ca^{+2} fluorescence imaging. The present invention also includes agents identified by such methods.

The present invention includes a method of treating a neurodegenerative disease and/or a cognitive disorder in a subject, the method comprising administering to the subject an effective amount of an agent that alters the antagonistic effect of a soluble assembly of amyloid β protein on the NMDA receptor.

The present invention includes an isolated Aβ*56/NMDA receptor complex.

The present invention includes agents that alter the inhibitory effect of a soluble assembly of amyloid β protein on the NMDA receptor.

The present invention includes antibodies that bind to a soluble assembly of amyloid β protein and prevent the formation of an amyloid β/NMDA receptor complex. The present invention also includes methods of treating a neurodegenerative disease and/or a cognitive disorder in a subject, the method including administering to the subject an effective amount of such an antibody.

The present invention includes antibodies that bind to a NMDA receptor and prevent the formation of an amyloid β/NMDA receptor complex. The present invention also includes methods of treating a neurodegenerative disease and/or a cognitive disorder in a subject, the method including administering to the subject an effective amount of such an antibody.

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In the methods of the present invention, the neurodegenerative disease and/or cognitive disorder may be Alzheimer's disease.

In any of the methods of the present invention, the soluble assembly of amyloid β protein may have a molecular weight of about 56 kDa as measured by SDS polyacrylamide gel electrophoresis.

In any of the methods of the present invention, the soluble assembly of amyloid β protein may be a dodecamer of amyloid β proteins.

In any of the methods of the present invention, the soluble assembly of amyloid β protein may be Aβ*56.

Unless otherwise specified, "a," "an," "the," and "at least one" are used interchangeably and mean one or more than one.

BRIEF DESCRIPTION OF THE FIGURES

Figures 1A-1C. Soluble Aβ*56 physically interacts with NMDA-receptor subunit NR1 and NR2A in Tg2576 brains. Fig. 1A shows Aβ*56 is present in both extracellular-enriched and membrane extracts. Western blots (WB) using anti-Aβ(1-16) antibodies (6E10) show multiples of Aβ trimers in addition to soluble APP (sAPPα) in soluble extracts of 13-month Tg2576 mice (left panel). In membrane extracts, full-length APP (fl-APP) and its BACE-generated CTF fragment, CTF-β, are detected along with Aβ monomers and Aβ*56 (right panel). $Tg2576^{-/-}$ and $Tg2576^{+/-}$ denote mice harbouring zero (non-Tg) and one transgene array, respectively; their ages (in months) are indicated in bold characters below the corresponding genotype. Synthetic human Aβ1-42 peptide ($hA\beta_{42}$) was used as a size marker and positive control. Arrows indicate respective migration positions of monomers (1-mer), dimers (2-mer), trimers (3-mer), tetramers (4-mer), hexamers (6-mer), nonamers (9-mer) and dodecamers (12-mer), as well as sAPPβ and fl-APP. Fig. 1B shows Aβ*56 physically binds NR1 subunits in Tg2576 mice in an age-dependent manner. Immunoprecipitations (IP) using NR1 specific antibodies were used to capture potential Aβ*56-NMDA receptor complexes. NR1 receptor subunit levels after IP are shown in the lower panels as loading controls, and also to confirm the fidelity of the protein extraction protocol. Fig. 1C shows Aβ*56-NMDA receptor complexes are immunocaptured using anti-Aβ(17-24) antibodies (4G8). NR1 and NR2A, but not

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NR2B, complexes are readily pulled down and detected by both N-ter (N-terminus specific) and C-ter (C-terminus specific) NMDA receptor antibodies.

Figures 2A-2C. Human-derived Aβ*56 physically binds NMDA receptors. Fig. 2A shows Aβ*56 coimmunoprecipitates with NR1 NMDA receptor subunits in brain tissue from Alzheimer (AD) patients but not from control subjects with no cognitive impairment (NCI), or extracts containing no brain proteins (NP). Fig. 2B shows Aβ*56 co-immunoprecipitates with NR2A, but much less readily with NR2B, NMDA receptor subunits in brain tissue from subjects with AD but not from control subjects (NCI). Fig. 2C shows Aβ*56 does not coimmunoprecipitate with α nicotinic acetylcholine receptors (α7nAChR). Panels below each blot confirm the ability of the various receptor antibodies to immunoprecipitate the respective receptors or receptor subunits.

Figures 3A-3D. Aβ*56 increases calcium signaling and inhibits NMDA-evoked currents in cultured cortical neurons. Fig. 3A shows calcium fluorescence measured from a cluster of neurons. Addition of 7 nM Aβ*56 increases Ca^{2+} signaling in the cells, indicating a rise in circuit activity within the network of neurons in culture. Fig. 3B shows the Aβ*56-induced increase in Ca^{2+} signaling is blocked by an NMDA receptor antagonist (CPP) but not by an mGluR antagonist (E4CPG). Calcium signaling is not increased when the vehicle alone is added. Bars show means ± SD. Fig. 3C shows calcium fluorescence measured from a single neuron. Calcium signaling increases within seconds after addition of 7 nM Aβ*56 and remains raised for greater than 10 minutes following Aβ*56 washout. Fig. 3D shows the inward current evoked by focal ejection of 50 μM NMDA onto a neuron is reduced by addition of 1 nM Aβ*56.

Figures 4A-4B. Aβ*56 does not bind AMPA receptors. Fig. 4A shows antibodies to GluR1 and GluR2 subunits of AMPA receptors fail to immunoprecipitate Aβ*56 from soluble or membrane-enriched fractions generated from brains of 13-month Tg2576 mice. Fig. 4B shows GluR1 and GluR2 AMPA receptor subunits are immunoprecipitated by the GluR1 and GluR2 antibodies in the membrane-enriched but not the soluble fractions, excluding the possibility that the inability to detect Aβ*56 was due to failure of the antibodies to immunoprecipitate the AMPA receptor subunits. $Tg2576^{-/-}$ and $Tg2576^{+/-}$ denote mice harboring zero (non-Tg) and one transgene array, respectively. Synthetic human $A\beta_{1-42}$ peptide ($hA\beta_{42}$) was loaded in parallel as a size

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marker and positive control. Arrows indicate respective migration positions of monomers (1-mer), dimers (2-mer), trimers (3-mer), tetramers (4-mer), hexamers (6-mer), nonamers(9-mer) and dodecamers (12-mer), as well as full-length APP (fl-APP) and soluble APP (sAPPα). Western blot (WB), immunoprecipitation (IP), soluble extracellular-enriched fraction (Sol), membrane-enriched fraction (MB).

Figure 5. Similar levels of immunoglobulin G in immunoprecipitates from membrane enriched fractions indicate consistent loading between samples. $Tg2576^{+/-}$ denotes mice harboring one transgene array; their ages (in months) are indicated above each gel. Arrows indicate the migration position of immunoglobulin G (IgG). Western blot (WB), immunoprecipitation (IP), Alzheimer brain (AD), brain from subjects with no cognitive impairment (NCI), no brain protein added (NP), no antibody added (No Ab).

Figures 6A-6B. Preparation of purified Aβ*56. Fig. 6A shows Tg2576 brain proteins from a 24-month mouse eluted after immunoaffinity purification in columns packed with 200 μg 6E10 (IPC 6E10-200) or 4G8 (IPC 4G8-200) antibodies, and probed with 6E10 antibodies by western blot (WB). Fig. 6B shoes optical density (A595) of fractions collected by size-exclusion chromatography of immunoaffinity-purified Tg2576 brain proteins using 4G8-packed columns. Silver stain of selected fractions corresponding to Aβ*56 (dodecamers) and Aβ trimers. Synthetic human $A\beta_{1-42}$ peptide ($hA\beta_{42}$) was loaded in parallel as a size marker and positive control. Arrows indicate respective migration positions of monomers (1-mer), dimers (2-mer), trimers (3-mer), tetramers (4-mer), hexamers (6-mer), nonamers (9-mer) and dodecamers (12-mer), as well as APP (combined full-length and soluble forms).

DETAILED DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS OF THE INVENTION

The present invention demonstrates for the first time that soluble assemblies of amyloid ß (Aß) protein bind to the NMDA receptor and function as an antagonist of the NMDA receptor. In particular, the present invention shows that Aß*56, a soluble assembly of Aß proteins, binds to NMDA receptors in both memory-impaired Tg2576 mice (a plaque-forming mice modeling Alzheimer disease) and patients with Alzheimer's disease (AD). The present invention also shows that Aß*56 inhibits NMDA-evoked currents. NMDA receptors are critical mediators of long lasting

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synaptic plasticity and memory and the present invention defines a new mechanism by which Aß proteins impair memory function in neurodegenerative disorders and cognitive disorders, such as Alzheimer's disease.

A major class of receptors for the neurotransmitter glutamate is referred to as N-methyl-D-aspartate receptors (NMDAR) since the receptor binds preferentially to N-methyl-D-aspartate (NMDA). NMDA is a chemical analog of aspartic acid. It normally does not occur in nature, and NMDA is not present in the brain. When molecules of NMDA contact neurons having NMDARs, they strongly activate the NMDAR (that is, they act as a powerful receptor agonist), causing the same type of neuronal excitation that glutamate does. The NMDA receptor is an excitatory, ionotropic receptor which plays a critical role in synaptic plasticity mechanisms and is necessary for several types of learning and memory. The NMDA receptor is a heteromeric, integral membrane protein formed by the assembly of obligatory NR1 subunits together with two modulatory NR2 subunits. The NRI subunit is the glycine binding subunit and exists as 8 splice variants of a single gene. The glutamate binding subunit is the NR2 subunit, which is generated as the product of four distinct genes, and provides most of the structural basis for heterogeneity in NMDA receptors. A related gene family of NR3 A-C subunits can substitute for NR2 subunits in specific brain regions and has an inhibitory effect on receptor activity. Multiple receptor isoforms with distinct brain distributions and functional properties arise by selective splicing of the NR1 transcripts and differential expression of the NR2 subunits. See, for example, Dingledine et al., "The glutamate receptor ion channels," Pharmacol Rev. 1999 51(1):7-61. As used herein, a NMDA receptor has an NR1 subunit and at least one of four different NR2 and NR3 subunits (designated as NR2A, NR2B, NR2C, and NR2D, NR3A and NR3B). An exemplary NR1 subunit is the human NMDAR1 polypeptide. The sequence of the polypeptide and corresponding nucleic acid may be obtained at Genbank, accession number L05666, and is published in Planells-Cases et al. (1993) P.N.A.S. 90(11):5057-5061. An exemplary NR2 subunit is the human NMDAR2A polypeptide. The sequence of the polypeptide and corresponding nucleic acid may be obtained at Genbank, accession number U09002, and is published in Foldes et al. (1994) Biochim. Biophys. Acta 1223 (1):155-159. Another NR2 subunit is the human NMDAR2B polypeptide. The sequence of the polypeptide and corresponding nucleic acid may be obtained at Genbank, accession number U11287,

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and is published in Adams et al. (1995) Biochim. Biophys. Acta 1260 (1):105-108. NMDA receptors are "ionotropic" receptors since they flux ions, such as Ca^{2+}. These ion channels allow ions to flow into a neuron upon depolarization of the postsynaptic membrane, when the receptor is activated by glutamate, aspartate, or an agonist drug.

As used herein an "amyloid-beta protein," also referred to as an "amyloid-beta polypeptide," an "amyloid-beta peptide," an amyloid-beta molecule," an "amyloid-β protein," an "amyloid-β polypeptide," an "amyloid-β peptide," and "amyloid-β molecule," an "Aβ protein," an "Aβ polypeptide," an "Aβ peptide," an "Aβ molecule," "amyloid-beta," "amyloid β," or "Aβ," is the major constituent of amyloid plaques in the brains of individuals afflicted with Alzheimer's disease, a polypeptide of 39 to 43 amino acid residue first identified by Glenner and Wong (see, for example, Glenner et al., (1984) Biochem Biophys Res Commun 120, 885-890; and Glenner and Wong (1984) Biochem Biophys Res Commun 122, 1131-1135) and Masters et al. (See Masters et al., (1985) Embo J 4, 2757-2764; and Masters et al., (1985) Proc Natl Acad Sci USA 82, 4245-4249). The gene for the amyloid precursor protein (APP) of the amyloid-beta protein has been cloned and sequenced (see, for example, Kang et al., (1987) Nature 325, 733; Tanzi et al., (1987) Science 235, 880-884; and Selkoe (1994) Annual Review of Neuroscience Vol, 17, 489-517). As used herein, an amyloid-beta protein may be any of the various known allelic variants and mutations of the amyloid-beta protein.

Amyloid beta peptide is generated from the beta-amyloid precursor protein (beta APP) in a two-step process. The first step involves cleavage of the extracellular, amino-terminal domain of beta APP. Protein cleavage is performed by an aspartyl protease termed beta-secretase (BACE). This enzyme is synthesized as a propeptide that must be modified to the mature and active form by the prohormone convertase, furin. Beta APP cleavage by the mature form of BACE results in the cellular secretion of a segment of beta APP and a membrane-bound remnant. This remnant is then processed by another protease termed gamma-secretase. Gamma-secretase cleaves an intra-membrane site in the carboxyl-terminal domain of beta APP, thus generating the amyloid beta peptide. Gamma-secretase is believed to be a multi-subunit complex containing presenilin-1 and 2 as central components. Found associated with the presenilins is the transmembrane glycoprotein nicastrin. Nicastrin has been found to bind to the carboxyl-terminus of betaAPP and helps to modulate the production of the

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amyloid beta peptide. Also found in the neurofibrillary lesions in Alzheimer's disease is the protein termed Tau. Tau is a neuronal microtubule-associated protein found predominantly on axons. The function of tau is to promote tubulin polymerization and stabilize microtubules. Tau, in its hyperphosphorylated form, is the major component of paired helical filaments (PHF), which is the building block of neurofibrillary lesions in Alzheimer's disease brain. See, for example, J. Neurosci. 18:1743-1752, 1998 and Neuron, 19:939-945, 1997.

As used herein, an amyloid-beta protein is a monomeric polypeptide, made up of one polypeptide chain. A monomeric polypeptide is also referred to herein as a "monomer."

As used herein, an oligomer of amyloid β, also referred to an oligomeric form of amyloid β, is a detergent-stable configuration of more than one amyloid-beta protein. An oligomer is not necessarily polymerized. An oligomer of amyloid β may be soluble. As used herein a "dimer" is a detergent-stable configuration of two amyloid-beta proteins. As used herein a "trimer" is a detergent-stable configuration of three amyloid-beta proteins. As used herein a "tetramer" is a detergent-stable configuration of four amyloid-beta proteins. As used herein a "pentamer" is a detergent-stable configuration of five amyloid-beta proteins. As used herein a "hexamer" is a detergent-stable configuration of six amyloid-beta proteins.

As used herein, an "assembly" is a configuration of one or more oligomers of Aβ proteins. In a preferred embodiment, an assembly is a configuration of more than one Aβ protein oligomer. An assembly of oligomers of Aβ proteins may be, for example, an assembly of two oligomers of Aβ proteins, three oligomers of Aβ proteins, four oligomers of Aβ proteins, five oligomers of Aβ proteins, six oligomers of Aβ proteins, or more oligomers of Aβ proteins. In some embodiments, an assembly of oligomers of Aβ proteins may be, for example, a nanomer of nine amyloid β proteins or a dodecamer of twelve amyloid β proteins. In some embodiments, an assembly of oligomers of Aβ proteins may be, for example, an assembly of more than one hexamer of amyloid β proteins, more than one pentamer of amyloid β proteins, more than one tetramer of amyloid β proteins, more than one trimer of amyloid β proteins, or more than one dimer of amyloid β proteins. In some embodiments, an assembly of oligomers of Aβ proteins may be, for example, an assembly of two hexamers of amyloid β proteins, three hexamers of amyloid β proteins, two tetramers of amyloid β

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proteins, three tetramers of amyloid β proteins, four tetramers of amyloid β proteins, two trimers amyloid β proteins, three trimers amyloid β proteins, four trimers of amyloid β proteins, five trimers amyloid β proteins, two dimers of amyloid β proteins, three dimers of amyloid β proteins, four dimers of amyloid β proteins, five dimers of amyloid β proteins, six dimers of amyloid β proteins, seven dimers of amyloid β proteins, or eight dimers of amyloid β proteins.

In some embodiments, amyloid-β protein assemblies may include detergent-stable dimers of amyloid-β protein. In some embodiments, amyloid-β protein assemblies may include detergent-stable trimers of amyloid-β protein. In some embodiments, amyloid-β protein assemblies may include detergent-stable tetramers of amyloid-β protein. In some embodiments, amyloid-β protein assemblies may include detergent-stable pentamers of amyloid-β protein. In some embodiments, amyloid-β protein assemblies may include detergent-stable hexamers of amyloid-β protein.

The present invention also includes isolated, soluble amyloid-β protein assemblies having one or more amyloid-β protein trimers. As used herein an "amyloid-β protein trimer" is a detergent-stable configuration of three Aβ molecules. In some embodiments, a soluble amyloid-β protein assembly has more than one amyloid-β protein trimer. In some embodiments, the amyloid-β protein assembly includes three amyloid-β protein trimers. In some embodiments, the amyloid-β protein assembly is a nonamer of amyloid-β proteins. In some embodiments, an amyloid-β protein assembly has a molecular weight of about 40 kDa as measured by SDS polyacrylamide gel electrophoresis. In some embodiments, the amyloid-β protein assembly includes four amyloid-β protein trimers. In some embodiments, the amyloid-β protein assembly has a molecular weight of about 56 kDa as measured by SDS polyacrylamide gel electrophoresis.

In some embodiments, amyloid-β protein assemblies may be a dodecamer of amyloid-β proteins. Such dodecamers of amyloid-β proteins may be six dimers of amyloid-β protein, four trimers of amyloid-β protein, three tetramers of amyloid-β protein, or two hexamers of amyloid-β protein. In some embodiments, the dodecamer of amyloid-β proteins has a molecular weight of about 56 kDa as measured by SDS polyacrylamide gel electrophoresis.

As used herein, a detergent-stable, also referred to herein as "detergent stable," configuration does not disassemble or disassociate into its component subunits in a

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detergent solution. Such a detergent solution may be, for example, a 1% solution Triton X-100 or a 2% solution of SDS. Thus, a detergent stable oligomer of amyloid-β protein does not disassociate into separate amyloid-β protein monomers in a detergent solution.

The assemblies of amyloid β protein of the present invention are soluble. As used herein, the term "soluble" means remaining in aqueous solution. In some embodiments, soluble assemblies of amyloid β protein remain in the supernatant after centrifugation, including, for example, ultracentrifugation. Soluble assemblies of amyloid β protein may remain in solution in a wide range of solutions, including, but not limited to, water, in an isotonic solution, tissue culture medium, a buffered solution, a detergent buffer, an organic buffer, or a body fluid, including, for example, plasma or cerebrospinal fluid. Assemblies of amyloid β protein may remain in solution in a physiological buffer.

Assemblies of amyloid β protein may remain in solution in range of temperatures. For example, the assemblies of amyloid β protein may remain in solution at a temperature greater than 0°C. Assemblies of amyloid β protein may remain in solution, for example, at a temperature of at least about 4°C, at a temperature of at least about 10°C, at a temperature of at least about 15°C, at a temperature of at least about 25°C, at a temperature of at least about 37°C, at a temperature of at least about 42°C, at a temperature of at least about 50°C, at a temperature of at least about 55°C, at a temperature of at least about 60°C, at a temperature of at least about 70°C, at a temperature of at least about 75°C, at a temperature of at least about 80°C, at a temperature of at least about 85°C, at a temperature of at least about 90°C, at a temperature of at least about and/or at a temperature of at least about 95°C.

Assemblies of amyloid β protein may remain in solution, for example, at a temperature of less than about 4°C, at a temperature of less than about 10°C, at a temperature of less than about 15°C, at a temperature of less than about 25°C, at a temperature of less than about 37°C, at a temperature of less than about 42°C, at a temperature of less than about 50°C, at a temperature of less than about 55°C, at a temperature of less than about 60°C, at a temperature of less than about 70°C, at a temperature of less than about 75°C, at a temperature of less than about 80°C, at a temperature of less than about 85°C, at a temperature of less about 90°C, at a temperature of less than about 95°C, and/or at a temperature of less than about 100°C.

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Assemblies of amyloid β protein may remain in solution, for example, at a temperature of about 4°C, at a temperature of about 10°C, at a temperature of about 15°C, at a temperature about 25°C, at a temperature of about 37°C, at a temperature of about 42°C, at a temperature of at about 50°C, at a temperature of about 55°C, at a temperature of about 60°C, at a temperature of about 70°C, at a temperature of about 75°C, at a temperature of at about 80°C, at a temperature of about 85°C, at a temperature of about 90°C, and/or at a temperature of about 95°C.

Assemblies of amyloid β protein may remain in solution in a range of any of the various temperatures discussed above.

Soluble assemblies of Aß include isolated, soluble, non-fibrillar amyloid-β protein (Aβ) assemblies having one or more detergent-stable oligomers of amyloid-β protein. The soluble, non-fibrillar amyloid-β protein (Aβ) assemblies of the present invention may be made up of one or more detergent-stable oligomers of amyloid-β protein. The soluble, non-fibrillar amyloid-β protein (Aβ) assemblies of the present invention may also be referred to herein as Aβ* assemblies, Aβ* molecules, Aβ star assemblies, Aβ star molecules, A-beta* assemblies, A-beta* molecules, A-beta star assemblies, A-beta star molecules, Aß*56, or A*56 (PCT/US2005/037828, "Assemblies of Oligomeric Amyloid Beta Protein and Uses Thereof" and Lesne et al., Nature. 2006 Mar 16;440(7082):352-7, "A specific amyloid-beta protein assembly in the brain impairs memory"). In some embodiments, a soluble, non-fibrillar amyloid-β protein (Aβ) assembly has more than one detergent-stable oligomers of amyloid-β protein. In some embodiments, the soluble, non-fibrillar amyloid-β protein (Aβ) assemblies of the present invention may be isolated and purified.

