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(54) Title: ANTIBODY DIRECTED AGAINST THE APOE AMINO-TERMINAL FRAGMENT OF 12 KDA

(57) Abstract: The disclosure relates to an antibody or antigen binding portion thereof, which binds to a neo-epitope of a C-terminal fragment of apolipoprotein E, to methods of producing such an antibody or antigen binding portion thereof, and to therapeutic and diagnostic uses thereof.



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NEW ANTIBODY

Field of the invention

The present invention relates to an antibody or antigen binding portion thereof, which binds to a neo-epitope of a C-terminal fragment of apolipoprotein E, to methods of producing such an antibody or antigen
5 binding portion thereof, and to therapeutic and diagnostic uses thereof.

Background

Alzheimer's disease (AD) is a progressive neurodegenerative dementia disorder, which exists in a more common late-onset form and an early-onset
10 familial form. AD is characterized by progressive loss of memory and cognitive function. At present, AD treatments are limited to symptomatic management and the prognosis is poor for AD patients. It is estimated that about 18 million people worldwide are presently suffering from AD, and the number of people suffering from AD is expected to increase due to the aging
15 population. The prevalence of AD doubles approximately every 5 years from the age of 60, from 10% of individuals at the age of 65 to 50% of individuals at the age of 85 or more (Solomon, Expert Opin. Investig. Drugs (2007) 16(6): 819-828).

A known genetic risk factor for late-onset AD is the APOE ϵ 4 allele,
20 although its precise role in the disease remains unclear. The APOE gene encodes apolipoprotein E (ApoE), which is a glycoprotein of 35 kDa expressed at high levels in the brain. ApoE exists in three different isoforms, ApoE2, ApoE3 and ApoE4, of which ApoE3 is the most common, ApoE2 has been shown to decrease the risk of AD, and ApoE4 increases it. Although it is
25 not clear how ApoE contributes to the onset and progression of AD, several studies have shown that the protein is subject to fragmentation in the human brain. The specific functions of ApoE fragments and their possible links with AD are reviewed by Muñoz *et al* in Neurochem Res (2019) 44(6): 1297-1305. With regards to the neurotoxicity of ApoE fragments, Muñoz *et al* conclude
30 that the existing research shows that both the N-terminal LDL-binding domain

and the C-terminal lipid-binding domain of ApoE are necessary for a neurotoxic effect (see e.g. page 1300 lines 4-8). With regards to ApoE fragments from the C-terminal, lipid-binding domain, Table 2 and Figure 2 of Muñoz *et al* indicate that one such fragment has been studied previously and shown to have a stabilizing effect on hexamers of amyloid beta (A β) peptide. The study in question was reported by Wellnitz *et al* in J Neurochem (2005) 94: 1351-1360, and discloses a 13 kDa fragment of ApoE with an N-terminal start at amino acid position 187 of ApoE. No antibodies specific for this fragment are disclosed, but it is studied using an antibody designated 3H1.

10 The antibody 3H1 is disclosed as binding to an epitope in the C-terminal part of ApoE formed by amino acid residues 243-273.

A similar conclusion regarding the neurotoxicity of ApoE fragments was reached in an earlier review by Mahley and Huang in Neuron (2012) 76: 871-885. This article focuses primarily on the potential neurotoxicity of fragments of the specific isoform ApoE4. Whereas it does show that an ApoE4 fragment of 12 kDa is generated (see e.g. Figure 6), the conclusion is drawn that neurotoxic ApoE fragments comprise both the LDL receptor binding region (aa 136-150) and the lipid binding region (aa 240-270) (see e.g. Figure 7 and page 876, right col, lines 13-21). Mahley and Huang do not disclose any antibodies specific for the reviewed ApoE4 fragments.

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Finally, a study by Mouchard *et al* in Sci. Rep. (2019) 9(1): 3989 investigated the association between fragments of ApoE and amyloid beta (A β) peptides of different sizes. An ApoE fragment of 12 kDa was identified, but was found to not interact with A β . The antibodies used in the Mouchard *et al* study are listed in Supplementary Table 2, and do not include any antibody specific for the 12 kDa ApoE fragment.