Assemblies of amyloid β protein may be obtained from a wide variety of sources. Assemblies of amyloid β protein may be obtained from natural sources; for example, from natural fluids, cells, or tissues, including, but not limited to, plasma, brain tissue, and cerebrospinal fluid. Assemblies of amyloid β may be isolated from the culture medium of cells expressing endogenous or transfected amyloid β protein precursor genes. For example, assemblies of oligomers of amyloid β protein may be obtained from the culture medium of Chinese hamster ovary (CHO) cells stably transfected to express amyloid β protein (Podlinsky et al., J Biol. Chem., 1995, 270(16):9564-9570). Assemblies of amyloid β protein may be synthetically produced. Assemblies of amyloid β protein may be produced recombinantly.

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Assemblies of amyloid-β protein disrupt cognitive functioning, representative of a cognitive disorder. Such cognitive disorders include, but are not limited to, mild cognitive impairment, memory deficits, age related memory decline, age associated memory impairment, and Alzheimer's disease, including, but not limited to presymptomatic Alzheimer's disease and early Alzheimer's disease. Disruptions of cognitive function may be representative of any phase of a neurological disorder, including, but not limited to, a presymptomatic phase, a preclinical phase, or an early phase of a neurological disorder. The disruption of cognitive function may be representative of age-related memory decline or age-associated memory impairment (see Craik, F. I. in Handbook of the Psychology of Aging (eds. Birren, J. E. & Schall, K.) 384-420 (Van Nostrand-Reinhold, New York, 1977) and Morrison and Hof, Science 1997, 278;412-9). Such functional deficiencies may be transient or permanent. Such functional deficiencies may be observed in the absence of neuropathological damage. Such neuropathologies may include, for example, amyloid plaque formation, amyloid deposits, oxidative stress, astrogliosis, microgliosis, cytokine production, dystrophic neurons, formation of neurobifillary tangles, neurodegeneration, gross neuronal atrophy, neuronal loss, synaptic loss, and other manifestations of neuropathology.

Methods for assaying disruption of learned behavior and/or cognitive functioning can include, for example, those described in Cleary et al., Nat. Neuroscience 8, 79-84 (2005); U.S. Provisional Application 60/584,695 (filed June 30, 2004); U.S. Provisional Application 60/621,549 (filed October 22, 2004); and PCT Application "Soluble Oligomers of Amyloid Beta Disrupt Memory of Learned Behavior" (filed June 30, 2005).

Cognitive disruption may be assayed by any of a variety of methods. One means of assessing cognitive functioning is the Alternating Lever Cyclic Ratio (ALCR) test, which has proven to be sensitive for measuring cognitive function (O'Hare et al., Behav Pharmacol 1996, 7:742-753; and Richardson et al., Brain Res 2002, 954:1). Under ALCR, rats learn a complex sequence of lever-pressing requirements for food reinforcement in a two-lever experimental chamber. Rats must alternate between the two levers, switching to the other lever after pressing the first lever enough to get a food pellet. The number of presses required for each food reward proceeds from low (2 presses) to high (56 presses), incorporating intermediate values

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based on the quadratic function, $x^2 - x$. One cycle is an entire ascending and descending sequence of these response requirements (for example, 2, 6, 12, 20, 30, 42, 56, 56, 42, 30, 20, 12, 6, and 2 presses per food reward). Six such full cycles are presented during each session. Errors are scored when the subject perseveres on a lever after reward, that is, does not alternate (a perseveration error), or when a subject switches levers before completing the response requirement on that lever (a switching error).

Other procedures that may be used to assess cognitive functioning, include, but are not limited to, a delayed non-matching to place test, a morris water maze (commonly used to assess working memory in rats and mice), a delayed matching to sample test (an operant procedure for testing working memory), and a fixed-interval operant responding test (a sensitive procedure to assess non-specific cognitive effects, for example, when the type and anatomical location of the cognition being tested is unknown), a delayed conditioning procedure (representing a variety of operant or non-operant tests under which animals are exposed to stimuli paired with a reward or punishment and, after a delay, their ability to respond appropriately to the stimulus-reward combination is assessed), or a repeated acquisition procedure (an operant test, under which subjects are required to repeatedly learn a new stimulus sequence).

The present invention includes a method for detecting the presence of assemblies of amyloid β protein in a sample taken from a subject by contacting a sample with an antibody to the NMDA receptor, a NMDA receptor subunit and/or an antibody to a complex of the NMDA receptor and soluble assemblies of amyloid β and detecting binding of the antibody. The sample may be, for example, serum, blood, cerebrospinal fluid (CSF), or brain tissue.

The present invention includes a method of detecting a neurodegenerative disease and/or cognitive disorder in a subject, the method including obtaining a sample from the subject; immunoprecipitating the sample with an antibody to a NMDA receptor or receptor subunit; wherein the coprecipitation of amyloid β along with the NMDA receptor indicates the subject has a neurodegenerative disease and/or cognitive disorder. In some aspect, the amyloid β is a soluble assembly of amyloid β protein, including any of the soluble assemblies of amyloid β described herein, including, but not limited to, Aβ*56. In some aspects, the antibody to an NMDA receptor is an

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antibody that binds to a NMDA receptor subunit selected from NR1, NR2A, and/or NR2B.

The present invention also includes a method of detecting a presymptomatic neurodegenerative disease and/or cognitive disorder in a subject, the method including obtaining a sample from the subject; immunoprecipitating the sample with an antibody to a NMDA receptor or receptor subunit; wherein the coprecipitation of amyloid β along with the NMDA receptor indicates the subject has a presymptomatic neurodegenerative disease and/or cognitive disorder disease. In some embodiments, the amyloid β is a soluble assembly of amyloid β protein, including any of the soluble assemblies of amyloid β described herein, including, but not limited to, Aβ*56. In some aspects, the antibody to an NMDA receptor is an antibody that binds to a NMDA receptor subunit selected from NR1, NR2A, and/or NR2B. The method may be used for detecting Alzheimer's disease. The method may be used for detecting presymptomatic Alzheimer's disease.

The present invention includes a method of inhibiting NMDA receptor function, the method including contacting a NMDA receptor with a soluble assembly of amyloid β protein, including any of the soluble assemblies of amyloid β described herein. In some embodiments, the soluble assembly of amyloid β protein is Aβ*56.

The present invention includes methods of screening for agents that alter or modulate the antagonistic effect of soluble assemblies of amyloid β on NMDA receptor function. Such a method may include contacting a NMDA receptor with both an agent and a soluble assembly of amyloid β, determining NMDA receptor function for the NMDA receptor contacted with both the agent and the soluble assembly of amyloid β, comparing NMDA receptor function for the NMDA receptor contacted with both the agent and the soluble assembly amyloid β to NMDA receptor function for a NMDA receptor contacted with the soluble assembly of amyloid β and not contacted with the agent. A difference in the level of NMDA receptor function in the NMDA receptor contacted with both the agent and the soluble assembly of amyloid β compared to NMDA receptor function in the NMDA receptor contacted with the soluble assembly of amyloid β and not contacted with the agent indicates the agent alters the antagonistic effect of the soluble assembly of amyloid β on NMDA receptor function. A soluble assembly of amyloid β can include any of those described herein, including, but not limited to, Aβ*56.

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As used herein, an antagonistic effect is an inhibition or decrease in the normal physiological function of a receptor. An antagonist that competes with an agonist for a receptor is a competitive antagonist. An antagonist that antagonize by other means is a non-competitive antagonist.

The present invention includes methods of screening for agents effective for the treatment of neurodegenerative diseases and/or cognitive disorders. Such a method may include contacting a NMDA receptor with an agent and a soluble assembly of amyloid β, determining NMDA receptor function for the NMDA receptor contacted with the agent and the soluble assembly of amyloid β, comparing NMDA receptor function for the NMDA receptor contacted with the agent and the soluble assembly of amyloid β to NMDA receptor function for an NMDA receptor contacted with the soluble assembly of amyloid β and not contacted with the agent. An altered level of NMDA receptor function in the NMDA receptor contacted with the agent and the soluble assembly of amyloid β compared to the NMDA receptor function of the NMDA receptor contacted with the soluble assembly of amyloid β and not contacted with the agent indicates the agent may be effective for the treatment of neurodegenerative disease and/or cognitive disorder. A soluble assembly of amyloid β can include any of those described herein, including, but not limited to, Aβ*56. The present invention also includes agents identified by the screening methods described herein and methods of treatment that include the administration of such agents.

NMDA receptors used in such methods may be provided in any of a wide variety of formats, including for example, as isolated receptors, reconstituted membranes, cell membrane preparations, whole cells, or tissues. For example, neuronal cell cultures, neurocortical cell cultures, and nerve and brain tissues. NMDA receptor function may be determined by any of a wide variety of means. For example, NMDA receptor function may be determined by assaying for changes in concentrations of intracellular calcium in cells or tissues maintained in tissue culture. Calcium concentration may be determined, for example, by the use of a calcium indicator dye.

Calcium indicator dyes (also called calcium ion probes) are widely used intracellular indicators (Cellular Calcium, A Practical Approach, (1991) McCormack, J.G. and P.H. Cobbold eds. IRL Press at Oxford Press, New York, New York). Calcium ion detection may be accomplished by using a dye that has a recognition portion as well as a region that confers fluorescence. One commonly used structure for

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calcium specific binding is 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid, (BAPTA). Calcium indicator dyes can be categorized into at least two groups; the first are the dyes that increase their fluorescence in the presence of calcium, while the second group are dyes that have different excitation and/or emission wavelengths in the presence of calcium than they have in its absence. The calcium indicator dyes, calcium green-1, calcium green-2, and Fluo-4 are representative of the dyes that increase their fluorescence in the presence of calcium ion without changing wavelengths. Fura-2 and Indo-1 are ratiometric Ca^{2+} indicators that are generally considered interchangeable in most experiments. Fura-2, upon binding Ca2+, exhibits a shift in its absorption or excitation peak from 338 nm to 366 nm (Haughland, R., (2002) Handbook of Fluorescent Compounds and Research Products, ninth Ed., Molecular Probes, Inc), making Fura-2 the dye a common choice for microscopy, where it is easier to change excitation wavelengths than emission. Indo-1 on the other hand has a shift in the emission from 485 nm to 405 nm in the presence of calcium. Thus, Indo-1 has a greater utility with flow cytometry where it is easier to use a single argon-ion laser for excitation and to monitor two different emissions. Calcium indicator dyes can be efficiently measured using filter based microplate readers. See, also, Principles of Fluorescence Spectroscopy 2nd Edition (1999) Lakowicz, J.R. Editor, Kluwer Academic/Plenum Publishers, New York, New York; The Encyclopedia of Molecular Biology (1994) Kendrew, J Editor, Blackwell Science Ltd. Cambridge, MA; Cellular Calcium, A Practical Approach, (1991) McCormack, J.G. and P.H. Cobbold eds. IRL Press at Oxford Press, New York, New York; Haughland, R., (2002) Handbook of Fluorescent Compounds and Research Products, ninth Ed., Molecular Probes, Inc; and P. Held, (June 6, 2003) "Detection of Calcium Concentration Changes Using the FLx800 Fluorescence Microplate Reader," Applications Department, Bio-Tek Instruments, Inc., available on the world wide web at biotek.com/resources/ tech_res_detail.php?id.

Alternatively, NMDA receptor function may be determined in whole cell patch clamp assays, as described in more detail herein. Alternatives to whole cell patch clamp assays may be employed, including, for example, discontinuous single electrode voltage-clamp (dSEVC) (Roelfsema et al., J Exp Bot. 2001 Sep;52(362):1933-9) and high-throughput methods utilizing multielectrode

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extracellular recordings of cell-electrode hybrids (Natarajan et al., Toxicol In Vitro. 2006 Apr;20(3):375-81. Epub 2005 Sep 29).

Agents of the present invention alter the antagonistic effect of a soluble assembly of amyloid β on NMDA receptor function. Altering an antagonistic effect includes inhibiting or decreasing the antagonistic effect. Altering an antagonistic effect includes increasing or enhancing the antagonistic effect. As used herein, an "agonist" or "activator" is a molecule which, when interacting with a target receptor protein prolongs the amount or duration of the effect of the biological activity of the target protein. By contrast, the term "antagonist," or "inhibitor" as used herein, refers to a molecule which, when interacting with a target protein, decreases the amount or the duration of the effect of the biological activity of the target protein. Agonists and antagonists include, but are not limited to, proteins, nucleic acids, carbohydrates, antibodies, or any other molecules that decrease the effect of a protein.

The term "analog" is used herein to refer to a molecule that structurally resembles a molecule of interest but which has been modified in a targeted and controlled manner, by replacing a specific substituent of the reference molecule with an alternate substituent. Compared to the starting molecule, an analog may exhibit the same, similar, or improved utility. Synthesis and screening of analogs, to identify variants of known compounds having improved traits (such as higher potency at a specific receptor type, or higher selectivity at a targeted receptor type and lower activity levels at other receptor types) is an approach that is well known in pharmaceutical chemistry. The present invention includes analogs of the agents described herein.

The terms "modulation" or "alteration," as used herein, refer to both up regulation, (activation or stimulation), for example by agonizing; and down regulation (i.e. inhibition or suppression), for example by antagonizing, of a bioactivity. Modulators include, but are not limited to, both "activators" and "inhibitors" of function. An "activator" is a substance that directly or indirectly enhances function, causing the NMDA receptor to become more active. Conversely, an "inhibitor" directly or indirectly decreases NMDA receptor function, causing the NMDA receptor to become less active. The reduction may be complete or partial. As used herein, modulators of NMDA-R signaling encompass antagonists and agonists.

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In some embodiments, the ability of an agent to enhance or inhibit NMDA-R activity is assayed in an *in vitro* system. In general, the in vitro assay format involves adding an agent and NMDA-R, and measuring the biological activity of the NMDA receptor.

In the assays and methods of the present invention, receptor function of NMDA receptors contacted with both an agent and a soluble assembly of amyloid β may be compared to receptor function of NMDA receptors contacted with only a soluble assembly of amyloid β and not contacted with the agent. NMDA receptor function of NMDA receptors contacted with only the soluble assembly of amyloid β and not contacted with the agent may be determined within the assay, by contacting NMDA receptors with the soluble assembly of amyloid β and determining receptor function. Alternatively, receptor function of NMDA receptors contacted with only the soluble assembly of amyloid β and not contacted with the agent may be provided as a value determined in a separate assay.

The present invention includes antibodies that bind to a soluble assembly of amyloid β protein and prevent the formation of an amyloid β/NMDA receptor complex. Such antibodies may be used in methods of detecting and treating a neurodegenerative disease and/or a cognitive disorder in a subject, the method including administering to the subject an effective amount of such an antibody.

The present invention includes antibodies that bind to a NMDA receptor and prevent the formation of an amyloid β/NMDA receptor complex. Such antibodies may be used in methods of detecting and treating a neurodegenerative disease and/or a cognitive disorder in a subject, the method including administering to the subject an effective amount of such an antibody.

The present invention includes antibodies that bind to an amyloid β/NMDA receptor complex NMDA receptor but do not bind to isolated amyloid β and do not bind to an isolated NMDA. Such antibodies may be used in methods of detecting and treating a neurodegenerative disease and/or a cognitive disorder in a subject, the method including administering to the subject an effective amount of such an antibody.

The antibodies of the present invention can be produced and characterized by any of many methods, including, but not limited to, any of the methods described herein. The ability of an antibody to inhibit the Aβ*56-mediated inhibition of NMDA-

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evoked currents may be determined by any of many available assays, including, but not limited to, any of the assays described herein.

Also included in the present invention are compositions including one or more of the antibodies as described herein. Such compositions may include a pharmaceutically acceptable carrier. Also included in the present invention are kits with one or more of the antibodies of the present invention.

As used herein, the terms "antibody" or "antibodies" includes polyclonal antibodies, affinity-purified polyclonal antibodies, monoclonal antibodies, and antigen-binding fragments thereof, such as $F(ab')_2$ and Fab proteolytic fragments. Genetically engineered intact antibodies or fragments, such as chimeric antibodies, Fv fragments, single chain antibodies and the like, as well as synthetic antigen-binding peptides and polypeptides, are also included. The term "polyclonal antibody" refers to an antibody produced from more than a single clone of plasma cells; in contrast "monoclonal antibody" refers to an antibody produced from a single clone of plasma cells. Polyclonal antibodies may be obtained by immunizing a variety of warm-blooded animals such as horses, cows, goats, sheep, dogs, chickens, rabbits, mice, hamsters, guinea pigs and rats as well as transgenic animals such as transgenic sheep, cows, goats or pigs, with an immunogen. Monoclonal antibodies can be obtained by various techniques familiar to those skilled in the art.

A therapeutically useful antibody may be derived from a "humanized" monoclonal antibody. Humanized monoclonal antibodies are produced by transferring one or more CDRs from the heavy and light variable chains of a mouse (or other species) immunoglobulin into a human variable domain, then substituting human residues into the framework regions of the murine counterparts. The use of antibody components derived from humanized monoclonal antibodies obviates potential problems associated with immunogenicity of murine constant regions. Techniques for producing humanized monoclonal antibodies can be found, for example, in Jones et al., *Nature* (1986);321: 522 and Singer et al., *J. Immunol.*, (1993);150: 2844.

Antibodies of the present invention may, for example, be administered following any of the procedures used for the administration of the antibodies to tumor necrosis fact (TNF) adalimumab (also known as HUMIRA) (see, for example, http://www.rxabbott.com/pdf/ humira.pdf or Baker, DE, "Adalimumab: human recombinant immunoglobulin G1 anti-tumor necrosis factor monoclonal antibody,"

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Rev Gastroenterol Disord. 2004 Fall;4(4):196-210) or infliximab (also known as REMICADE) (see, for example, http://www.remicade.com/pdf/ IN04810.pdf, Harriman et al., "Summary of clinical trials in rheumatoid arthritis using infliximab, an anti-TNFalpha treatment," Ann Rheum Dis. 1999 Nov;58 Suppl 1:I61-4, or Hochberg et al., "Comparison of the efficacy of the tumour necrosis factor alpha blocking agents adalimumab, etanercept, and infliximab when added to methotrexate in patients with active rheumatoid arthritis," Ann Rheum Dis. 2003 Nov;62 Suppl 2:ii13-6).

In addition, chimeric antibodies can be obtained by splicing the genes from a mouse antibody molecule with appropriate antigen specificity together with genes from a human antibody molecule of appropriate biological specificity; see, for example, Takeda et al., *Nature* (1985);314: 544-546. A chimeric antibody is one in which different portions are derived from different animal species.

The phrase "specifically binds" or "specifically immunoreactive with," when referring to an antibody, refers to a binding reaction that is determinative of the presence of a protein in a heterogeneous population of proteins and other biologics. Thus, under designated immunoassay conditions, the specified antibodies bind to a particular protein at least about two times the background and do not substantially bind in a significant amount to other proteins present in the sample. Typically a specific or selective reaction will be at least about twice background signal or noise and more typically more than about 10 to about 100 times background. Specific binding to an antibody under such conditions may require an antibody that is selected for its specificity for a particular protein.

"Binding affinity" or "affinity binding" refers to the strength of the sum total of noncovalent interactions between a single binding site of a molecule (e.g., an antibody) and its binding partner (e.g., an antigen or antigenic epitope). The affinity of a molecule X for its partner Y is represented by the dissociation constant (Kd), which can generally be determined by using methods known in the art, for example, using the BIAcore biosensor, commercially available from BIAcore Inc., Piscataway, NJ. Antibodies of the present invention can also be described in terms of their binding affinity for the amyloid β, Aβ*56, the NMDS receptor and/or a complex of the NMDA receptor and amyloid β.

Antibodies of the present invention can be assayed for specific binding by any suitable method known in the art. The immunoassays that can be used include but are

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not limited to competitive and non-competitive assay systems using techniques such as BIAcore analysis, FACS (Fluorescence activated cell sorter) analysis, immunofluorescence, immunocytochemistry, Western blots, radio-immunoassays, ELISA (enzyme linked immunosorbent assay), "sandwich" immunoassays, immunoprecipitation assays, precipitin reactions, gel diffusion precipitin reactions, immunodiffusion assays, agglutination assays, complement-fixation assays, immunoradiometric assays, fluorescent immunoassays, protein A immunoassays, to name but a few. Such assays are routine and well known in the art (see e.g., Ausubel et al, eds, Current Protocols in Molecular Biology, Vol. 1, John Wiley & Sons, Inc., N.Y. (1994)).

The present invention includes hybridoma cell lines, transformed B-cell lines, host cells, and progeny, derivatives or equivalents thereof producing the antibodies of the present invention. The present invention also includes polynucleotides encoding an antibody of the present invention, or antigen-binding fragment thereof.

Various delivery systems are known and can be used to administer the agents, antibodies or pharmaceutical compositions of the invention. Methods of introduction include but are not limited to intradermal, intramuscular, intraperitoneal, intravenous, subcutaneous, intranasal, epidural, and oral routes. The compounds or compositions can be administered by any convenient route, for example, by infusion or bolus injection, by absorption through epithelial or mucocutaneous linings (e.g., oral mucosa, rectal and intestinal mucosa, etc.) and can be administered together with other biologically active agents. Administration can be systemic or local. In some embodiments, it can be desirable to administer the pharmaceutical compounds or compositions of the invention locally to the area in need of treatment; this can be achieved, for example, by local infusion during surgery, by topical application, in conjunction with a wound dressing after surgery, by injection, by means of a catheter, by means of a suppository, or by means of an implant. In other embodiments, the compound or composition can be delivered in a vesicle, for example, a liposome. In yet other embodiments, the compound or composition can be delivered in a controlled release system. In some embodiments a pump can be used. In other embodiments polymeric materials can be used.

The present invention also provides pharmaceutical compositions. Such compositions comprise a therapeutically effective amount of compound of the

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invention, and a pharmaceutically acceptable carrier. In some embodiments, the term "pharmaceutically acceptable" means approved by a regulatory agency of the Federal or a state government or listed in the U.S. Pharmacopeia or other generally recognized international pharmacopeia for use in animals, and more particularly in humans. The term "carrier" refers to a diluent, adjuvant, excipient, or vehicle with which the therapeutic is administered. Such pharmaceutical carriers can be sterile liquids, such as water and oils, including those of petroleum, animal, vegetable or synthetic origin, such as peanut oil, soybean oil, mineral oil, sesame oil and the like. In some embodiments, water can be used as a carrier when the pharmaceutical composition is administered intravenously. Saline solutions and aqueous dextrose and glycerol solutions can also be employed as liquid carriers, particularly for injectable solutions. Suitable pharmaceutical excipients include starch, glucose, lactose, sucrose, gelatin, malt, rice, flour, chalk, silica gel, sodium stearate, glycerol monostearate, talc, sodium chloride, dried skim milk, glycerol, propylene, glycol, water, ethanol and the like. The composition, if desired, can also contain minor amounts of wetting or emulsifying agents, or pH buffering agents. These compositions can take the form of solutions, suspensions, emulsions, tablets, pills, capsules, powders, sustained-release formulations and the like. The composition can be formulated as a suppository, with traditional binders and carriers such as triglycerides. Oral formulation can include standard carriers such as pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, or magnesium carbonate. Examples of suitable pharmaceutical carriers are described in Remington's Pharmaceutical Sciences by E. W. Martin. Such compositions will contain a therapeutically effective amount of the compound, preferably in purified form, together with a suitable amount of carrier so as to provide the form for proper administration to the patient. The formulation should suit the mode of administration.

In other embodiments, the composition is formulated in accordance with routine procedures as a pharmaceutical composition adapted for intravenous administration to a subject. Typically, compositions for intravenous administration are solutions in sterile isotonic aqueous buffer. Where necessary, the composition can also include a solubilizing agent and a local anesthetic such as lignocaine to ease pain at the site of the injection. Generally, the ingredients are supplied either separately or mixed together in unit dosage form, for example, as a dry lyophilized powder or water free

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concentrate in a hermetically sealed container such as an ampoule or sachette indicating the quantity of active agent. Where the composition is to be administered by infusion, it can be dispensed with an infusion bottle containing sterile pharmaceutical grade water or saline. Where the composition is administered by injection, an ampoule of sterile water for injection or saline can be provided so that the ingredients can be mixed prior to administration.

The agents and/or antibodies of the present invention can be used either alone or in combination with other compounds or compositions. The agents and/or antibodies of the present invention can be used in both *in vitro* and *in vivo* diagnostic and therapeutic methods. Also included in the present invention are such *in vitro* and *in vivo* diagnostic and therapeutic methods.

An agent or antibody may be administered by any of a wide variety of means. For example, delivered orally, subcutaneaously, intramuscularly, intravenously, intrathecally, and/or intracranially. Delivery may be by local delivery or injection. Delivery may be by pump or extended release composition. An agent or antibody may be delivered prior to, during, and/or after delivery of another therapeutic agent. One or more agents may be administered.

The invention also provides a kit including the agents and/or antibodies of the present invention. The kit can include one or more containers filled with one or more of the agents and/or antibodies of the invention. Additionally, the kit may include other reagents such as buffers and solutions needed to practice the invention are also included. Optionally associated with such container(s) can be a notice or printed instructions. As used herein, the phrase "packaging material" refers to one or more physical structures used to house the contents of the kit. The packaging material is constructed by well known methods, preferably to provide a sterile, contaminant-free environment. As used herein, the term "package" refers to a solid matrix or material such as glass, plastic, paper, foil, and the like, capable of holding within fixed limits a polypeptide. Thus, for example, a package can be a glass vial used to contain milligram quantities of an antibody.

The present invention also includes agents identified by the screening methods described herein and methods of treatment that include the administration of such agents. Such agents may be administered to a subject for the treatment of a neurodegenerative disease and/or cognitive disorder, including, but not limited to

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Alzheimer's disease. Suitable agents include any of a wide variety of molecules. The term "agent" includes any substance, molecule, element, compound, entity, or a combination thereof. It includes, but is not limited to, e.g., polypeptides, oligopeptide, small organic molecule, polysaccharide, polynucleotide, antisense molecules, ribozymes, antibodies, and the like. It can be a natural product, a synthetic compound, or a chemical compound, or a combination of two or more substances. Unless otherwise specified, the terms "agent," "substance," and "compound" can be used interchangeably.

As used herein "treating" or "treatment" includes both therapeutic and prophylactic treatments. Desirable effects of treatment include preventing occurrence or recurrence of disease, alleviation of symptoms, diminishment of any direct or indirect pathological consequences of the disease, decreasing the rate of disease progression, amelioration or palliation of the disease state, and remission or improved prognosis.

The present invention includes an isolated complex of soluble assemblies of amyloid β and the NMDA receptor. In a preferred embodiment, the isolated complex is a complex between a NMDA receptor and Aβ*56. Isolated complexes may be isolated from natural sources or prepared synthetically, for example, by incubation of isolated soluble assemblies of amyloid β, such as, for example Aβ*56, and one or NMDA receptors or one or more NMDA receptor subunits. Such complexes are useful, for example, as positive controls in any of a variety of assays, including, but not limited to, methods to detect to coprecipitation of an amyloid β and a NMDA receptor and methods to detect agents that alter the antagonistic effect soluble assemblies of amyloid β on NMDA receptor function. The present invention also includes compositions of isolated complexes of soluble assemblies of amyloid β and NMDA receptors.

Isolated complexes of soluble assemblies of amyloid β protein and the NMDA receptor may serve as an antigen or vaccine to immunize an animal to elicit an immune response. The preparation and use of such antigens and vaccines is well known in the art. Immunization may be accomplished in the presence or absence of an adjuvant, e.g., Freund's adjuvant. Booster immunizations may be given at intervals, for example, at two to eight weeks.

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As used herein, "isolated" refers to material removed from its original environment (e.g., the natural environment if it is naturally occurring), and thus is altered "by the hand of man" from its natural state.

Any of the methods described in WO 2004/031400 ("Amyloid Beta-Derived Diffusible Ligands (ADDLS), ADDL-Surrogates, ADDL-Binding Molecules, and Uses Thereof") and Lacor et al., Neurobiology of Disease 23(45):101991-10200, 2004 ("Synaptic Targeting by Alzheimer's-Related Amyloid β Oligomers") may be used in the present invention.

The present invention is illustrated by the following examples. It is to be understood that the particular examples, materials, amounts, and procedures are to be interpreted broadly in accordance with the scope and spirit of the invention as set forth herein. All headings are for the convenience of the reader and should not be used to limit the meaning of the text that follows the heading, unless so specified.

EXAMPLES

Example 1

Aβ*56 is an NMDA Receptor Antagonist

Amyloid-ß (Aß) proteins accumulate in Alzheimer's disease (AD), but the mechanism by which they disrupt cognitive function is unclear. This example shows that Aß*56 (Aß star 56), a 56-kD complex of Aß molecules, specifically binds NR1 and NR2A subunits of NMDA receptors in Tg2576 and AD brains. Purified Aß*56 increases NMDA-mediated spontaneous circuit activity and inhibits NMDA evoked currents in cultured neurons. From these result, it can be concluded that Aß*56 is an NMDA receptor antagonist. Because NMDA receptors are critical mediators of long lasting synaptic plasticity and memory, the data define a new mechanism by which Aß may impair cognitive function associated with AD.

The effects of synthetic soluble Aß oligomers and Aß oligomers secreted by cultured cells include impairment of neuronal survival (Lambert et al., Proc Natl Acad Sci USA 95, 6448 (1998); Kayed et al., Science 300, 486 (2003)), inhibition of long-lasting synaptic plasticity (Wang et al., Brain Res 924, 133 (2002); Walsh et al., Nature

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416, 535 (2002); and Wang et al., J Neurosci 24, 3370 (2004)), and disruption of behaviour (Cleary et al., Nat Neurosci 8, 79 (2005)), as well as up-regulation of the synaptic immediate-early gene Arc (Lacor et al., J Neurosci 24, 10191 (2004)), and endocytosis of NMDA receptors (Snyder et al., Nature Neuroscience 8, 1051 (2005)). However, the effects of endogenous soluble Aß protein assemblies generated *in vivo* have only recently been elucidated (WO 2006/047254). The endogenous Aß assembly, Aß*56, correlates strongly with spatial memory in Tg2576 mice and disrupts cognitive function in rats, in the absence of neuronal loss or amyloid plaque deposition (WO 2006/047254). Because glutamate receptors are important elements in synaptic plasticity and memory (Collingridge, Nature 330, 604 (1987); Malinow et al., Annu Rev Neurosci 25, 103 (2002); and Dudai, "Memory from A to Z: Keywords, Concepts and Beyond" (Oxford University Press, Oxford, 2002)), and down-regulation of Arc and other synaptic genes critical for memory consolidation occurs in Tg2576 and other APP transgenic mice (Dickey et al., J Neurosci 23, 5219 (2003); Palop et al., Proc Natl Acad Sci USA 100, 9572 (2003)), with this example the possibility that Aß*56 impairs memory by interacting directly with glutamate receptors was examined.

Materials and Methods

Transgenic animals. Tg2576 mice (Hsiao et al., Science 274, 99-102 (1996)) were the offspring of mice backcrossed successively to B6SJLF1 breeders.

Human brain tissue. Frozen specimens of cerebral cortex were obtained from three Alzheimer's disease patients and two cognitively intact control subjects from the Rush Alzheimer's Disease Center (Chicago, IL), and one Alzheimer's disease patient from the Regions Hospital Alzheimer's Treatment and Research Center (St. Paul, MN).

Cultured neurons. Cultures of cortical neurons from P1 Sprague Dawley rat pups were prepared according to previously published protocols (Dubinsky, J Neurosci 13, 623-631 (1993)). Dissections of cortical mantels excluded meninges, hippocampus, septal regions, and basal ganglia. Cortical neurons were plated at an approximate density of 50,000-100,000/centimeters squared (cm^2) onto preplated cortical astroglial feeder layers (Dubinsky, J Neurosci 13, 623-631 (1993)) on Themanox plastic coverslips (Electron Microscopy Sciences, Ft. Washington, PA) in Minimal Essential Medium without glutamine, supplemented with 10% NuSerum (Life

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Technologies, Grand Island NY), 27 millimolar (mM) glucose, 50 units/milliliter (U/ml) penicillin, and 50 micrograms/milliliter (μg/ml) streptomycin and maintained at 37°C in 5% CO_2. Cultures were used between twelve and sixteen days in vitro at a time when synaptic networks were well developed (Dichter, Brain Res 149, 279-293 (1978)).

Antibodies. The following primary antibodies were used: 6E10 and 4G8 [1:100-10,000] respectively against Aβ1-17 and Aβ17-25 (Signet Laboratories, USA), APPCter-C17 [1:5000] against APP C-terminus (Sergeant et al., J. Neurochem 81(4):663-72 (2002)), antibodies raised against PSD95, NR1, NR2 subunits (A-D), GluRs [1:200] (SantaCruz Biotechnologies Inc, USA).

Protein extractions. Soluble, extracellular-enriched fractions were generated from hemi-forebrains harvested in 500 microliter (μl) of solution containing 50 millimolar (mM) Tris-HCl (pH 7.6), 0.01% NP-40, 150 mM NaCl, 2mM EDTA, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride (PMSF), and protease inhibitor cocktail (Sigma). Soluble, extracellular enriched proteins were collected from mechanically homogenized lysates (1 milliliter (ml) syringe, gauge 20 needle [ten repeats]) following centrifugation for five minutes at 3,000 rpm. Membrane-enriched fractions were generated from hemi-forebrains harvested in 500 μl of solution containing 50 mM Tris-HCl (pH 7.6), 0.1% NP-40, 150 mM NaCl, 2mM EDTA, 1% SDS, 1 mM PMSF, 2 mM 1,10-PTH and protease inhibitor cocktail (Sigma). Lysates were mechanically homogenized (1 ml syringe and needle, guage 20 [ten repeats]) and centrifuged for 90 minutes at 13,000 rpm. Membrane-associated proteins were generated from the pellets re-suspended with 500 μl of buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 0.5% Triton X-100, 1 mM EGTA, 3% SDS, 1% deoxycholate, 1 mM of PMSF) following centrifugation for 90 minutes at 13,000 rpm.

All supernatants were clarified by centrifuging for 90 minutes at 13,000 rpm prior to western blot analysis. Protein amounts were determined (BCA Protein Assay, Pierce). Western blot and immunoprecipitations were performed as described in WO 2006/047254.

Imunoaffinity chromatography. Tg2576 brain proteins remaining in the supernatant following tissue lysis with RIPA buffer and centrifugation for 90 minutes at 13,000 rpm at 4°C were incubated overnight with affinity columns packed with 200 micrograms (μg) of purified antibody (6E10 or 4G8). Columns were created using the

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Seize™ Primary Immunoprecipitation Kit (Pierce) following the manufacturer's instructions.

Size-exclusion chromatography (SEC). Immunoaffinity purified protein extracts were loaded on Tricorn Superdex® 75 columns (Amersham Life Sciences, Piscataway, NJ) and run at a flow rate of approximately 0.3 milliliter per minute (ml/min). Fractions of 500 µl of eluate in 50 mM Ammonium Acetate, pH 8.5, were diluted 1:100 or 1:750 in neurophysiology experiments, or were concentrated using a vacuum system (VacuFugeTM, Brinkmann- Eppendorf) and analyzed by silver staining.

Silver staining. Following SEC fractionation and SDS polyacrylamide gel electrophoresis (SDS-PAGE), gels were subjected to silver staining to control the purity of the samples. For sensitivity purposes, proteins were stained using the SilverXpress® Silver Staining Kit (Invitrogen™ Life Technologies, USA) with adapted protocol. Changes are as follows: all washing steps were repeated four times and gels were incubated in Developing Solution for fifteen minutes.

Calcium imaging. Neuronal cultures were incubated in the Ca^{2+}-indicator dye Fluo-4 AM (31 µg/ml) and pluronic acid (2.6 mg/ml) for 30 minutes at room temperature. Calcium indicator dye fluorescence was monitored with 488 nanometer (nm) excitation, a 500 nm long-pass barrier filter and confocal microscopy (Odyssey scanner, Noran, Middleton, WI). Cultures were perfused with HEPES-buffered saline equilibrated with air, pH 7.4. Glycine was not added to the perfusate. To quantify neuronal Ca^{2+} responses, Ca^{2+} fluorescence was measured from single neurons or clusters of neurons. Baseline fluorescence was normalized to one and the area between the curve and the baseline calculated using Origin software analysis routines (OriginLab Corporation, Northampton, MA).

Whole cell patch recordings. Neuronal cultures were perfused with HEPES-buffered saline supplemented with 10 µM glycine and 200 nanomolar (nM) tetrodotoxin. Individual neurons were stimulated by focal ejection of 50 µM NMDA and 10 µM glycine. Cells were whole cell voltage clamped and NMDA-evoked inward currents recorded at a holding potential of -35 mV. The HEPES-buffered saline solution contained 135.5 mM NaCl, 3.0 mM KCl; 2.0 mM $CaCl_2$; 1.0 mM $MgSO_4$; 0.5 mM NaH_2PO_4; 15.0 mM D-glucose; 10 mM HEPES; pH 7.4. The intracellular pipette solution contained 5.0 mM Na-methanesulfonate; 128.0 mM K-methanesulfonate; 2.0

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mM $MgCl_2$; 5.0 mM K-EGTA; 1.0 mM glutathione; 2.0 mM MgATP; 0.2 mM NaGTP; 5.0 mM HEPES; pH 7.4.

Results and Discussion

In Tg2576 mice, Aß*56 is found in soluble, extracellular-enriched protein fractions in the brain (WO 2006/047254). To test the hypothesis that Aß*56 is a receptor ligand, membrane-enriched fractions were examined by western blotting with 6E10 monoclonal antibodies (raised against Aß1-16). An immunoreactive band corresponding in molecular weight to Aß*56 was found, in addition to the expected bands corresponding to full length APP and its derivatives CTF-ß and monomeric Aß (Fig. 1A). Importantly, although Aß*56 is one of several species of Aß oligomers in the soluble, extracellular enriched protein fraction, Aß*56 was the only Aß species detected after modest film exposure times, indicating it is the dominant Aß species associated with cell membranes. The possibility that the 56 kilodalton (kD) band was a degradation product of APP was excluded by the demonstration that 22C11 (raised against the N-terminal region of APP) and APP17Cter (raised against the C-terminal regions of APP) failed to bind the 56-kD species.

Next, cross-immunoprecipitations were performed against ionotropic glutamate receptor subunits and Aß in membrane-enriched Tg2576 brain extracts. When proteins were captured with antibodies directed against the NMDA receptor subunit NR1, Aß*56, but no other Aß species, was detected with either 6E10 (Fig. 1B) or 4G8 antibodies. Aß*56 was not immunoprecipitated by NR1 antibodies in soluble, extracellular-enriched extracts containing similar amounts of Aß*56 (Figs. 1A and 1B). This is because NR1 immunoreactivity after NR1 immunoprecipitation was present only in the membrane-enriched extracts, as expected (Fig. 1B). Aß*56 was immunoprecipitated with NR1 antibodies in nine and twenty-four month, but not two month Tg2576 brain extracts (Fig. 1B), which is consistent with the absence of Aß*56 in less than six-month-old mice exhibiting normal spatial memory (WO 2006/047254). NR2A antibodies also immunoprecipitated Aß*56, and were considerably more effective than NR2B antibodies in immunoprecipitating Aß*56. The 4G8 antibody (raised against Aß17-24) immunoprecipitated NR1, NR2A, but not NR2B, receptor subunits from membrane-enriched Tg2576 brain extracts (Fig. 1C), confirming immunoprecipitation experiments with NR1, NR2A and NR2B antibodies. When

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proteins were captured with antibodies to the AMPA receptor subunits, GluR1 and GluR2, neither Aß*56 nor any other Aß oligomers were immunoprecipitated (Fig. 4A-4B), indicating that Aß*56 does not bind AMPA receptors.

The interaction of Aß assemblies and ionotropic glutamate receptors in brain tissue from patients with AD and control individuals without dementia was examined. NR1, NR2A and, to a significantly lesser extent, NR2B antibodies immunoprecipitated a 56-kD 6E10-immunoreactive protein co-migrating with Aß*56 in brain tissue samples from all four patients with Alzheimer's disease, but in neither of two samples from control individuals with no cognitive impairment (NCI) (Figs. 2A and 2B). The unequal levels of NR2A subunits were not due to inconsistencies in loading samples (Fig. 5), and therefore reflected actual receptor subunit levels in the brain specimens. These data indicate that Aß*56 or an Aß*56-like molecule binds NMDA receptors selectively in Alzheimer's disease.

Soluble Aβ oligomers have been postulated to bind α7-nicotinic acetylcholine (nACh) receptors (Dineley et al., J Neurosci 21, 4125 (2001)) and thereby mediate increased endocytosis of NMDA receptors (Snyder et al., Nature Neuroscience 8, 1051 (2005)). Whether As*56 binds α7-nACh receptors was therefore examined. Aβ*56 was not immunoprecipitated from membrane-enriched Tg2576 brain extracts using anti-α7-nACh receptor antibodies (Fig. 2C). The receptors were detected with α7-nACh receptor antibodies in the immunoprecipitated material, excluding failure to immunoprecipitate the receptors as a possible explanation for the absence of Aβ*56. The data indicate that Aβ*56 does not bind α7-nACh receptors in human or Tg2576 brain tissue.

To ascertain whether the physical interaction of Aß*56 and NMDA receptors is functionally relevant, purified Aß*56 from Tg2576 brain was applied to primary cultured neurons and measured changes in intraneuronal ionic calcium (Ca^{2+}) and NMDA evoked currents. Aβ*56 was purified using immunoaffinity chromatography followed by size-exclusion chromatography, which yielded preparations of Aβ*56 that ran as a single band on silver-stained gels (Figs. 6A and 6B). Changes in intracellular Ca^{2+} were monitored in primary cultured neurons in response to external applications of Aβ*56, and quantified Ca^{2+} concentrations by integrating Ca^{2+} indicator dye fluorescence over a 100 second period using confocal microscopy. The addition of 7-8.5 nM Aβ*56 led to an approximately 3-fold (2.95 ± 1.83, $n = 26$ cell clusters from

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five cultures) elevation of the resting Ca^{2+} signal (Figs. 3A and 3B), which was completely abolished when the competitive NMDA receptor antagonist, (+/-)-3-(*2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid* (CPP), was co-applied with Aβ*56 (Fig. 3B). In contrast, the Group I/Group II mGluR antagonist *(RS)-a-Ethyl-4-carboxyphenylglycine* (E4CPG) failed to block the increase in Ca^{2+} triggered by Aβ*56 (Fig. 3B). In single cell measurements, Aβ*56 induced a rapid Ca^{2+} increase within seconds that remained well above baseline for longer than twenty-five minutes (min) (Fig. 3C). The increase in Ca^{2+} remained elevated for at least ten minutes after Aβ*56 was washed out of the culture dish. These results indicate that Aß*56 acts at the NMDA receptor within seconds to increase the circuit activity of neuronal networks in culture, and suggest that the Aß*56-NMDA receptor complex remains functionally active at the neuronal membrane for relatively long periods of time after initial exposure (that is, at least twenty-five minutes). The increased electrical activity of the neuronal network may be explained in a couple of ways: Aß*56 may be an NMDA receptor agonist which depolarizes neurons expressing NMDA receptors, in turn leading to synaptic activation of connected neurons; or Aß*56 may be an NMDA receptor antagonist in a network in which inhibitory interneurons are activated by neurons expressing NMDA receptors, inhibition of which leads to an overall increase in network activity.

To test the effects of Aβ*56 on NMDA-evoked currents directly, whole cell patch clamp recordings were preformed on cultured neurons (Fig. 3D). The addition of 1nM Aβ*56 significantly reduced NMDA-evoked currents to 70 ± 8% of control values ($n = 15$, $p < 0.005$) within five minutes of exposure to Aβ*56, while application of vehicle alone did not alter NMDA-evoked currents (106 ± 7% of control, $n = 7$). These results indicate that Aβ*56 is an NMDA receptor antagonist at the concentration (1 nM) tested, which is the concentration of Aβ measured by standard enzyme-linked immunosorbent assays in human cerebral spinal fluid (Walsh et al., Nature 416, 535 (2002)). There are several possible mechanisms by which Aβ*56 could inhibit NMDA receptor function. It could be a competitive NMDA receptor antagonist. It could induce NMDA receptor endocytosis, probably not via the α7-nACh receptor (Snyder et al., Nature Neuroscience 8, 1051 (2005)), but possibly by mimicking the priming of NMDA receptor endocytosis by glycine (Nong et al., Nature 422, 302 (2003)). Or, it

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could act by a combination of these activities. It will be important in future studies to elucidate the mechanism by which Aß*56 antagonizes NMDA receptor function.

Aß*56 is the sole endogenous Aß protein complex whose disruptive effects on cognitive function have been described (WO 2006/047254). The binding of Aß*56 to synaptic NMDA receptors in Tg2576 and AD brain and its identification as an NMDA receptor antagonist at physiologic, nanomolar concentrations suggest that it functions like a pharmacological agent in the brain. It is proposed that Aß*56 disrupts memory and cognitive function by altering the intrinsic physiological function of NMDA receptors, which are critical mediators of long-lasting synaptic plasticity (Collingridge, Nature 330, 604 (1987); Collingridge et al., Nat Rev Neurosci 5, 952 (2004); and Bliss and Collingridge, Nature 361, 31 (1993)) and memory (Dudai, "Memory from A to Z: Keywords, Concepts and Beyond" (Oxford University Press, Oxford, 2002); Morris et al., Nature 319, 774 (1986)). The reversibility of pre-existing memory deficits in Tg2576 and other APP transgenic mice (Kotilinek et al., J Neurosci 22, 6331 (2002); Dodart et al., Nat Neurosci 5, 452 (2002)), and the transience of cognitive disruption induced by Aß*56 in normal rats (Cleary et al., Nat Neurosci 8, 79 (2005)), is consistent with the pharmacological properties of Aß*56. The selective down-regulation of genes important for long-lasting synaptic plasticity in Tg2576 and other APP transgenic mice (Dickey et al., J Neurosci 23, 5219 (2003); Palop et al., Proc Natl Acad Sci USA 100, 9572 (2003)) may be a result of the NMDA receptor antagonistic properties of Aß*56. The present example defines a mechanism by which Aß causes reversible neuronal dysfunction rather than irreversible structural degeneration. Targeting reversible neuronal dysfunction with drugs aimed at the interaction between Aß*56 and NMDA receptors may prevent dementia in persons at risk for AD and restore brain function in the early stages of Alzheimer's disease.

Example 2

Antibodies to Aß block Aß*-mediated inhibition of NMDA-evoked currents

Candidate anti-Aß clones will be screened comprehensively using methods that ensure that the anti-Aß* monoclonals specifically recognize natively folded Aß*56, and do not bind fibrillar or monomeric Aß. Dot blot methods followed by

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confirmatory liquid-phase immunoprecipitation and immunoblotting experiments can be used for this purpose. Direct liquid phase ELISA methods can also be used. The dot blot method is advantageous due to its rapid throughput and minimal potential for steric hindrance preventing detection of suitable clones. The ELISA method is useful due to its ability to detect natively folded Aß*56 directly.

Various methods may be used to screen candidate monoclonals for antibodies that specifically detect Aß*56. In the Dot Blot assay, Aß*56, synthetic monomeric Aß(1-42), soluble Aß(1-42) oligomers and fibrillar Aß(1-42) can be spotted at known concentrations on nitrocellulose or nylon filters. The filters can be overlaid with candidate monoclonals. Clones that selectively stain Aß*56 at low concentrations can be selected. In the Western Blot assay, which is a confirmatory test for the Dot Blot assay, Aß*56, synthetic monomeric Aß(1-42), soluble Aß(1-42) oligomers and fibrillar Aß(1-42) can be size-fractionated by polyacrylamide gel electrophoresis and transferred to nitrocellulose or nylon filters. The filters can be overlaid with candidate monoclonals. Clones that selectively stain Aß*56 but no other forms of Aß can be selected. In the liquid phase ELISA method, monoclonal anti-Aß antibodies 6E10 or 4G8 can be immobilized onto the wells of plastic plates, overlaid with Aß*56. Candidate monoclonals can be applied to wells. Clones that bind Aß*56 can be detected with goat anti-mouse antibodies conjugated to a fluorescent marker.

To generate anti-Aß* monoclonals, mice will be immunized with purified Aß*56 from the brains of Tg2576 mice greater than six months old, AD patients, or Down syndrome patients, or with synthetic Aß oligomers that include species which are ~56 kDa. Aß*56 can be purified by immunoaffinity chromatography followed by size-exclusion chromatography so that it runs as a single band on silver stained gels. Biochemical methods can also be used to purify Aß*56, taking advantage of the stability of Aß*56 in 8M urea, which denatures most globular proteins.

To determine that the purified immunogen is biologically active, it can be assayed for its ability to inhibit NMDA-evoked currents in cultured neurons, prior to injection as an immunogen. However, this is not an essential step. It is expected that these methods will successfully lead to the generation of specific antibodies to Aß*56, particularly since multimerized proteins tend to be better immunogens than monomeric proteins, because they crosslink immunoglobulins on B-cells.

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It is possible that Aß*56 in human brain (from AD patients) and mouse brain (from Tg2576 mice) may differ subtly in conformation. Therefore, both natively folded Aß*56 purified from human AD brains and Tg2576 mouse brain tissue can be used to screen monoclonals. The results will generate a two by two catalogue of clones showing specific recognition of AD-Aß*56, Tg2576-Aß*56, both AD-Aß*56 and Tg2576-Aß*56, or neither protein complex. This catalogue will aid in selecting the most appropriate anti-Aß* monoclonals for use in humans.

Assessing the ability of Aß*-monoclonals to block Aß*-mediated inhibition of NMDA-evoked currents in cultured rat cortical neurons and human neuronal cell lines. Confirmation of the ability of anti-Aß* monoclonals to block the inhibitory effects of Tg2576-Aß*56 or AD-Aß*56 on NMDA-evoked currents in cultured cortical neurons may be accomplished using the methods described in Example 1, assessing the effects of Tg2576-Aß*56 on NMDA-evoked currents. These experiments will ensure that the anti-Aß* monoclonals specifically block the interaction of Tg2576-Aß*56 or AD-Aß*56 with rat and human NMDA receptors. Anti-Aß* monoclonals which have been functionally and biochemically validated may then undergo further screening at lower concentrations to define dose-effect curves, which will serve to identify the most potent monoclonal inhibitors of the effects of Aß*56 on memory, cognitive function and NMDA receptor function.

Example 3

NMDA receptor function determined by Ca^{2+} fluorescence imaging

The assay will use Ca^{2+} fluorescence imaging to monitor the electrical activity of neurons in culture. The assay determines the effective dose of Aβ*56 in binding to NMDA receptors. An advantage of the assay is that the effect of Aβ*56 on NMDA receptor function can be determined quickly and easily.

Cultured neurons. Cultures of cortical neurons from rat pups are prepared according to established protocols. Neurons are co-cultured with glial cells on glass coverslips. Cultured neurons between twelve and sixteen days in vitro are used, when synaptic networks are well developed and spontaneous electrical activity occurs.

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Calcium imaging. Cultured neurons on coverslips are incubated in the Ca^{2+}-indicator dye Fluo-4 AM (31 μg/ml) and pluronic acid (2.6 mg/ml) for 30 minutes at room temperature. The coverslips are then rinsed and placed in a Petri dish containing HEPES-buffered saline equilibrated with air, pH 7.4. Calcium indicator dye fluorescence is monitored with 488 nm excitation, a 500 nm long-pass barrier filter and either confocal microscopy or epifluorescence microscopy.

Analysis. Calcium fluorescence is measured from single neurons or clusters of neurons to quantify neuronal activity. Baseline fluorescence is normalized and the area between the curve and the baseline calculated using Origin software analysis routines. The resulting integrated Ca^{2+} signal is used as a measure of neuronal activity.

Experimental protocol for determining the Aβ*56 dose-response relation. Baseline neuronal activity in control solution will be determined using the Ca^{2+} fluorescence assay. The concentration of Aβ*56 in the solution will then be raised stepwise and neuronal activity will be determined at each Aβ*56 concentration. A vehicle control will also conducted to ensure that changes in neuronal activity are not due to the buffer solution that contains the Aβ*56.

The complete disclosure of all patents, patent applications, and publications, and electronically available material (including, for instance, nucleotide sequence submissions in, e.g., GenBank and RefSeq, and amino acid sequence submissions in, e.g., SwissProt, PIR, PRF, PDB, and translations from annotated coding regions in GenBank and RefSeq) cited herein are incorporated by reference. The foregoing detailed description and examples have been given for clarity of understanding only. No unnecessary limitations are to be understood therefrom. The invention is not limited to the exact details shown and described, for variations obvious to one skilled in the art will be included within the invention defined by the claims.

For any method disclosed herein that includes discrete steps, the steps may be conducted in any feasible order. And, as appropriate, any combination of two or more steps may be conducted simultaneously.

All headings are for the convenience of the reader and should not be used to limit the meaning of the text that follows the heading, unless so specified.

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What is claimed is:

1. A method of detecting a neurodegenerative disease and/or cognitive disorder in a subject, the method comprising:

obtaining a sample from the subject;

immunoprecipitating the sample with an antibody to a NMDA receptor;

wherein the coprecipitation of amyloid β along with the NMDA receptor indicates the subject has a neurodegenerative disease and/or cognitive disorder.

2. A method of detecting a presymptomatic neurodegenerative disease and/or cognitive disorder in a subject, the method comprising:

obtaining a sample from the subject;

immunoprecipitating the sample with an antibody to a NMDA receptor;

wherein the coprecipitation of amyloid β along with the NMDA receptor indicates the subject has a presymptomatic neurodegenerative disease and/or cognitive disorder disease.

3. The method of claim 1 or 2, wherein the antibody to an NMDA receptor is an antibody that binds to a NMDA receptor subunit selected from the group consisting of NR1, NR2A, and NR2B.

4. A method of inhibiting NMDA receptor function, the method comprising contacting a NMDA receptor with a soluble assembly of amyloid β protein.

5. A method of screening for an agent that alters the antagonistic effect of a soluble assembly of amyloid β protein on NMDA receptor function, the method comprising:

contacting a NMDA receptor with the agent and a soluble assembly of amyloid β protein;

determining NMDA receptor function for the NMDA receptor contacted with the agent and the soluble assembly of amyloid β protein;

comparing NMDA receptor function for the NMDA receptor contacted with the

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agent and the soluble assembly of amyloid β protein to NMDA receptor function for a NMDA receptor contacted with the soluble assembly of amyloid β protein and not contacted with the agent;

wherein a difference in the level of NMDA receptor function in the NMDA receptor contacted with the agent and the soluble assembly of amyloid β protein compared to NMDA receptor function in the NMDA receptor contacted with the soluble assembly of amyloid β protein and not contacted with the agent indicates the agent alters the antagonistic effect of the soluble assembly of amyloid β protein on NMDA receptor function.

6. A method of screening for an agent for the treatment of neurodegenerative disease and/or cognitive disorder, the method comprising:

contacting a NMDA receptor with the agent and a soluble assembly of amyloid β protein;

determining NMDA receptor function for the NMDA receptor contacted with the agent and the soluble assembly of amyloid β protein;

comparing NMDA receptor function for the NMDA receptor contacted with the agent and the soluble assembly of amyloid β protein to NMDA receptor function for an NMDA receptor contacted with the soluble assembly of amyloid β protein and not contacted with the agent;

wherein an altered level of NMDA receptor function in the NMDA receptor contacted with the agent and the soluble assembly of amyloid β protein compared to the NMDA receptor function of the NMDA receptor contacted with the soluble assembly of amyloid β protein and not contacted with the agent indicates the agent as an agent for the treatment of neurodegenerative disease and/or cognitive disorder.

7. The method of claim 5 or 6, wherein the agent inhibits the antagonistic effect of the soluble assembly of amyloid β protein on NMDA receptor function.

8. The method of any one of claims 5-7, wherein NMDA receptor function is determined by whole cell patch clamp recording.

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9. The method of any one of claims 5-7, wherein NMDA receptor function is determined by Ca^{+2} fluorescence imaging.

10. A method of treating a neurodegenerative disease and/or a cognitive disorder in a subject, the method comprising administering to the subject an effective amount of an agent that alters the antagonistic effect of a soluble assembly of amyloid β protein on the NMDA receptor.

11. The method of any one of claims 1 to 3 or 6 to 10, wherein the neurodegenerative disease and/or cognitive disorder is Alzheimer's disease.

12. The method of any one of claims claim 1-11, wherein the soluble assembly of amyloid β protein has a molecular weight of about 56 kDa as measured by SDS polyacrylamide gel electrophoresis.

13. The method of any one of claims claim 1-11, wherein the soluble assembly of amyloid β protein comprises a dodecamer of amyloid β proteins.

14. The method of any one of claims claim 1-11, wherein the soluble assembly of amyloid β protein is Aβ*56.

15. An isolated Aβ*56/NMDA receptor complex.

16. An agent that alters the inhibitory effect of a soluble assembly of amyloid β protein on the NMDA receptor.

17. An agent identified by any one of the methods of claims 5-9.

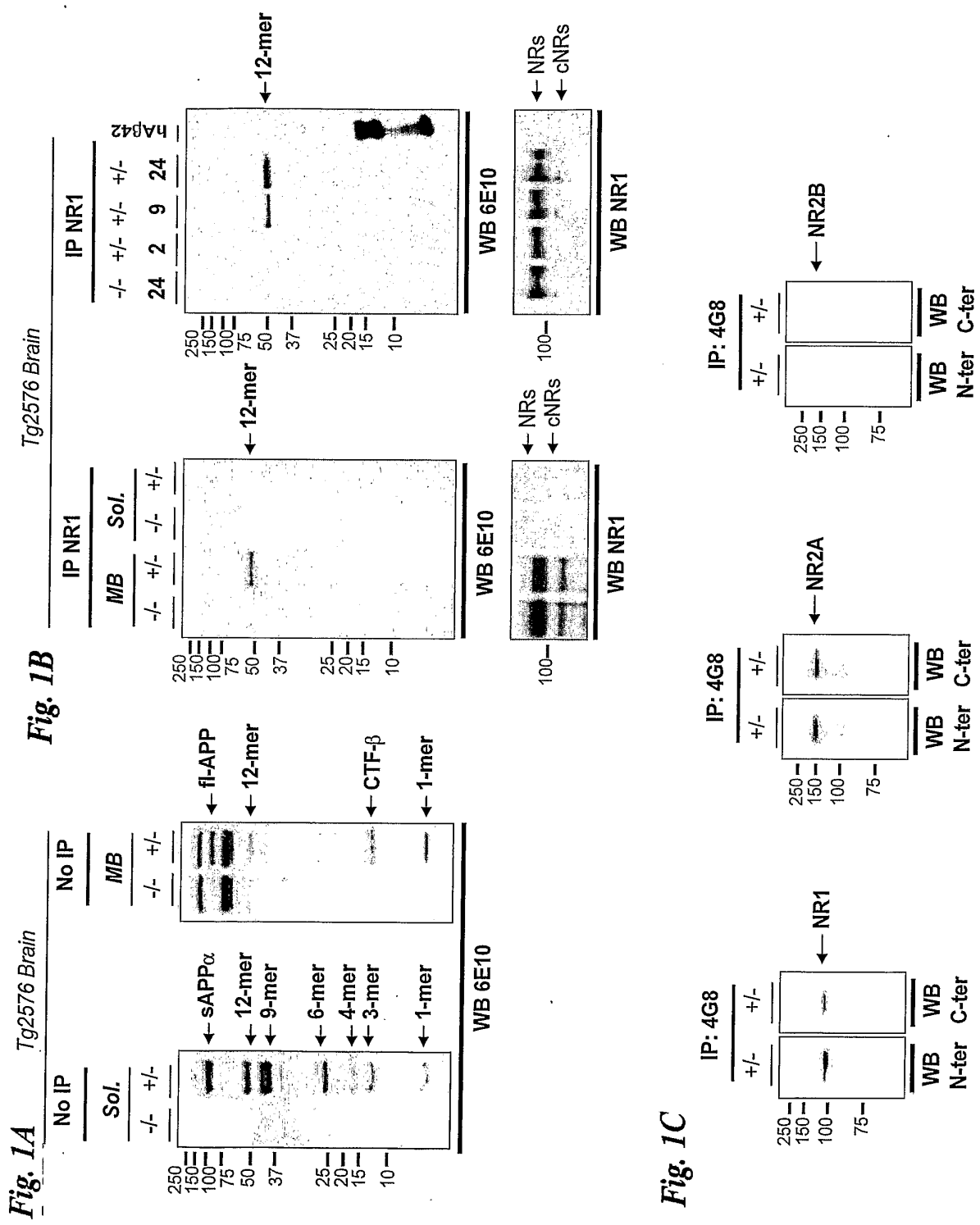
18. An antibody that binds to a soluble assembly of amyloid β protein and prevents the formation of an amyloid β/NMDA receptor complex.

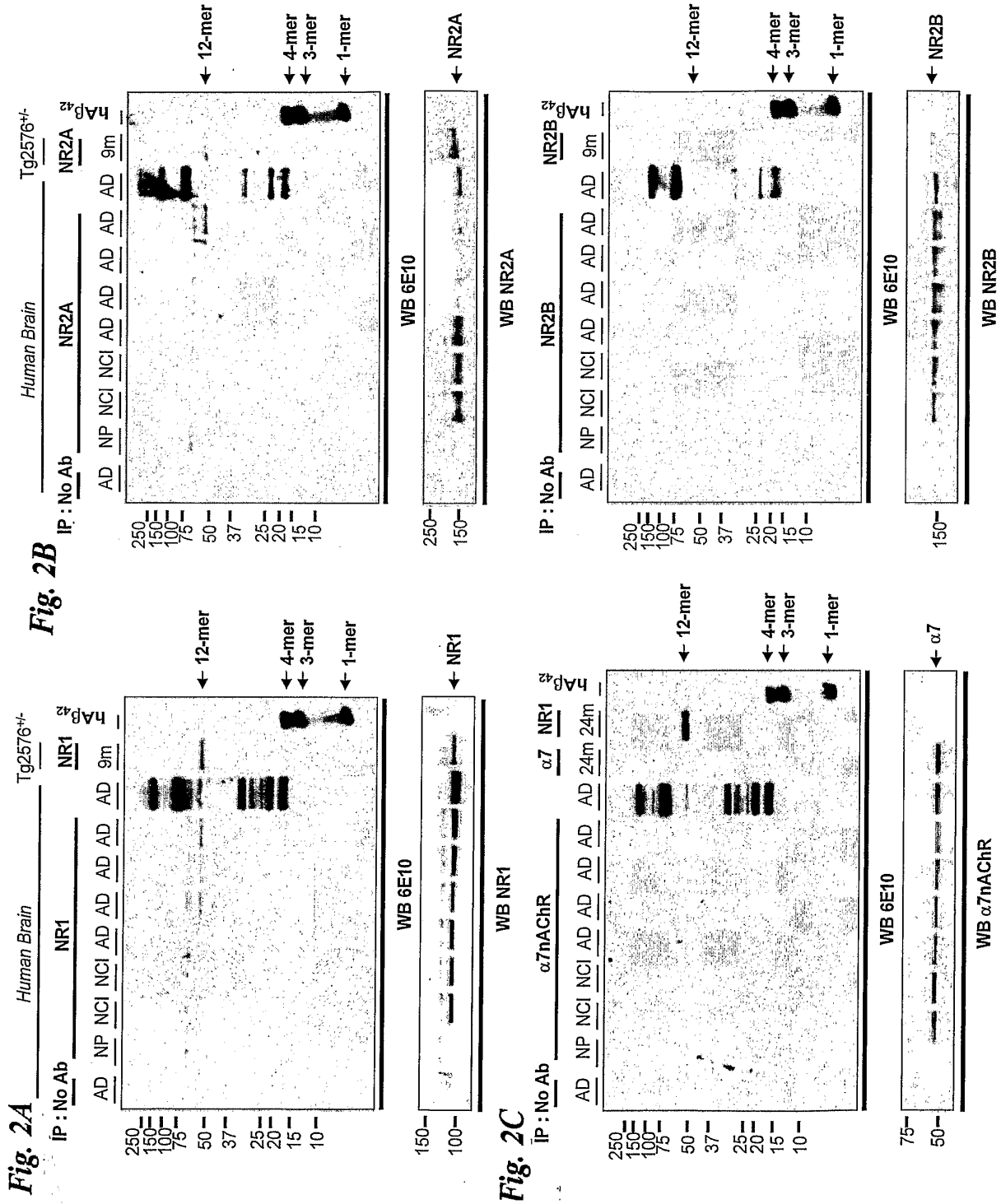
19. An antibody that binds to a NMDA receptor and prevents the formation of an amyloid β/NMDA receptor complex.

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20. A method of treating a neurodegenerative disease and/or a cognitive disorder in a subject, the method comprising administering to the subject an effective amount of an antibody of claim 18 or 19.

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Fig. 1A
Tg2576 Brain
No IP
Sol.
-/- +/-
No IP
MB
-/- +/-
sAPPα
12-mer
9-mer
6-mer
4-mer
3-mer
1-mer
fl-APP
12-mer
CTF-β
1-mer
WB 6E10
250
150
100
75
50
37
25
20
15
10
Fig. 1B
Tg2576 Brain
IP NR1
MB Sol.
-/- +/- -/- +/-
12-mer
WB 6E10
NRs
cNRs
WB NR1
IP NR1
-/- +/- +/- +/-
24 2 9 24
hAβ42
12-mer
WB 6E10
NRs
cNRs
WB NR1
Fig. 1C
IP: 4G8
+/- +/-
NR1
WB WB
N-ter C-ter
IP: 4G8
+/- +/-
NR2A
WB WB
N-ter C-ter
IP: 4G8
+/- +/-
NR2B
WB WB
N-ter C-ter


Fig. 2A
Human Brain
Tg2576+/-
IP : No Ab
AD NP NCI NCI AD AD AD AD AD 9m
NR1
hAβ42
12-mer
4-mer
3-mer
1-mer
WB 6E10
NR1
WB NR1
Fig. 2B
Human Brain
Tg2576+/-
IP : No Ab
AD NP NCI NCI AD AD AD AD AD 9m
NR2A
hAβ42
12-mer
4-mer
3-mer
1-mer
WB 6E10
NR2A
WB NR2A
IP : No Ab
AD NP NCI NCI AD AD AD AD AD 9m
NR2B
hAβ42
12-mer
4-mer
3-mer
1-mer
WB 6E10
NR2B
WB NR2B
Fig. 2C
IP : No Ab
AD NP NCI NCI AD AD AD AD AD 24m 24m
α7nAChR
α7
NR1
hAβ42
12-mer
4-mer
3-mer
1-mer
WB 6E10
α7
WB α7nAChR

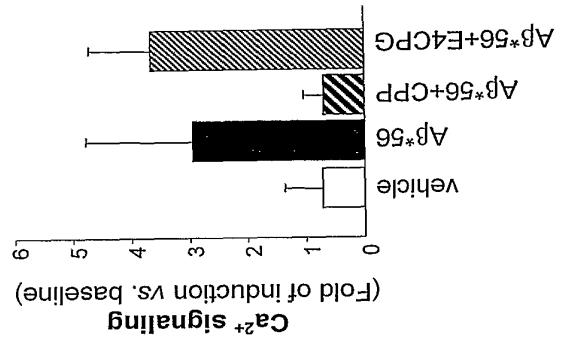


Fig. 3B

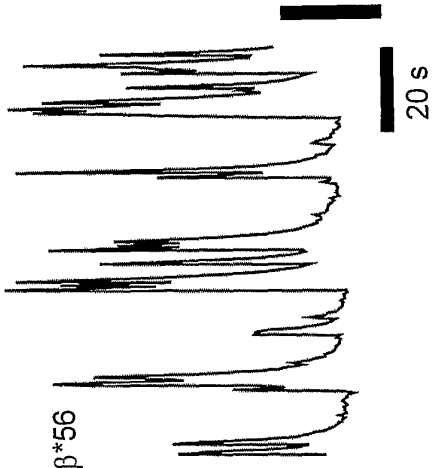


Fig. 3A

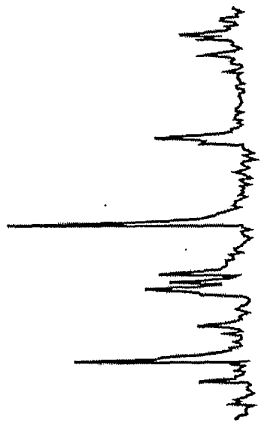
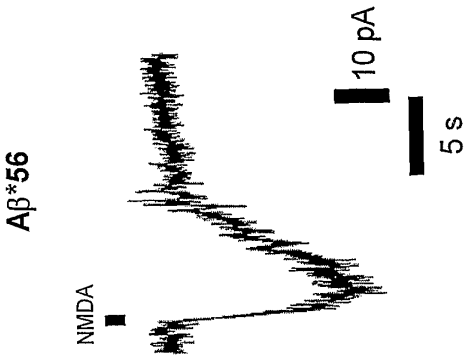


Fig. 3D



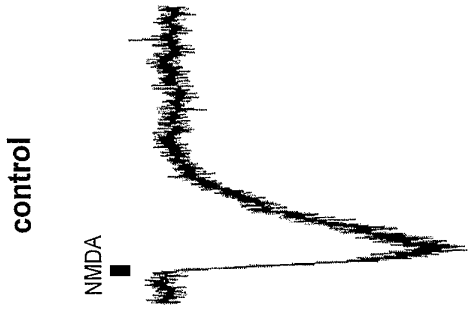
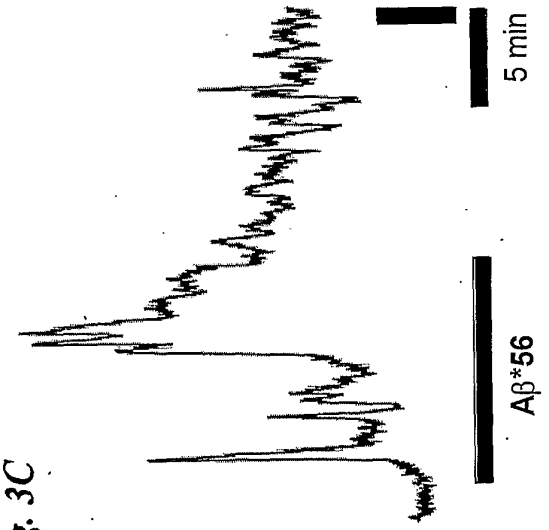


Fig. 3C



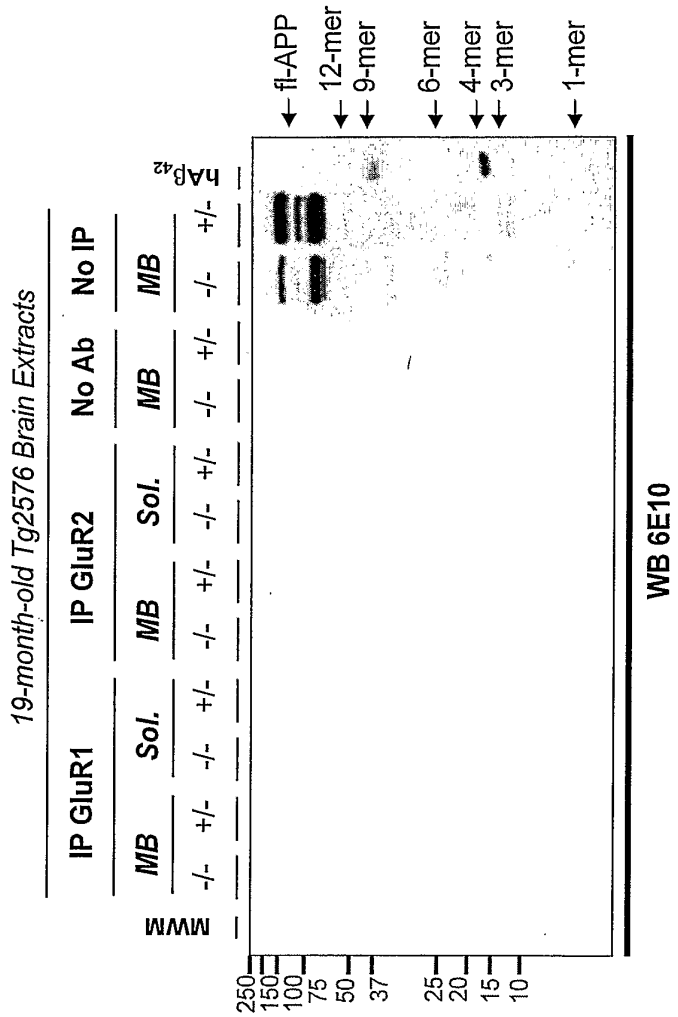

19-month-old Tg2576 Brain Extracts
IP GluR1
IP GluR2
No Ab
No IP
MB
Sol.
MB
Sol.
MB
MB
-/- +/- -/- +/- -/- +/- -/- +/-
MWM
hAβ42
250
150
100
75
50
37
25
20
15
10
fl-APP
12-mer
9-mer
6-mer
4-mer
3-mer
1-mer
WB 6E10

Fig. 4A

IP GluR1
MB
Sol.
-/- +/- -/- +/-
GluR1
150
100
WB GluR1
IP GluR2
MB
Sol.
-/- +/- -/- +/-
GluR2
150
100
WB GluR2

Fig. 4B

Fig. 5

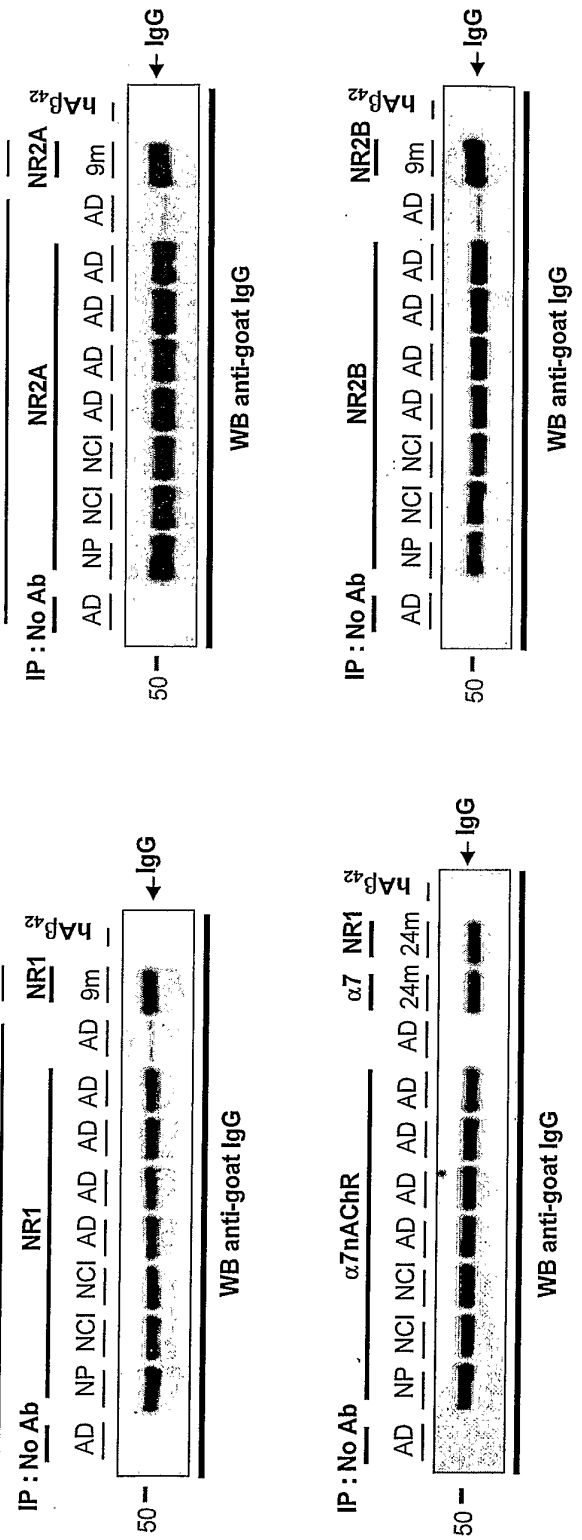

Human Brain
Tg2576+/-
IP : No Ab
NR2A
NR2A
hAβ42
AD NP NCI NCI AD AD AD AD AD AD 9m
50
IgG
WB anti-goat IgG
Human Brain
Tg2576+/-
IP : No Ab
NR2B
NR2B
hAβ42
AD NP NCI NCI AD AD AD AD AD AD 9m
50
IgG
WB anti-goat IgG
Human Brain
Tg2576+/-
IP : No Ab
NR1
NR1
hAβ42
AD NP NCI NCI AD AD AD AD AD AD 9m
50
IgG
WB anti-goat IgG
IP : No Ab
α7nAChR
α7 NR1
hAβ42
AD NP NCI NCI AD AD AD AD 24m 24m
50
IgG
WB anti-goat IgG

Fig. 6A

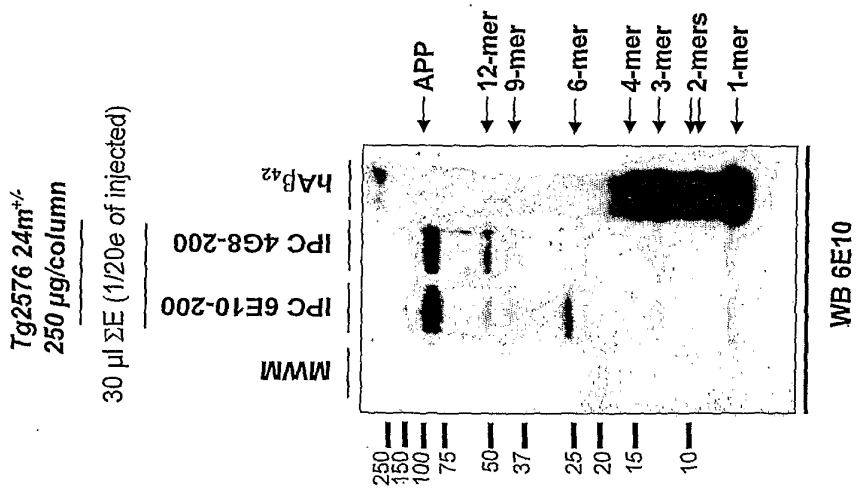
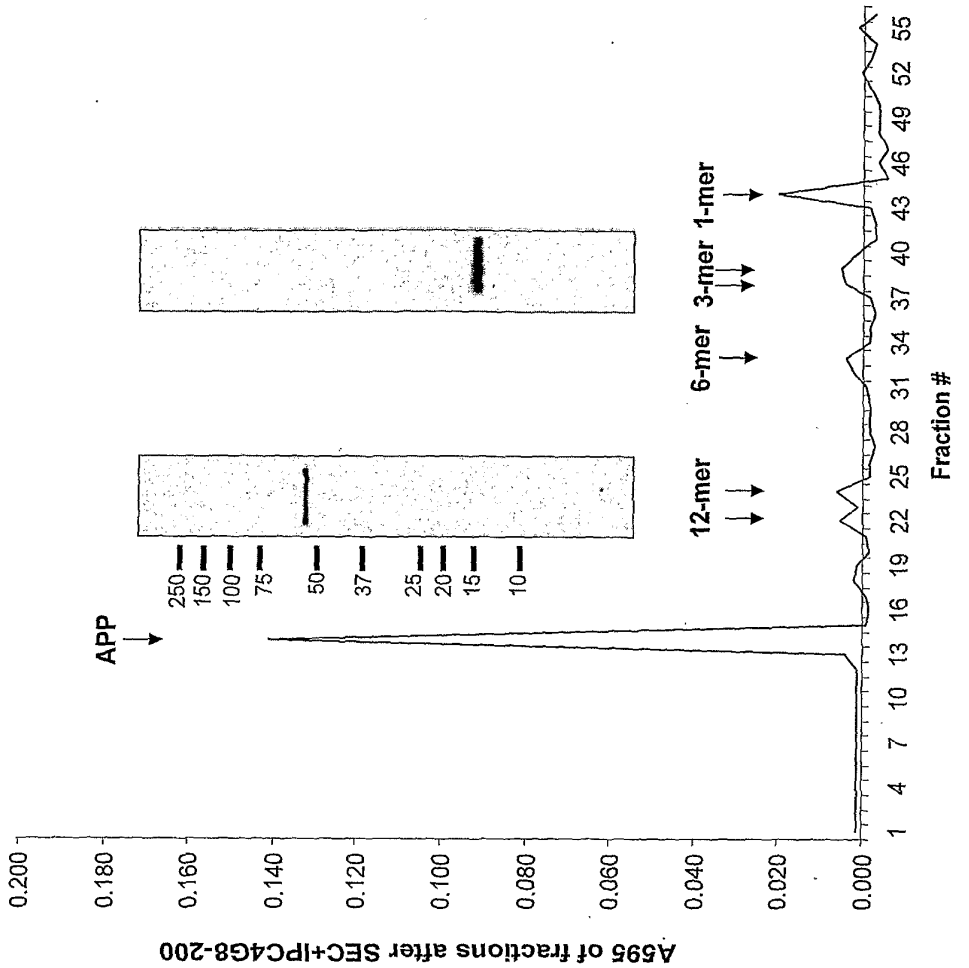

Tg2576 24m+/-
250 µg/column
30 µl ΣE (1/20e of injected)
MWM
IPC 6E10-200
IPC 4G8-200
hAβ42
APP
12-mer
9-mer
6-mer
4-mer
3-mer
2-mers
1-mer
250
150
100
75
50
37
25
20
15
10
WB 6E10

Fig. 6B


A595 of fractions after SEC+IPC4G8-200
0.200
0.180
0.160
0.140
0.120
0.100
0.080
0.060
0.040
0.020
0.000
1 4 7 10 13 16 19 22 25 28 31 34 37 40 43 46 49 52 55
Fraction #
APP
12-mer
6-mer
3-mer
1-mer
250
150
100
75
50
37
25
20
15
10

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2006/029452

A. CLASSIFICATION OF SUBJECT MATTER
INV. G01N33/68 A61K39/00 A61K31/00

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 A61K

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 practical, search terms used)
EPO-Internal, BIOSIS, WPI Data, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CULLEN WILLIAM K ET AL: "Beta-Amyloid produces a delayed NMDA receptor-dependent reduction in synaptic transmission in rat hippocampus" NEUROREPORT, vol. 8, no. 1, 1996, pages 87-92, XP008071648 ISSN: 0959-4965 abstract figure 3	4,10-14, 16,17
X	US 2003/105152 A1 (INGRAM VERNON M [US] ET AL) 5 June 2003 (2003-06-05) the whole document in particular: abstract example 1 paragraph [0170]	4

-/--

[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

"E" earlier document 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
20 November 2006	13/12/2006

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, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Tuynman, Antonin

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

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2006/029452

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	MOLNAR ZSOLT ET AL: "Enhancement of NMDA responses by beta-amyloid peptides in the *hippocampus in vivo*" NEUROREPORT, vol. 15, no. 10, 19 July 2004 (2004-07-19), pages 1649-1652, XP008071651 ISSN: 0959-4965 abstract page 1651, right-hand column, line 38 - line 40	5-7
X	WO 01/77170 A2 (UNIV EDINBURGH [GB]; GRANT SETH GARRAN NIELS [GB]; HUSI HOLGER [GB] *UN*) *18 October 2001* (2001-10-18) the whole document in particular: abstract examples 1,2	1-3
A	COWBURN RICHARD F ET AL: "Effects of beta-amyloid-(25-35) peptides on radioligand binding to excitatory amino acid receptors and voltage-dependent calcium channels: Evidence for a selective affinity for the glutamate and glycine recognition sites of the NMDA receptor" NEUROCHEMICAL RESEARCH, vol. 22, no. 12, 1997, pages 1437-1442, XP002408132 ISSN: 0364-3190 abstract	1-3
P,X	LESNE SYLVAIN ET AL: "A specific amyloid-beta protein assembly in the brain impairs memory" NATURE (LONDON), vol. 440, no. 7082, March 2006 (2006-03), pages 352-357, XP002408133 ISSN: 0028-0836 cited in the application the whole document	15-17
P,X	WO 2006/047254 A (UNIV MINNESOTA [US]; LESNE SYLVAIN E [US]; ASHE KAREN H [US]; CLEARY J) 4 May 2006 (2006-05-04) cited in the application the whole document	1-17

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

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box II.1

Although claims 4,10-14 and 20 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

Continuation of Box II.1

Claims Nos.: 4,10-14 (in part); 18-20

Rule 39.1(iv) PCT - Method for treatment of the human or animal body by therapy

---

Continuation of Box II.2

Claims Nos.: 18-20

Present claim 18-20 relate to antibodies or the use thereof (cliam20), which have a given desired property or effect, namely an antibody that binds to a soluble assembly of amyloid b protein and prevents the formation of an amyloid b /NMDA receptor complex (claim 18) or an antibody that binds to a NMDA receptor and prevents the formation of an amyloid b /NMDA receptor complex (claim 19). However, the description does not provide support and disclosure in the sense of Article 6 and 5 PCT for any such antibody having the said property or effect and there is no common general knowledge of this kind available to the person skilled in the art. This non-compliance with the substantive provisions is to such an extent, that taking into consideration the non-compliance in determining the extent of the search of the claim (PCT Guidelines 9.19 and 9.20), no search was performed for these claims.

The applicant's attention is drawn to the fact that claims relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure. If the application proceeds into the regional phase before the EPO, the applicant is reminded that a search may be carried out during examination before the EPO (see EPO Guideline C-VI, 8.5), should the problems which led to the Article 17(2) declaration be overcome.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2006/029452

Box II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: 4,10-14 (in part); 18-20
because they relate to subject matter not required to be searched by this Authority, namely:

Although claims 4,10-14 and 20 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [X] Claims Nos.: 18-20
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

see FURTHER INFORMATION sheet PCT/ISA/210

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

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

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

Fujirebio Diagnostics, Inc. v. Quanterix Corp.
EX-1045
7925

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/US2006/029452

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 2003105152 A1	05-06-2003	NONE	
WO 0177170 A2	18-10-2001	AU 4441101 A	23-10-2001
		CA 2405311 A1	18-10-2001
		EP 1272517 A2	08-01-2003
		JP 2003530125 T	14-10-2003
		US 2003176651 A1	18-09-2003
WO 2006047254 A	04-05-2006	NONE	

Fujirebio Diagnostics, Inc. v. Quanterix Corp.
EX-1045
7926

Doc Code: TRAN.LET
Document Description: Transmittal Letter

USED IN LIEU OF PTO/SB/21 (07-09)

TRANSMITTAL FORM

(to be used for all correspondence after initial filing)

Total Number of Pages in This Submission	

Application Number	14/111,326-Conf. #3274
Filing Date	June 24, 2014
First Named Inventor	David Wilson
Art Unit	1649
Examiner Name	A. M. Weidner
Attorney Docket Number	Q0052.70018US01

ENCLOSURES (*Check all that apply*)

- [ ] Fee Transmittal Form
- [ ] Fee Attached
- [ ] Amendment/Reply
- [ ] After Final
- [ ] Affidavits/declaration(s)
- [ ] Extension of Time Request
- [ ] Express Abandonment Request
- [x] Information Disclosure Statement
- [ ] Certified Copy of Priority Document(s)
- [ ] Reply to Missing Parts/ Incomplete Application
- [ ] Reply to Missing Parts under 37 CFR 1.52 or 1.53
- [ ] Drawing(s)
- [ ] Licensing-related Papers
- [ ] Petition
- [ ] Petition to Convert to a Provisional Application
- [ ] Power of Attorney, Revocation Change of Correspondence Address
- [ ] Terminal Disclaimer
- [ ] Request for Refund
- [ ] CD, Number of CD(s) _____
- [ ] Landscape Table on CD
- [ ] After Allowance Communication to TC
- [ ] Appeal Communication to Board of Appeals and Interferences
- [ ] Appeal Communication to TC **(Appeal Notice, Brief, Reply Brief)**
- [ ] Proprietary Information
- [ ] Status Letter
- [x] Other Enclosure(s) (please Identify below):

-Form PTO-1449
-Copies of Cited References

Remarks

SIGNATURE OF APPLICANT, ATTORNEY, OR AGENT

Firm Name	WOLF, GREENFIELD & SACKS, P.C.		
Signature	/Andrea J. Gabert/		
Printed name	Andrea J. Gabert, Ph.D.		
Date	February 22, 2016	Reg. No.	68,904

Certificate of Electronic Filing Under 37 CFR 1.8

I hereby certify that this paper (along with any paper referred to as being attached or enclosed) is being transmitted via the Office electronic filing system in accordance with 37 CFR § 1.6(a)(4).

Dated: February 22, 2016 Signature: ____/Nathaniel W. Taylor/_____ (Nathaniel W. Taylor)

PTO/SB/17 (03-13)
Approved for use through 01/31/2014. OMB 0651-0032
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995 no persons are required to respond to a collection of information unless it displays a valid OMB control number

FEE TRANSMITTAL

		Complete if known	
		Application Number	14/111,326-Conf. #3274
		Filing Date	June 24, 2014
☐	Applicant asserts small entity status. See 37 CFR 1.27.	First Named Inventor	David Wilson
☐	Applicant certifies micro entity status. See 37 CFR 1.29. Form PTO/SB/15A or B or equivalent must either be enclosed or have been submitted previously.	Examiner Name	A. M. Weidner
		Art Unit	1649
TOTAL AMOUNT OF PAYMENT	($) 1,800.00	Practitioner Docket No.	Q0052.70018US01

METHOD OF PAYMENT (check all that apply)

☐ Check ☒ Credit Card ☐ Money Order ☐ None ☐ Other (please identify): _____

☐ Deposit Account Deposit Account Number: 23/2825 Deposit Account Name: Wolf, Greenfield & Sacks, P.C.

For the above-identified deposit account, the Director is hereby authorized to (check all that apply):

☐ Charge fee(s) indicated below ☐ Charge fee(s) indicated below, **except for the filing fee**

☒ Charge any additional fee(s) or underpayment of fee(s) under 37 CFR 1.16 and 1.17 ☒ Credit any overpayment of fee(s)

WARNING: Information on this form may become public. Credit card information should not be included on this form. Provide credit card information and authorization on PTO-2038.

FEE CALCULATION

1. BASIC FILING, SEARCH, AND EXAMINATION FEES (U = undiscounted fee; S = small entity fee; M = micro entity fee)

	FILING FEES			SEARCH FEES			EXAMINATION FEES			
Application Type	**U ($)**	**S ($)**	**M ($)**	**U ($)**	**S ($)**	**M ($)**	**U ($)**	**S ($)**	**M ($)**	**Fees Paid ($)**
Utility	280	140*	70	600	300	150	720	360	180	
Design	180	90	45	120	60	30	460	230	115	
Plant	180	90	45	380	190	95	580	290	145	
Reissue	280	140	70	600	300	150	2,160	1,080	540	
Provisional	260	130	65	0	0	0	0	0	0	

* The $140 small entity status filing fee for a utility application is further reduced to $70 for a small entity status applicant who files the application via EFS-Web.

2. EXCESS CLAIM FEES

Fee Description	**Undiscounted Fee ($)**	**Small Entity Fee ($)**	**Micro Entity Fee ($)**
Each claim over 20 (including Reissues)	80	40	20
Each independent claim over 3 (including Reissues)	420	210	105
Multiple dependent claims	780	390	195

Total Claims	**Extra Claims**	**Fee ($)**	**Fee Paid ($)**	**Multiple Dependent Claims**	
				Fee ($)	**Fee Paid ($)**
_____ - 20 or HP =	_____ x	_____ =	_____	_____	_____

HP = highest number of total claims paid for, if greater than 20.

Indep. Claims	**Extra Claims**	**Fee ($)**	**Fee Paid ($)**
_____ - 3 or HP =	_____ x	_____ =	_____

HP = highest number of independent claims paid for, if greater than 3.

3. APPLICATION SIZE FEE

If the specification and drawings exceed 100 sheets of paper (excluding electronically filed sequence or computer listings under 37 CFR 1.52(e)), the application size fee due is $400 ($200 for small entity) ($100 for micro entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).

Total Sheets	**Extra Sheets**	**Number of each additional 50 or fraction thereof**	**Fee ($)**	**Fee Paid ($)**
_____ - 100 =	_____ /50 =	_____ (round **up** to a whole number) x	_____ =	_____

4. OTHER FEE(S)

	Fees Paid ($)
Non-English specification, $130 fee (no small or micro entity discount)	
Non-electronic filing fee under 37 CFR 1.16(t) for a utility application, $400 fee ($200 small or micro entity)	
Other (e.g., late filing surcharge): 1801 Request for RCE - 1st request (see 37 CFR 1.114)	1,200.00
1252 Extension for response within second month	600.00

SUBMITTED BY

Signature	/Andrea J. Gabert/	Registration No. (Attorney/Agent)	68,904	Telephone	617.646.8000
Name (Print/Type)	Andrea J. Gabert, Ph.D.			Date	February 22, 2016

Certificate of Electronic Filing Under 37 CFR 1.8

I hereby certify that this paper (along with any paper referred to as being attached or enclosed) is being transmitted via the Office electronic filing system in accordance with 37 CFR § 1.6(a)(4).

Dated: February 22, 2016 Signature: /Ellen J. Paul/ (Ellen J. Paul)

4604551.1

Electronic Patent Application Fee Transmittal

Application Number:	14111326
Filing Date:	24-Jun-2014
Title of Invention:	METHODS OF DETERMINING A TREATMENT PROTOCOL FOR AND/OR A PROGNOSIS OF A PATIENT'S RECOVERY FROM A BRAIN INJURY
First Named Inventor/Applicant Name:	David Wilson
Filer:	Andrea J. Gabert/Ellen Paul
Attorney Docket Number:	Q0052.70018US01

Filed as Large Entity

Filing Fees for U.S. National Stage under 35 USC 371

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
Pages:				
Claims:				
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				
Extension-of-Time:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Extension - 2 months with $0 paid	1252	1	600	600
Miscellaneous:				
Request for Continued Examination	1801	1	1200	1200
		Total in USD ($)		**1800**

PTO/SB/06 (09-11)
Approved for use through 1/31/2014. OMB 0651-0032
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

PATENT APPLICATION FEE DETERMINATION RECORD Substitute for Form PTO-875	Application or Docket Number 14/111,326	Filing Date 06/24/2014	☐ To be Mailed

ENTITY: ☒ LARGE ☐ SMALL ☐ MICRO

APPLICATION AS FILED – PART I

	(Column 1)	(Column 2)		
FOR	NUMBER FILED	NUMBER EXTRA	RATE ($)	FEE ($)
☐ BASIC FEE (37 CFR 1.16(a), (b), or (c))	N/A	N/A	N/A	
☐ SEARCH FEE (37 CFR 1.16(k), (i), or (m))	N/A	N/A	N/A	
☐ EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A	N/A	
TOTAL CLAIMS (37 CFR 1.16(i))	minus 20 =	*	X $ =	
INDEPENDENT CLAIMS (37 CFR 1.16(h))	minus 3 =	*	X $ =	
☐ APPLICATION SIZE FEE (37 CFR 1.16(s))	If the specification and drawings exceed 100 sheets of paper, the application size fee due is $310 ($155 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).			
☐ MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))				
* If the difference in column 1 is less than zero, enter "0" in column 2.			TOTAL	

APPLICATION AS AMENDED – PART II

AMENDMENT	02/22/2016	(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	RATE ($)	ADDITIONAL FEE ($)
	Total (37 CFR 1.16(i))	* 20	Minus	** 20	= 0	X $80 =	0
	Independent (37 CFR 1.16(h))	* 2	Minus	***3	= 0	X $420 =	0
	☐ Application Size Fee (37 CFR 1.16(s))						
	☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))						
						TOTAL ADD'L FEE	0

AMENDMENT		(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	RATE ($)	ADDITIONAL FEE ($)
	Total (37 CFR 1.16(i))	*	Minus	**	=	X $ =	
	Independent (37 CFR 1.16(h))	*	Minus	***	=	X $ =	
	☐ Application Size Fee (37 CFR 1.16(s))						
	☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))						
						TOTAL ADD'L FEE	

* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.
** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20".
*** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3".
The "Highest Number Previously Paid For" (Total or Independent) is the highest number found in the appropriate box in column 1.

LIE
/VANESSA HODGES/

This collection of information is required by 37 CFR 1.16. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.

Electronic Acknowledgement Receipt

EFS ID:	24980920
Application Number:	14111326
International Application Number:	
Confirmation Number:	3274
Title of Invention:	METHODS OF DETERMINING A TREATMENT PROTOCOL FOR AND/OR A PROGNOSIS OF A PATIENT'S RECOVERY FROM A BRAIN INJURY
First Named Inventor/Applicant Name:	David Wilson
Customer Number:	23628
Filer:	Andrea J. Gabert/Ellen Paul
Filer Authorized By:	Andrea J. Gabert
Attorney Docket Number:	Q0052.70018US01
Receipt Date:	22-FEB-2016
Filing Date:	24-JUN-2014
Time Stamp:	15:49:27
Application Type:	U.S. National Stage under 35 USC 371

Payment information:

Submitted with Payment	yes
Payment Type	Credit Card
Payment was successfully received in RAM	$1800
RAM confirmation Number	2343
Deposit Account	232825
Authorized User	GABERT, ANDREA J.
The Director of the USPTO is hereby authorized to charge indicated fees and credit any overpayment as follows:	

Charge any Additional Fees required under 37 CFR 1.21 (Miscellaneous fees and charges)

Charge any Additional Fees required under 37 CFR 1.492(a) (basic national fee only)

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1		Q005270018US01-AMN-AJG.pdf	46931 6b2042464ef139d750b661fdd88e57c241b2343b	yes	9

Multipart Description/PDF files in .zip description

Document Description	Start	End
Amendment Submitted/Entered with Filing of CPA/RCE	1	1
Claims	2	6
Applicant Arguments/Remarks Made in an Amendment	7	9

Warnings:					
Information:					
2	Extension of Time	Q005270018US01-EXT-AJG.pdf	23736 8e13fa47e60755854fdb9682f80a3b3455a3b820	no	1
Warnings:					
Information:					
3	Fee Worksheet (SB06)	Q005270018US01-FEE-AJG.pdf	31717 1841078e40295af9d471d8637e6a37f46e9f89a8	no	1
Warnings:					
Information:					
4	Request for Continued Examination (RCE)	Q005270018US01-RCE-AJG.pdf	26407 09a86a2976878cd4a5cfaa668e57acff87a2f229	no	1
Warnings:					
This is not a USPTO supplied RCE SB30 form.					
Information:					
5	Fee Worksheet (SB06)	fee-info.pdf	33193 91774824b0d6b065dd964b5541129d10b9cbc35d	no	2
Warnings:					
Information:					
		Total Files Size (in bytes):	161984		

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111
If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371
If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office
If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

Electronic Acknowledgement Receipt

EFS ID:	24975304
Application Number:	14111326
International Application Number:	
Confirmation Number:	3274
Title of Invention:	METHODS OF DETERMINING A TREATMENT PROTOCOL FOR AND/OR A PROGNOSIS OF A PATIENT'S RECOVERY FROM A BRAIN INJURY
First Named Inventor/Applicant Name:	David Wilson
Customer Number:	23628
Filer:	Andrea J. Gabert/Nathaniel Taylor
Filer Authorized By:	Andrea J. Gabert
Attorney Docket Number:	Q0052.70018US01
Receipt Date:	22-FEB-2016
Filing Date:	24-JUN-2014
Time Stamp:	17:01:41
Application Type:	U.S. National Stage under 35 USC 371

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Foreign Reference	EP1160256_QC.pdf	1107665 88e01e54dda1b97bc5e3faeaf0273f4257aaf67e	no	25

Warnings:	
Information:	

2	Foreign Reference	EP1298436_QC.pdf	2108030 d9baa00c9a3276fc023f53c3690999932bfd1eb5	no	30
Warnings:					
Information:					
3	Foreign Reference	EP1978035_QC.pdf	2309953 2e687cdde4e3dc8740be81f996676e6e22b4243b	no	40
Warnings:					
Information:					
4	Foreign Reference	WO9519178A1_QC.pdf	4063642 2b19470a148ef7940c083b11264f3c6f9d458504	no	98
Warnings:					
Information:					
5	Foreign Reference	WO9604309A1_QC.pdf	1793123 5386d0d695c041e95228fe6ed1559d5a7c1c5de7	no	44
Warnings:					
Information:					
6	Foreign Reference	WO0190757A1_QC.pdf	1345059 67d0416a094ffab2c427cdd9d75f2ad183222052	no	28
Warnings:					
Information:					
7	Foreign Reference	WO0246222A2_QC.pdf	3100097 6e3be79e9d13ee39256550ab33e1fa2f6f57207d	no	53
Warnings:					
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8	Foreign Reference	WO0247466A2_QC.pdf	5039499 e8b82ae42a519c5756d5aec9d5f12535e737d52d	no	94
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9	Foreign Reference	WO04043226A2_QC.pdf	1990658 5b3ce9d859d97cb480d64117b72ada1070acca91	no	43
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10	Foreign Reference	WO05011599A2_QC.pdf	2680500 49e8bb9a8f83299681cf4f91d8580ab2bd47dd0d	no	49
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11	Foreign Reference	WO05047484A2_QC.pdf	6489806 64589e6b10e1349846bce6e33ff315f966bee2ac	no	145
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12	Foreign Reference	WO05075507A1_QC.pdf	5036916 cd047a5ccb3ae8c1000ba99964a794a7b9f990a0	no	137
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13	Foreign Reference	WO05123775A1_QC.pdf	2048145 57d2cee13b2210f26fdedbc6af478c0250edd033	no	42
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14	Foreign Reference	WO06103116A1_QC.pdf	7043071 76fd5dbe3fe7e8e08432c7f0ec68266302e70cb0	no	133
Warnings:					
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15	Foreign Reference	WO07016357A1_QC.pdf	3012566 544a6099b7e0a47b202b34c0d77200ae636cf16b	no	52
Warnings:					
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16	Foreign Reference	WO07050359A2_QC.pdf	5202410 1a69cf7a5c55e71c4b39e765f93092f6d6c1fce9	no	141
Warnings:					
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18	Foreign Reference	WO07068429A1_QC.pdf	7425516 291af0956af4ea559a61bdb39360fb5269d599ff	no	169
Warnings:					
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19	Foreign Reference	WO07082750A1_QC.pdf	1548731 4b3d2938053ddb7bebc3534599ab6c7d722f0f3a	no	44
Warnings:					
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20	Foreign Reference	WO07090126A2_QC.pdf	3032859 98560e9371df8453899d018fec439b816435a843	no	54
Warnings:					
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21	Foreign Reference	WO07106762A2_QC.pdf	2905177 9d694eb6fba73f3a9c73e6bc4fdc55aae683e55e	no	55
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Warnings:					
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23	Foreign Reference	WO08027017A1_QC.pdf	5983364 3d6bd3648c229832ba4e2afdc8ad0b5e7b0eda5f	no	127
Warnings:					
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31	Miscellaneous Incoming Letter	Q005270018US01-TRN-AJG.pdf	24374 5b4050e935b75fb2c4df7cb2f87e0445fee7fd36	no	1
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32		Q005270018US01-IDS-AJG.pdf	48171 86da92477f9c564aaebe8c3d4316a11a59a5779a	yes	6

	Multipart Description/PDF files in .zip description		
	Document Description	**Start**	**End**
	Transmittal Letter	1	3
	Information Disclosure Statement (IDS) Form (SB08)	4	6
Warnings:			
Information:			
	Total Files Size (in bytes):	126364933	

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111
If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371
If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office
If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
14/111,326	06/24/2014	David Wilson	Q0052.70018US01	3274

23628 7590 03/18/2016
WOLF GREENFIELD & SACKS, P.C.
600 ATLANTIC AVENUE
BOSTON, MA 02210-2206

EXAMINER
WEIDNER, ADAM M

ART UNIT	PAPER NUMBER
1649	

NOTIFICATION DATE	DELIVERY MODE
03/18/2016	ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

Patents_eOfficeAction@WolfGreenfield.com
WGS_eOfficeAction@WolfGreenfield.com

Fujirebio Diagnostics, Inc. v. Quanterix Corp.
EX-1045
7940

Office Action Summary	Application No. 14/111,326	Applicant(s) WILSON ET AL.	
	Examiner ADAM M. WEIDNER	Art Unit 1649	AIA (First Inventor to File) Status No

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTHS FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☒ Responsive to communication(s) filed on *2/22/16*.

☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on _____.

2a)☐ This action is **FINAL**. 2b)☒ This action is non-final.

3)☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on _____; the restriction requirement and election have been incorporated into this action.

4)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims*

5)☒ Claim(s) *55,92-102 and 108-115* is/are pending in the application.

5a) Of the above claim(s) _____ is/are withdrawn from consideration.

6)☐ Claim(s) _____ is/are allowed.

7)☒ Claim(s) *55,92-102 and 108-115* is/are rejected.

8)☐ Claim(s) _____ is/are objected to.

9)☐ Claim(s) _____ are subject to restriction and/or election requirement.

* If any claims have been determined allowable, you may be eligible to benefit from the **Patent Prosecution Highway** program at a participating intellectual property office for the corresponding application. For more information, please see http://www.uspto.gov/patents/init_events/pph/index.jsp or send an inquiry to PPHfeedback@uspto.gov.

Application Papers

10)☐ The specification is objected to by the Examiner.

11)☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

Certified copies:

a)☐ All b)☐ Some** c)☐ None of the:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

** See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☐ Notice of References Cited (PTO-892)

2) ☒ Information Disclosure Statement(s) (PTO/SB/08a and/or PTO/SB/08b) Paper No(s)/Mail Date _____.

3) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date. _____ .

4) ☐ Other: _____.

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The present application is being examined under the pre-AIA first to invent provisions.

In the event the determination of the status of the application as subject to AIA 35 U.S.C. 102 and 103 (or as subject to pre-AIA 35 U.S.C. 102 and 103) is incorrect, any correction of the statutory basis for the rejection will not be considered a new ground of rejection if the prior art relied upon, and the rationale supporting the rejection, would be the same under either status.

DETAILED ACTION

Continued Examination Under 37 CFR 1.114

A request for continued examination under 37 CFR 1.114, including the fee set forth in 37 CFR 1.17(e), was filed in this application after final rejection. Since this application is eligible for continued examination under 37 CFR 1.114, and the fee set forth in 37 CFR 1.17(e) has been timely paid, the finality of the previous Office action has been withdrawn pursuant to 37 CFR 1.114. Applicant's submission filed on 2/22/16 has been entered.

Claims 55, 92-102, and 108-115 are pending and under examination.

Withdrawn Rejections

The objection to the specification is withdrawn.

The rejection under §112, second paragraph is withdrawn in light of the amendments

The rejection under §112, first paragraph for written description is withdrawn in light of the amendments. Specifically, the independent claims now recite specific steps that are drawn to a method demonstrated to possess such sensitivity and no longer encompass any and all current or future developed assays with the given sensitivity. Further, claims 104-107 have been cancelled, rendering this rejection moot.

Current Rejections

Claim Rejections - 35 USC § 101

35 U.S.C. 101 reads as follows:

> Whoever invents or discovers any new and useful process, machine, manufacture, or composition of matter, or any new and useful improvement thereof, may obtain a patent therefor, subject to the conditions and requirements of this title.

Claims 55, 92-102, and 108-110 are rejected under 35 U.S.C. 101 because the claimed invention is directed to a judicial exception (i.e., a law of nature, a natural phenomenon, or an abstract idea) without significantly more. The claims are directed to a series of steps (a method) for "determining a method" of treatment by measuring a natural concentration of naturally occurring tau protein. The claims do not include additional elements that are sufficient to amount to significantly more than observing a natural phenomenon.

Claim 55 is an independent claim. Step (c) of the claim is drawn to a natural correlation/abstract step: "determining" (mental) as well as the correlation between tau levels as calculated (area under the curve) and an indication of prognosis/treatment (natural correlation).

Step (c) is recited at a high degree of generality; the user must determine for themselves what the treatment should be or what the prognosis is "based at least in part" on the area under the curve and/or the second peak in tau concentration. No actual treatment is enacted and the claims do not apply the gathered data in any particular way, other than to inform a relevant audience that the data is important in some way. Thus, step (c) is no more than generic instructions to observe the correlation and "apply it".

Step (b) is also an abstract step (determine the area under the curve) and represents no more than a necessary data gathering step (analysis) required to use the correlation. Data gathering is explicitly discussed in the guidelines for an analysis under §101 as "insignificant extrasolution activity". Regardless of how simple or complex the natural correlation is, it remains a description of the correlation itself and does not provide significantly more. See also the decision in *Alice v CLS Bank* in which the Court held that merely requiring generic computer implementation fails to transform an abstract idea into a patent-eligible invention. Thus, even if the calculation is arguably sufficiently difficult that it could not be performed mentally, such calculations still require no more than a generic or general use computer to perform the calculations and is therefore still considered "abstract" in that it is not enough to confer patent eligibility.

Step (a) has been amended to require a series of steps (1-5) for performing the assay. However, these steps are also part of the necessary data gathering step and do not add significantly more than instructions to observe the phenomenon in a particular way and then "apply it". Moreover, steps 1-5 represent no more than what was routine and conventional in the art. See for example Rissen ("single molecule enzyme linked immunosorbent assay detects serum proteins as subfemtomolar concentrations"; cited on IDS 4/28/15). Here, Rissen describes antibodies attached (covalently associated) to beads (a plurality of monomeric beads), which then bind a target antigen (generating a bead comprising at least one immobilized protein), followed by binding the antigen with a biotin-labeled antibody, where only some of the beads bind the biotin-antibody (a statistically significant fraction of the beads are not associated) dividing these beads into wells (spatially segregating them into a plurality of locations), and imaging (addressing) them to determine the number of locations containing the biotin-antibody by exposure to a streptavidin-ß-gal (streptavidin-enzyme) conjugate in order to determine the concentration of the antigen (e.g., figure 1). While Rissen does not explicitly use the technique to measure tau, Rissen teaches the technology is broadly applicable to detecting single protein molecules in blood for sensitive diagnostic assays (abstract). The instant claims do not require any particular, novel antibody or other unique reagent. Thus, once informed of the relevant correlation, the "digital ELISA" technique would have been a well-known choice using conventional, commercially available monoclonal antibodies in order to measure tau concentration. Thus, step (a) does not provide significantly more than instructions to a relevant audience to use known techniques to observe a natural phenomenon and to "apply it" somehow. This is not patent eligible.

See also (all cited on IDS):

- Harma 2000 *Clinical Chemistry*
- Gorris 2010 "analytical chemistry on the femtoliter scale"
- Ekins 2010 "*Clinical Chemistry*
- Duffy 2011 "immunoassays with broad dynamic ranges based on combining digital and digitally enhanced analog detection of enzyme labels"
- Chen 2009 *AIChE*

- Chao 2009 *JACS*
- Burton 2010 *Lab Chip*
- Agrawal 2007 *J Agric Food Chem1*
- Quaternix 2010 *IQT Technology Focus Day*
- 2011 "Quanterix discovers link between heart attack-induced hypoxia suspected Alzheimer's disease pathway"
- US 20110245097
- US 20100075407
- US 20110212537
- US 20110212848

This list is not exhaustive, but illustrates that at the time of or before the instant invention, the instantly claimed assay was already publicly known and had been disclosed as measuring numerous different proteins from cancer antigens to Aß to tau. For example, the Quaternix document above ("single molecule arrays for digital detection in complex samples" *IQT technology focus day* presented 25 March 2010, more than one year before the instant priority date of 12 April 2011) describes using antibodies bound to beads to capture a "target protein", followed by a biotin-labeled detection antibody, performed in a plurality of wells (p.13), suggests its use relative to neurological diseases (p.19), and explicitly states that the method was used to measure tau in blood of AD patients and age-matched controls (p.22). The document also teaches measuring tau in relation to TBI (p.29). This document (also discussed below) teaches the assay of instant step (a) in the same way as the Rissen document above, but also directly teaches that tau can and should be measured using this assay.

The instant claims are similar to the claims at issue in Mayo, in which the Supreme Court indicated that a correlation that is a consequence of how a certain compound is metabolized was patent ineligible. Observation that the natural expression of tau is somehow correlated to brain injury with general instructions that the information should be somehow "applied" does not add significantly more to the exception itself and is thus patent ineligible. In Mayo, the data was also gathered in a way already

known and used in the prior art and it was only the observation of the correlation that was “inventive”, which the Court deemed was not enough to render a claim patent eligible. In Mayo, the claims also included specific metabolite levels where a value above or below that value indicated a need to alter treatment; this is similar to the instant claims which indicate levels above or below a certain value represents a good/poor prognosis. In both cases, this is not considered a particular application of the natural correlation and does confer eligibility.

With respect to claim 92, limiting the injury to one which results from a hypoxic event is simply further defining the population from which to gather data, i.e., further describing where the correlation is found. This does not add significantly more to the correlation itself.

With respect to claim 93, introduction of an assay detection limit does not relate to the correlation itself as the claim does not require any particular value of tau is measured, only that the assay should be capable of detecting tau at a certain threshold. This is "insignificant extrasolution activity", e.g., data gathering, and does not add significantly more to the correlation.

With respect to claims 94-97, these claims describe certain threshold values where a prognosis is “good” or “poor”. However, these claims are descriptions of the data itself. This is similar to the fact pattern in Mayo. In Mayo, a level of the metabolite "less than about 230 pmol per $8x10^8$ red blood cells [indicated] a need to increase the amount of said drug”. Such claim limitations were found to be a description of the correlation itself and not “significantly more”. It was also found that steps prior to this constituted routine data gathering, instructing the artisan to engage in conventional, well-known practices and then "warning" the user of the implications of the data; this was not eligible in Mayo and thus the instant claim(s) are not patent eligible.

With respect to claims 98-99, these claims describe the baseline concentration. As this only serves to further define data gathering/the correlation, these claims do not offer significantly more.

With respect to claims 100-102, these claims indicate the sample should be a body fluid, particularly blood or “not CSF” (e.g., blood). However, these are well-known fluids in which to measure tau protein and represent a routine, conventional practice. The assay steps now recited were also known to measure proteins in blood (see the Rissen and Quaternix documents discussed above). The claimed

steps represent “mere data gathering” in conjunction with a law of nature or abstract idea, and is considered “insignificant extrasolution activity” (see guidelines in Federal Register indicated above).

With respect to claims 108-110, these claims are also directed to the data gathering step. Limitations regarding how tau is to be measured represents insignificant data-gathering steps, insignificant pre/extra-solution activity, and an attempt to limit the use of the abstract idea/correlation to a particular technological environment (See Federal Register p.74632). Viewed both individually and as an ordered combination, the claimed steps are conventional and specified at a high level of generality. There is no suggestion that these data gathering steps are themselves novel and the instant specification points to numerous examples of existing technology for this purpose (e.g., p.22-23, 25, 34; citations above).

In summary, the above analysis conducted in line with the guidance issued on 12/15/2014 sets forth the rationale for deeming the above claims patent ineligible. It is appreciated that all inventions at some level embody, use, reflect, rest upon, or apply a law of nature, natural phenomenon, or abstract idea. However, the instant claims do not improve another technology, apply the judicial exception with any specific machine, transform the gathered data into a different state or thing, add a limitation that is anything more than what is well-understood, conventional, or routine in the field, or any other meaningful limitation beyond generally linking the use of the exception to a particular technological environment. In other words, the claims as a whole instruct a user to observe tau using conventional samples and a known methodology and then informs this relevant audience about a correlation that exists between the gathered data and a prognosis. The added steps of determining the prognosis “based at least in part” on the gathered data is no more than instructions to “apply it”.

Response to Arguments

Applicant's arguments filed 2/22/16 have been fully considered but they are not persuasive. Applicant argues the assay components are not conventional and were “specifically developed to facilitate the detection of tau antibodies in the context of the presently claim inventions” (Remarks 2/22/16, p.8). Applicant argues the five newly added method steps are "new and innovative". This has been fully considered but is not persuasive.

Applicant is reminded that arguments of counsel cannot take the place of factually supported objective evidence (MPEP §2145). Applicant does no more than make the above assertions without providing any factually based evidence. Rather, the evidence of record contradicts Applicant's arguments. As listed on the IDS or otherwise provided, several documents describe similar or identical technology to that which is instantly claimed. Given the breadth of proteins the technology has been used to measure, it does not appear that it was “specifically developed” for use in the instant claims but rather represents a) a predictable variation of the technology, utilizing tau antibodies to measure tau where it was disclosed that the assay could be used for a wide variety of proteins, or b) the same as that shown in the Quaternix *IQT* document. Applicant does not provide any evidence that these steps represent more than "insignificant extrasolution activity, e.g., data gathering" and as such the claims are still considered patent ineligible.

The natural correlation, even if complex, remains a judicial exception. Thus, claims describing methods of analysis (area under the curve, peaks to measure, baselines to establish, thresholds which indicate information) are all part of the correlation itself. Thus, the limitations outside the judicial exception must provide "significantly more". However, the instant limitations beyond the correlation itself are largely drawn to the data gathering step. This is discussed in the guidelines cited by Applicant, and generally does not add significantly more to the natural correlation itself. Rather, the guidelines look to the application of that law/correlation ("new and useful end", a robotic arm that is controlled by a mathematical relationship, *Bilski*, a machine that uses the Arrhenius equation to improve an existing process such that the gathered information instructs a machine to open at the appropriate time and results in the transformation of one product into another). In the instant case, the application of the correlation is no more than instructions to make a decision based at least in part on the gathered information. Such a broad, generic limitation is akin to the words "apply it" which does not offer significantly more in the same way that gathering metabolite information and informing the user of the possible need to alter treatment was not eligible.

Applicant further argues that the components are not naturally occurring. It is agreed that these components are not naturally occurring; however, the argument is moot as the rejection is not made on this basis. Applicant further argues that "it follows that the act of an assay using these components cannot

occur in nature". The guidelines for eligibility do not require the assay or act to occur in nature and therefore this argument is off-point. See for example the Court decisions such as in *Mayo* which also used non-natural products for an assay that would not occur in nature but was still deemed ineligible.

Therefore, claims 55, 92-102, and 108-110 are not patent eligible.

Claim Rejections - 35 USC § 103

The following is a quotation of pre-AIA 35 U.S.C. 103(a) which forms the basis for all obviousness rejections set forth in this Office action:

> (a) A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102 of this title, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains. Patentability shall not be negatived by the manner in which the invention was made.

This application currently names joint inventors. In considering patentability of the claims under pre-AIA 35 U.S.C. 103(a), the examiner presumes that the subject matter of the various claims was commonly owned at the time any inventions covered therein were made absent any evidence to the contrary. Applicant is advised of the obligation under 37 CFR 1.56 to point out the inventor and invention dates of each claim that was not commonly owned at the time a later invention was made in order for the examiner to consider the applicability of pre-AIA 35 U.S.C. 103(c) and potential pre-AIA 35 U.S.C. 102(e), (f) or (g) prior art under pre-AIA 35 U.S.C. 103(a).

Claims 111-115 are rejected under pre-AIA 35 U.S.C. 103(a) as being unpatentable over Quaternix "single molecule arrarys for digital detection in complex samples" *IQT technology Focus Day* (hereinafter Quaternix 2010) in view of 20100075439 (Duffy, hereinafter referred to as '439; cited on IDS).

Quaternix 2010 describes the bead-based SiMoA (single molecule array) immunoassay (p.13). Quaternix 2010 teaches a monomeric bead attached to an antibody (see bead at left of page 13) exposed to blood plasma ("100 µl of serum or plasma as test sample") to form a bead comprising at least one immobilized protein molecule (second bead image on page 13). Quaternix 2010 then discloses exposing

(mixing) the bead to an antibody/biotin conjugate followed by exposure to an antibody-enzyme complex. This is followed by using the enzyme to determine the concentration of the protein by spatially segregating the reporter. This can also be seen on page 5 where samples are partitioned into a plurality of well and each reporter is individually counted (where a statistically significant number of well are not associated), page 12 where the reporters are spatially separated and on page 14 where the number of "active wells" is measured. Quaternix 2010 teaches using this technology for diagnostic use in neurological diseases such as Alzheimer's (p.19) and explicitly uses this assay to measure tau in blood of AD patients and age matched controls (p.22). Quaternix 2010 states "You want a nice serum marker for TBI, you do it through tau" (p.29). Quaternix 2010 does not explicitly teach using monoclonal antibodies, covalent attachment of the antibody, or using a plurality of beads.

'439 teaches "exposing a substrate comprising a plurality of capture components to a plurality of analyte molecules" (paragraph 60). '439 teaches the substrate is a plurality of beads (paragraph 61) and the capture component is an antibody, including monoclonal antibodies (paragraph 90). '439 teaches attaching the capture component (antibody) though a variety of means including functional groups, crosslinkers, and other "coupling chemistries/techniques" known in the art (paragraph 94). These meet the criteria of covalent association. This is further supported by paragraph 97, which states that in certain embodiments covalent modification is unnecessary, indicating that such covalent attachment in the methods discussed prior to this paragraph involve such covalent modification. In paragraph 184 and figure 16, '439 discloses a plurality of particles [330] are exposed to particles and subsequently immobilize them. These particles are shown as discrete units and therefore clearly teach the plurality of beads are monomeric. See also [900] in figure 10A. In a preferred embodiment, the analyte is detect in blood plasma or serum (paragraph 142; figures 20-23). Such exposure leads to immobilization of the protein on the bead; see also figure 3 and paragraph 12. Further, figure 29 represents beads bound to antibodies which immobilize the protein of interest (paragraph 45). '439 clearly teaches subsequently mixing the prepared beads with antibody-biotin conjugates (paragraph 224 teaches covalent association of monoclonal anti-tnf-α antibody onto a plurality of beads, paragraph 226 teaches exposing the beads to the protein to form immunocomplexes, followed by incubating with detection antibody which, as disclosed

in paragraph 225, is an antibody-biotin conjugate). Note that while this embodiment uses a polyclonal detection antibody, the document otherwise clearly suggests that monoclonal antibodies can be used as '439 describes antibodies and indicates they can be monoclonal as well as teaching capture antibodies (explicitly monoclonal) can be used for detection (e.g., paragraph 105). Moreover, using a monoclonal antibody as the detection antibody represents a predictable variation of the disclosure of '439 as a monoclonal antibody would serve the same purpose of binding the target protein as a polyclonal antibody. '439 teaches spatially segregating the particles in a plurality of locations, wherein a statistically significant fraction of the beads do not bind the secondary detection antibody (e.g., paragraphs 183, 184, 196; figures 2, 5, 16). Paragraph 226 describes "addressing" (detecting) the antibody-biotin conjugate by exposure to streptavidin-ß-galactosidase (streptavidin-enzyme conjugate). The '439 document describes using streptavidin-ß-gal as a reporter or "dissociated species" (paragraph 231) and that these dissociated species are spatially segregated (e.g., paragraph 83) where such segregation and detection aids in "the determination of a measure of the concentration of analyte molecules in a fluid sample" (paragraph 83). While '439 discusses broad applications for the assay, '439 does not explicitly indicate measuring tau or using anti-tau antibodies.

Regarding claim 111, nevertheless one of ordinary skill in the art would have been motivated to modify the teachings of Quaternix 2010 to arrive at the instant method. The difference between Quanternix 2010 and the instant claims is in choosing monoclonal detection antibodies, covalently attaching the capture antibody, and using a plurality of beads. Quanternix 2010 teaches a well-based assay (p.9) as well as a bead-based assay (p.13). The document does not make clear which form was used when reporting results. For example, the graph on page 14 clearly requires multiple wells, but it is unclear if this represents a bead in each well or if the capture antibody is bound to the well itself. In one case, a plurality of beads was used. Alternately, it would have been obvious to use a plurality of beads as using a single bead would not provide the gradation to determine the analyte concentration, which is disclosed as the purpose of the assay. The diagram on page 13 shows the antibody attached to the bead. In one case, this is a covalent attachment. Alternately, covalently attaching the antibody represents a common, well-known technique in the art for attaching antibodies and would have been obvious to one of

ordinary skill in the art. The document does not disclose if the antibodies are monoclonal. In one case, the detection antibody was monoclonal. Alternately, selecting a monoclonal antibody would have been obvious at the time of the invention as such antibodies were readily available and known to be highly selective for their target. These conclusions are all also supported by the '439 document. '439 is concerned with the same problem (detecting low concentrations of analyte in blood plasma) and offers the same solution: capture antibodies covalently bound to beads, which immobilize the target analyte, followed by binding of a detection antibody (which includes monoclonal antibodies) conjugated to biotin, spatially segregating the complex, and using a streptavidin-enzyme reporter to determine the concentration of the analyte by addressing a plurality of locations where a statistically significant number of such locations are not associated with the antibody-biotin complex. Thus, one of ordinary skill in the art would have been motivated to make the known, predictable modifications to the assay of Quaternix 2010 to predictably measure tau concentration with a reasonable expectation of success.

This is true of the reverse analysis. The only difference between '439 and the instant claims is in the choice of analyte to detect (tau). However, '439 discloses the assay as capable of detecting very low concentrations of analyte and broadly applicable to a variety of target analytes. Quaternix 2010 provides the same assay (bead-based "digital ELISA") and further demonstrates its applicability across multiple proteins, including explicitly measuring tau.

As noted by the United States Supreme Court, if a person of ordinary skill can implement a predictable variation, § 103 likely bars its patentability. For the same reason, if a technique has been used to improve one method (i.e., the '439 detection assay or the assay of Quaternix 2010), and a person of ordinary skill would recognize that it would improve similar methods (i.e., the '439 assay could be predictably modified to measure tau; the Quaternix 2010 assay could be predictably modified to use monoclonal antibodies and covalent attachments) in the same way, using the technique is obvious unless its actual application is beyond his or her skill. KSR, 127 S. Ct. at 1740. "When there is a design need or market pressure to solve a problem and there are a finite number of identified, predictable solutions, a person of ordinary skill has good reason to pursue the known options within his or her technical grasp. If this leads to the anticipated success, it is likely the product is not of innovation but of ordinary skill and

common sense. In that instance the fact that a combination was obvious to try might show it was obvious under 35 U .S.C. 103." KSR Int'l Co. v. Teleflex Inc., 127 S.Ct. 1727, 1742, 82USPQ2d 1385, 1396 (2007).

Regarding claim 112, Quaternix 2010 (p.5, 12) and '439 (paragraph 83) both describe using a plurality of reaction vessels.

Regarding claim 113, Quaternix 2010 teaches the volume is 50 picoliters (50,000 femtoliter; p.5) and '439 teaches the volume is between 10 attoliters and 100 picoliters (paragraph 88). Thus, this range would have been obvious. Moreover, in the case where the claimed ranges "overlap or lie inside ranges disclosed by the prior art" a prima facie case of obviousness exists. In re Wertheim, 541 F.2d 257, 191 USPQ 90 (CCPA 1976); In re Woodruff, 919 F.2d 1575, 16 USPQ2d 1934 (Fed. Cir. 1990). See MPEP §2144.05(I).

Regarding claim 114, '439 discloses the use of calibration curves (paragraph 194) and explicitly states "in certain such embodiments, the measure of the concentration of the analyte molecules or particles in the fluid sample is determined at least in part by comparison of a measured parameter to a calibration standard" (paragraph 196), rendering the use of such a standard obvious.

Regarding claim 115, Quaternix 2010 teaches the sensitivity of the assay is well below 0.2 pg/ml (p. 11 states the limit of detection is 3 aM) and '439 teaches the limit of detection is in the femtomolar range (table 1).

Therefore, claims 111-115 would have been obvious over Quaternix 2010 in view of '439.

Conclusion

No claim is allowed.

Note that the Quaternix 2010 document discussed above provides motivation to use the "digital ELISA" technique to measure tau in a method of determining treatment or prognosis of a patient's recovery from brain injury. However, Quaternix 2010 clearly indicates that tau might be useful in this regard (p.29, "you may have a use for tau", "you may be able to get some assessment of the degree of

injury following a TBI"). Thus, while Quaternix 2010 demonstrates the usefulness of measuring tau in AD (which is not reasonably considered a "brain injury" in the art; see also specification at page 1) and clearly suggests doing the same for TBI, it does not provide a reasonable expectation that tau levels would actually be useful in this way, at best representing a "hope" that it would. This is insufficient to render a claim such as instant claim 55 obvious.

US 20100075407, US 20100075862, US 20110212462, US 20110212537, US 20110212848, and US 20110245097 (see IDS) all appear to be relevant to the assay steps, i.e., to the data gathering steps of the method. However, these documents fail to discuss tau in the same way as the '439 document and fail to correlate tau levels with brain injury in the same way as the '439/Quaternix 2010 combination discussed above.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to ADAM M. WEIDNER whose telephone number is (571)272-3045. The examiner can normally be reached on Monday - Friday, 800 - 1630 EST.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Jeffrey Stucker can be reached on 571-272-0911. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

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/ADAM M WEIDNER/

Examiner, Art Unit 1649

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INFORMATION DISCLOSURE STATEMENT BY APPLICANT				FILING DATE: October 11, 2013	CONFIRMATION NO.: 3274
				FIRST NAMED INVENTOR: David Wilson	
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EXAMINER:	DATE CONSIDERED:
/Adam Weidner/	03/10/2016

[#] EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609; Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to Applicant.

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /AMW/

FORM PTO-1449/A and B (modified PTO/SB/08)	APPLICATION NO.: 14/111,326	ATTY. DOCKET NO.: Q0052.70018US01
INFORMATION DISCLOSURE STATEMENT BY APPLICANT	FILING DATE: October 11, 2013	CONFIRMATION NO.: 3274
	FIRST NAMED INVENTOR: David Wilson	
Sheet 17 of 19	GROUP ART UNIT: 1649	EXAMINER: A. M. Weidner

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EXAMINER:	DATE CONSIDERED:
/Adam Weidner/	03/10/2016

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FORM PTO-1449/A and B (modified PTO/SB/08)	APPLICATION NO.: 14/111,326	ATTY. DOCKET NO.: Q0052.70018US01
INFORMATION DISCLOSURE STATEMENT BY APPLICANT	FILING DATE: October 11, 2013	CONFIRMATION NO.: 3274
	FIRST NAMED INVENTOR: David Wilson	
Sheet 18 of 19	GROUP ART UNIT: 1649	EXAMINER: A. M. Weidner

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EXAMINER: /Adam Weidner/	DATE CONSIDERED: 03/10/2016

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FORM PTO-1449/A and B (modified PTO/SB/08)	APPLICATION NO.: 14/111,326	ATTY. DOCKET NO.: Q0052.70018US01
INFORMATION DISCLOSURE STATEMENT BY APPLICANT	FILING DATE: October 11, 2013	CONFIRMATION NO.: 3274
	FIRST NAMED INVENTOR: David Wilson	
Sheet 19 of 19	GROUP ART UNIT: 1649	EXAMINER: A. M. Weidner

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[NOTE – No copies of U.S. patents, published U.S. patent applications, or pending, unpublished patent applications stored in the USPTO's Image File Wrapper (IFW) system, are included. See 37 CFR § 1.98 and 1287OG163. Copies of all other patent(s), publication(s), unpublished, pending U.S. patent applications, or other information listed are provided as required by 37 CFR § 1.98 unless 1) such copies were provided in an IDS in an earlier application that complies with 37 CFR § 1.98, and 2) the earlier application is relied upon for an earlier filing date under 35 U.S.C. § 120.]

EXAMINER:	DATE CONSIDERED:
/Adam Weidner/	03/10/2016

EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609; Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to Applicant.

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /AMW/

EAST Search History

EAST Search History (Prior Art)

Ref #	Hits	Search Query	DBs	Default Operator	Plurals	Time Stamp
L12	1	11 and tau	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO	ADJ	ON	2016/03/14 14:13
L11	57	10 and (traumatic or hypoxia or hypoxic or (brain injury))	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO	ADJ	ON	2016/03/14 14:13
L10	150	9 and (area under)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO	ADJ	ON	2016/03/14 14:13
L9	9502	L7 or L8 or L4	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO	ADJ	ON	2016/03/14 14:12
L8	23	quanterix.as.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO	ADJ	ON	2016/03/14 14:12
L7	8416	duffy.in. or rissen.in.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO	ADJ	ON	2016/03/14 14:12
L6	0	L5 and (area under)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO	ADJ	ON	2016/03/14 14:11
L5	13	L4 and femtoliter	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO	ADJ	ON	2016/03/14 14:11
L4	1086	tufts.as.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO	ADJ	ON	2016/03/14 14:11
S64	13	S61 and femtoliter	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO	ADJ	ON	2016/03/10 14:27
S63	0	S61 and (simoa)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO	ADJ	ON	2016/03/10 14:27
S62	0	S61 and (digital elisa)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO	ADJ	ON	2016/03/10 14:26
S61	1086	tufts.as.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO	ADJ	ON	2016/03/10 14:26
S60	23	quanterix.as.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO	ADJ	ON	2016/03/10 13:48
S59	119	S58 and elisa	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO	ADJ	ON	2016/03/10 13:48
S58	8416	duffy.in. or rissen.in.	US-PGPUB; USPAT; USOCR; FPRS; EPO;	ADJ	ON	2016/03/10 13:48

Fujirebio Diagnostics, Inc. v. Quanterix Corp.
EX-1045
7978

			JPO			

EAST Search History (Interference)

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Fujirebio Diagnostics, Inc. v. Quanterix Corp.
EX-1045
7979

Search Notes	Application/Control No.	Applicant(s)/Patent Under Reexamination
	14111326	WILSON ET AL.
	Examiner	Art Unit
	ADAM M WEIDNER	1649

CPC- SEARCHED		
Symbol	Date	Examiner

CPC COMBINATION SETS - SEARCHED		
Symbol	Date	Examiner

US CLASSIFICATION SEARCHED			
Class	Subclass	Date	Examiner

SEARCH NOTES		
Search Notes	Date	Examiner
inventor east	1/22/15	amw
text east/google	1/23/15	amw
PLUS search	1/23/15	amw
updated inventor/assignee east	9/14/15	amw
update text search (AOC, tau, brain injury, diagnosis/prognosis) east, internet	9/14/15	amw
inventor/assignee east update	3/14/16	amw
text and claims east	3/14/16	amw

INTERFERENCE SEARCH			
US Class/ CPC Symbol	US Subclass / CPC Group	Date	Examiner

USED IN LIEU OF PTO/SB/31 (02-14)

NOTICE OF APPEAL FROM THE EXAMINER TO THE PATENT TRIAL AND APPEAL BOARD	Docket Number (Optional) Q0052.70018US01
In re Application of David Wilson et al.	
Application Number 14/111,326-Conf. #3274	Filed June 24, 2014
For METHODS OF DETERMINING A TREATMENT PROTOCOL FOR AND/OR A PROGNOSIS OF A PATIENT'S RECOVERY FROM A BRAIN INJURY	
Art Unit 1649	Examiner A. M. Weidner

Applicant hereby **appeals** to the Patent Trial and Appeal Board from the last decision of the examiner.

The fee for this Notice of Appeal is (37 CFR 41.20(b)(1)) $ 800.00

☐ Applicant claims small entity status. See 37 CFR 1.27. Therefore, the fee shown above is reduced by half, and the resulting fee is: $ _____

☐ A check in the amount of the fee is enclosed.

☒ Payment by credit card. ~~Form PTO-2038 is attached~~.

☒ The Director is hereby authorized to charge any fees which may be required, or credit any overpayment to Deposit Account No. 23/2825 .

☒ A petition for an extension of time under 37 CFR 1.136(a) (PTO/SB/22) is enclosed.

WARNING: Information on this form may become public. Credit card information should not be included on this form. Provide credit card information and authorization on PTO-2038.

I am the

☐ applicant/inventor.

☐ assignee of record of the entire interest. See 37 CFR 3.71. Statement under 37 CFR 3.73(b) is enclosed. (Form PTO/SB/96)

☒ attorney or agent of record.
Registration number 68,904 .

☐ attorney or agent acting under 37 CFR 1.34.
Registration number if acting under 37 CFR 1.34. _____

/Andrea J. Gabert/
Signature

Andrea J. Gabert, Ph.D.
Typed or printed name

617.646.8000
Telephone number

September 12, 2016
Date

NOTE: Signatures of all the inventors or assignees of record of the entire interest or their representative(s) are required. Submit multiple forms if more than one signature is required, see below*.

☐ *Total of 1 forms are submitted.

Certificate of Electronic Filing Under 37 CFR 1.8

I hereby certify that this paper (along with any paper referred to as being attached or enclosed) is being transmitted via the Office electronic filing system in accordance with 37 CFR § 1.6(a)(4).

Dated: September 12, 2016 Electronic Signature for Ellen J. Paul: /Ellen J. Paul/

PTO/SB/22 (03-13)
Approved for use through 3/31/2013. OMB 0651-0031
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

PETITION FOR EXTENSION OF TIME UNDER 37 CFR 1.136(a)	Docket Number (Optional) Q0052.70018US01
Application Number 14/111,326-Conf. #3274	Filed June 24, 2014
For METHODS OF DETERMINING A TREATMENT PROTOCOL FOR AND/OR A PROGNOSIS OF A PATIENT'S RECOVERY FROM A BRAIN INJURY	
Art Unit 1649	Examiner A. M. Weidner

This is a request under the provisions of 37 CFR 1.136(a) to extend the period for filing a reply in the above-identified application.

The requested extension and fee are as follows (check time period desired and enter the appropriate fee below):

	Fee	Small Entity Fee	Micro Entity Fee	
☐ One month (37 CFR 1.17(a)(1))	$200	$100	$50	$
☐ Two months (37 CFR 1.17(a)(2))	$600	$300	$150	$
☒ Three months (37 CFR 1.17(a)(3))	$1,400	$700	$350	$ 1,400.00
☐ Four months (37 CFR 1.17(a)(4))	$2,200	$1,100	$550	$
☐ Five months (37 CFR 1.17(a)(5))	$3,000	$1,500	$750	$

☐ Applicant asserts small entity status. See 37 CFR 1.27.

☐ Applicant certifies micro entity status. See 37 CFR 1.29.
Form PTO/SB/15A or B or equivalent must either be enclosed or have been submitted previously.

☐ A check in the amount of the fee is enclosed.

☒ Payment by credit card. ~~Form PTO-2038 is attached~~.

☐ The Director has already been authorized to charge fees in this application to a Deposit Account.

☒ The Director is hereby authorized to charge any fees which may be required, or credit any overpayment, to Deposit Account Number 23/2825 .

☒ Payment made via EFS-Web.

WARNING: Information on this form may become public. Credit card information should not be included on this form. Provide credit card information and authorization on PTO-2038.

I am the

☐ applicant/inventor.

☐ assignee of record of the entire interest. See 37 CFR 3.71. 37 CFR 3.73(b) statement is enclosed (Form PTO/SB/96).

☒ attorney or agent of record. Registration number 68,904 .

☐ attorney or agent acting under 37 CFR 1.34. Registration number _____ .

/Andrea J. Gabert/	September 12, 2016
Signature	Date
Andrea J. Gabert, Ph.D.	617.646.8000
Typed or printed name	Telephone Number

NOTE: This form must be signed in accordance with 37 CFR 1.33. See 37 CFR 1.4 for signature requirements and certifications. Submit multiple forms if more than one signature is required, see below*.

☐ * Total of 1 forms are submitted.

Certificate of Electronic Filing Under 37 CFR 1.8

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Doc Code: TRAN.LET
Document Description: Transmittal Letter

USED IN LIEU OF PTO/SB/21 (07-09)

TRANSMITTAL FORM

(to be used for all correspondence after initial filing)

Total Number of Pages in This Submission	

Application Number	14/111,326-Conf. #3274
Filing Date	June 24, 2014
First Named Inventor	David Wilson
Art Unit	1649
Examiner Name	A. M. Weidner
Attorney Docket Number	Q0052.70018US01

ENCLOSURES (*Check all that apply*)

- [X] Fee Transmittal Form
- [ ] Fee Attached
- [ ] Amendment/Reply
- [ ] After Final
- [ ] Affidavits/declaration(s)
- [X] Extension of Time Request
- [ ] Express Abandonment Request
- [ ] Information Disclosure Statement
- [ ] Certified Copy of Priority Document(s)
- [ ] Reply to Missing Parts/ Incomplete Application
- [ ] Reply to Missing Parts under 37 CFR 1.52 or 1.53
- [ ] Drawing(s)
- [ ] Licensing-related Papers
- [ ] Petition
- [ ] Petition to Convert to a Provisional Application
- [ ] Power of Attorney, Revocation Change of Correspondence Address
- [ ] Terminal Disclaimer
- [ ] Request for Refund
- [ ] CD, Number of CD(s) _____
- [ ] Landscape Table on CD
- [ ] After Allowance Communication to TC
- [ ] Appeal Communication to Board of Appeals and Interferences
- [X] Appeal Communication to TC **(Appeal Notice, Brief, Reply Brief)**
- [ ] Proprietary Information
- [ ] Status Letter
- [ ] Other Enclosure(s) (please Identify below):

Remarks

SIGNATURE OF APPLICANT, ATTORNEY, OR AGENT

Firm Name	WOLF, GREENFIELD & SACKS, P.C.		
Signature	/Andrea J. Gabert/		
Printed name	Andrea J. Gabert, Ph.D.		
Date	September 12, 2016	Reg. No.	68,904

Certificate of Electronic Filing Under 37 CFR 1.8

I hereby certify that this paper (along with any paper referred to as being attached or enclosed) is being transmitted via the Office electronic filing system in accordance with 37 CFR § 1.6(a)(4).

Dated: September 12, 2016 Electronic Signature for Ellen J. Paul: /Ellen J. Paul/

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PTO/SB/17 (03-13)
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FEE TRANSMITTAL

	Complete if known
	Application Number: 14/111,326-Conf. #3274
	Filing Date: June 24, 2014
☐ Applicant asserts small entity status. See 37 CFR 1.27.	First Named Inventor: David Wilson
☐ Applicant certifies micro entity status. See 37 CFR 1.29. Form PTO/SB/15A or B or equivalent must either be enclosed or have been submitted previously.	Examiner Name: A. M. Weidner
	Art Unit: 1649
TOTAL AMOUNT OF PAYMENT ($) 2,200.00	Practitioner Docket No.: Q0052.70018US01

METHOD OF PAYMENT (check all that apply)

☐ Check ☒ Credit Card ☐ Money Order ☐ None ☐ Other (please identify): _____

☐ Deposit Account Deposit Account Number: 23/2825 Deposit Account Name: Wolf, Greenfield & Sacks, P.C.

For the above-identified deposit account, the Director is hereby authorized to (check all that apply):

☐ Charge fee(s) indicated below ☐ Charge fee(s) indicated below, **except for the filing fee**

☒ Charge any additional fee(s) or underpayment of fee(s) under 37 CFR 1.16 and 1.17 ☒ Credit any overpayment of fee(s)

WARNING: Information on this form may become public. Credit card information should not be included on this form. Provide credit card information and authorization on PTO-2038.

FEE CALCULATION

1. BASIC FILING, SEARCH, AND EXAMINATION FEES (U = undiscounted fee; S = small entity fee; M = micro entity fee)

	FILING FEES			SEARCH FEES			EXAMINATION FEES			
Application Type	**U ($)**	**S ($)**	**M ($)**	**U ($)**	**S ($)**	**M ($)**	**U ($)**	**S ($)**	**M ($)**	**Fees Paid ($)**
Utility	280	140*	70	600	300	150	720	360	180	
Design	180	90	45	120	60	30	460	230	115	
Plant	180	90	45	380	190	95	580	290	145	
Reissue	280	140	70	600	300	150	2,160	1,080	540	
Provisional	260	130	65	0	0	0	0	0	0	

* The $140 small entity status filing fee for a utility application is further reduced to $70 for a small entity status applicant who files the application via EFS-Web.

2. EXCESS CLAIM FEES

Fee Description	**Undiscounted Fee ($)**	**Small Entity Fee ($)**	**Micro Entity Fee ($)**
Each claim over 20 (including Reissues)	80	40	20
Each independent claim over 3 (including Reissues)	420	210	105
Multiple dependent claims	780	390	195

Total Claims	**Extra Claims**	**Fee ($)**	**Fee Paid ($)**	**Multiple Dependent Claims**	
				Fee ($)	**Fee Paid ($)**
- or HP =	x	=			

HP = highest number of total claims paid for, if greater than 20.

Indep. Claims	**Extra Claims**	**Fee ($)**	**Fee Paid ($)**
- or HP =	x	=	

HP = highest number of independent claims paid for, if greater than 3.

3. APPLICATION SIZE FEE

If the specification and drawings exceed 100 sheets of paper (excluding electronically filed sequence or computer listings under 37 CFR 1.52(e)), the application size fee due is $400 ($200 for small entity) ($100 for micro entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).

Total Sheets	**Extra Sheets**	**Number of each additional 50 or fraction thereof**	**Fee ($)**	**Fee Paid ($)**
- 100 =	/50 =	(round **up** to a whole number) x		=

4. OTHER FEE(S)

	Fees Paid ($)
Non-English specification, $130 fee (no small or micro entity discount)	
Non-electronic filing fee under 37 CFR 1.16(t) for a utility application, $400 fee ($200 small or micro entity)	
Other (e.g., late filing surcharge): 1253 Extension for response within third month	1,400.00
1401 Notice of appeal	800.00

SUBMITTED BY

Signature	/Andrea J. Gabert/	Registration No. (Attorney/Agent)	68,904	Telephone	617.646.8000
Name (Print/Type)	Andrea J. Gabert, Ph.D.			Date	September 12, 2016

Certificate of Electronic Filing Under 37 CFR 1.8

I hereby certify that this paper (along with any paper referred to as being attached or enclosed) is being transmitted via the Office electronic filing system in accordance with 37 CFR § 1.6(a)(4).

Dated: September 12, 2016 Electronic Signature for Ellen J. Paul: /Ellen J. Paul/

4972475.1

Electronic Patent Application Fee Transmittal

Application Number:	14111326
Filing Date:	24-Jun-2014
Title of Invention:	METHODS OF DETERMINING A TREATMENT PROTOCOL FOR AND/OR A PROGNOSIS OF A PATIENT'S RECOVERY FROM A BRAIN INJURY
First Named Inventor/Applicant Name:	David Wilson
Filer:	Andrea J. Gabert/Ellen Paul
Attorney Docket Number:	Q0052.70018US01

Filed as Large Entity

Filing Fees for U.S. National Stage under 35 USC 371

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
Pages:				
Claims:				
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				
Notice of Appeal	1401	1	800	800
Post-Allowance-and-Post-Issuance:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Extension-of-Time:				
Extension - 3 months with $0 paid	1253	1	1400	1400
Miscellaneous:				
		Total in USD ($)		**2200**

Electronic Acknowledgement Receipt

EFS ID:	26897048
Application Number:	14111326
International Application Number:	
Confirmation Number:	3274
Title of Invention:	METHODS OF DETERMINING A TREATMENT PROTOCOL FOR AND/OR A PROGNOSIS OF A PATIENT'S RECOVERY FROM A BRAIN INJURY
First Named Inventor/Applicant Name:	David Wilson
Customer Number:	23628
Filer:	Andrea J. Gabert/Ellen Paul
Filer Authorized By:	Andrea J. Gabert
Attorney Docket Number:	Q0052.70018US01
Receipt Date:	12-SEP-2016
Filing Date:	24-JUN-2014
Time Stamp:	16:26:05
Application Type:	U.S. National Stage under 35 USC 371

Payment information:

Submitted with Payment	yes
Payment Type	CARD
Payment was successfully received in RAM	$2200
RAM confirmation Number	091316INTEFSW16271201
Deposit Account	8543
Authorized User	Wolf Greenfield
The Director of the USPTO is hereby authorized to charge indicated fees and credit any overpayment as follows: 37 CFR 1.17 (Patent application and reexamination processing fees)	

37 CFR 1.21 (Miscellaneous fees and charges)

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Extension of Time	Q005270018US01-EXT-AJG.pdf	23596 d30fe2c7d82eca0837f366c1106ea12466849fb1	no	1
Warnings:					
Information:					
2	Fee Worksheet (SB06)	Q005270018US01-FEE-AJG.pdf	31651 d2a90223fd86b97a8f7794401fc739df3c6275d8	no	1
Warnings:					
Information:					
3	Notice of Appeal Filed	Q005270018US01-NAPL-AJG.pdf	20471 1317deac4b9074dcb816e5fcfff8a019ab14d7d8	no	1
Warnings:					
Information:					
4	Miscellaneous Incoming Letter	Q005270018US01-TRN-AJG.pdf	24772 23246dbd14fcf64c6622d05ca1ec70b90d9cc133	no	1
Warnings:					
Information:					
5	Fee Worksheet (SB06)	fee-info.pdf	33066 827679bd70335a0a0a6e6cdc074b4b789a41fab6	no	2
Warnings:					
Information:					
		Total Files Size (in bytes):	133556		

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111
If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371
If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office
If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

Electronic Acknowledgement Receipt

EFS ID:	26897048
Application Number:	14111326
International Application Number:	
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Title of Invention:	METHODS OF DETERMINING A TREATMENT PROTOCOL FOR AND/OR A PROGNOSIS OF A PATIENT'S RECOVERY FROM A BRAIN INJURY
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Payment information:

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Authorized User	Wolf Greenfield
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37 CFR 1.21 (Miscellaneous fees and charges)

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Extension of Time	Q005270018US01-EXT-AJG.pdf	23596 d30fe2c7d82eca0837f366c1106ea12466849fb1	no	1
Warnings:					
Information:					
2	Fee Worksheet (SB06)	Q005270018US01-FEE-AJG.pdf	31651 d2a90223fd86b97a8f7794401fc739df3c6275d8	no	1
Warnings:					
Information:					
3	Notice of Appeal Filed	Q005270018US01-NAPL-AJG.pdf	20471 1317deac4b9074dcb816e5fcfff8a019ab14d7d8	no	1
Warnings:					
Information:					
4	Miscellaneous Incoming Letter	Q005270018US01-TRN-AJG.pdf	24772 23246dbd14fcf64c6622d05ca1ec70b90d9cc133	no	1
Warnings:					
Information:					
5	Fee Worksheet (SB06)	fee-info.pdf	33066 827679bd70335a0a0a6e6cdc074b4b789a41fab6	no	2
Warnings:					
Information:					
		Total Files Size (in bytes):	133556		

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New International Application Filed with the USPTO as a Receiving Office
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UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
14/111,326	06/24/2014	David Wilson	Q0052.70018US01	3274

23628 7590 04/26/2017
WOLF GREENFIELD & SACKS, P.C.
600 ATLANTIC AVENUE
BOSTON, MA 02210-2206

EXAMINER
WEIDNER, ADAM M

ART UNIT	PAPER NUMBER
1649	

NOTIFICATION DATE	DELIVERY MODE
04/26/2017	ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

Patents_eOfficeAction@WolfGreenfield.com
WGS_eOfficeAction@WolfGreenfield.com

Fujirebio Diagnostics, Inc. v. Quanterix Corp.
EX-1045
7993

Notice of Abandonment	Application No.	Applicant(s)
	14/111,326	WILSON ET AL.
	Examiner	Art Unit
	ADAM M. WEIDNER	1649

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address--

This application is abandoned in view of:

1. ☒ Applicant's failure to timely file a proper reply to the Office letter mailed on *18 March 2016*.
   (a) ☐ A reply was received on _____ (with a Certificate of Mailing or Transmission dated _____), which is after the expiration of the period for reply (including a total extension of time of _____ month(s)) which expired on _____.
   (b) ☐ A proposed reply was received on _____, but it does not constitute a proper reply under 37 CFR 1.113 to the final rejection.
   (A proper reply under 37 CFR 1.113 to a final rejection consists only of: (1) a timely filed amendment which places the application in condition for allowance; (2) a timely filed Notice of Appeal (with appeal fee); or (3) if this is utility or plant application, a timely filed Request for Continued Examination (RCE) in compliance with 37 CFR 1.114. Note that RCEs are not permitted in design applications.)
   (c) ☐ A reply was received on _____ but it does not constitute a proper reply, or a bona fide attempt at a proper reply, to the non-final rejection. See 37 CFR 1.85(a) and 1.111. (See explanation in box 7 below).
   (d) ☒ No reply has been received.

2. ☐ Applicant's failure to timely pay the required issue fee and publication fee, if applicable, within the statutory period of three months from the mailing date of the Notice of Allowance (PTOL-85).
   (a) ☐ The issue fee and publication fee, if applicable, was received on _____ (with a Certificate of Mailing or Transmission dated _____), which is after the expiration of the statutory period for payment of the issue fee (and publication fee) set in the Notice of Allowance (PTOL-85).
   (b) ☐ The submitted fee of $_____ is insufficient. A balance of $_____ is due.
   The issue fee required by 37 CFR 1.18 is $_____. The publication fee, if required by 37 CFR 1.18(d), is $_____.
   (c) ☐ The issue fee and publication fee, if applicable, has not been received.

3. ☐ Applicant's failure to timely file corrected drawings as required by, and within the three-month period set in, the Notice of Allowability (PTO-37).
   (a) ☐ Proposed corrected drawings were received on _____ (with a Certificate of Mailing or Transmission dated _____), which is after the expiration of the period for reply.
   (b) ☐ No corrected drawings have been received.

4. ☐ The letter of express abandonment which is signed by the attorney or agent of record or other party authorized under 37 CFR 1.33(b). See 37 CFR 1.138(b).

5. ☐ The letter of express abandonment which is signed by an attorney or agent (acting in a representative capacity under 37 CFR 1.34) upon the filing of a continuing application.

6. ☐ The decision by the Board of Patent Appeals and Interference rendered on _____ and because the period for seeking court review of the decision has expired and there are no allowed claims.

7. ☒ The reason(s) below:

A message for Andrea Gabert was left on 4/19/17 to confirm no response had been filed.

/ADAM M WEIDNER/
Primary Examiner, Art Unit 1649

Petitions to revive under 37 CFR 1.137, or requests to withdraw the holding of abandonment under 37 CFR 1.181, should be promptly filed to minimize any negative effects on patent term.

Fujirebio Diagnostics, Inc. v. Quanterix Corp.
EX-1045
7994