25

There remains a need in the art for novel therapeutic and/or diagnostic tools for detecting and treating Alzheimer's disease. There is also a need to further elucidate and characterize the role of ApoE fragments in the onset and progression of neurodegenerative disease.

30

Summary of the invention

The present invention relates to antibodies and antigen binding portions thereof that bind to apolipoprotein E (ApoE). As reported herein, the antibodies and antigen binding portions of the invention bind to a neo-epitope
5 present within C-terminal fragments of ApoE.

Thus, in a first aspect, the present invention provides an antibody or antigen binding portion thereof that binds to a fragment of apolipoprotein E (ApoE), wherein the fragment has

- an apparent molecular weight of 12 kDa as measured by SDS-PAGE, and
- 10 - an N-terminus corresponding to an amino acid in full-length apolipoprotein E which is selected from the group consisting of amino acids L198, A199 and G200; and wherein the antibody or antigen binding portion thereof binds to an epitope comprising the N-terminus of the fragment. In certain embodiments, the ApoE fragment consists of the amino acid sequence of any one of SEQ ID
15 NOs: 1, 2 or 3.

In a further aspect, the present invention provides methods of producing antibodies or antigen binding portions thereof, the methods comprising a step of immunizing a host mammal with a peptide immunogen comprising an N-terminal amino acid sequence selected from the group
20 consisting of LAGQPL (SEQ ID NO:4), AGQPLQ (SEQ ID NO:5), GQPLQE (SEQ ID NO:6), LAGQPLQ (SEQ ID NO:7), AGQPLQE (SEQ ID NO:8) and LAGQPLQE (SEQ ID NO:9). Antibodies and antigen binding portions thereof obtainable by said methods are encompassed by a third aspect of the invention.

25 In a still further aspect, the present invention provides a pharmaceutical composition comprising an antibody or antigen binding portion thereof in accordance with the first or third aspect of the invention and a pharmaceutically acceptable excipient or carrier.

In further aspects, the present invention provides antibodies, antigen
30 binding portions thereof and/or pharmaceutical compositions comprising the same for use in methods of treatment or for use in methods of detection or diagnosis as described herein.

The invention can be further understood with reference to the following illustrative embodiments.

1. An antibody or antigen binding portion thereof that binds to a
5 fragment of apolipoprotein E (ApoE), wherein the fragment has
 - an apparent molecular weight of 12 kDa as measured by SDS-PAGE, and
 - an N-terminus corresponding to an amino acid in full-length apolipoprotein E which is selected from the group consisting of amino acids
10 L198, A199 and G200; and wherein the antibody or antigen binding portion thereof binds to an epitope comprising the N-terminus of the fragment.
2. The antibody or antigen binding portion thereof according to (1), wherein the antibody or antigen binding portion thereof binds selectively to
15 the ApoE fragment.
3. The antibody or antigen binding portion thereof according to (1) or (2), wherein the antibody or antigen binding portion thereof does not bind to full-length apolipoprotein E.
20
4. The antibody or antigen binding portion thereof according to any one of (1)-(3), wherein the fragment of apolipoprotein E has an N-terminus corresponding to amino acid G200 in full-length apolipoprotein E.
- 25 5. The antibody or antigen binding portion thereof according to any one of (1)-(3), wherein the fragment of apolipoprotein E has an N-terminus corresponding to amino acid A199 in full-length apolipoprotein E.
- 30 6. The antibody or antigen binding portion thereof according to any one of (1)-(3), wherein the fragment of apolipoprotein E has an N-terminus corresponding to amino acid L198 in full-length apolipoprotein E.

7. The antibody or antigen binding portion thereof according to any one of (1)-(4), wherein the antibody or antigen binding portion thereof binds to an epitope comprising amino acid residues 200-205 in full-length apolipoprotein E (GQPLQE).

5

8. The antibody or antigen binding portion thereof according to any one of (1)-(7), wherein the fragment of apolipoprotein E is selected from:

i) a fragment consisting of the amino acid sequence of any one of SEQ ID NOs:1-3; and

10 ii) a fragment having at least 80% identity to any one of SEQ ID NOs: 1-3.

9. The antibody or antigen binding portion thereof according to (8), wherein the fragment of apolipoprotein E is selected from a fragment
15 consisting of the amino acid sequence of any one of SEQ ID NOs:1, 2 and 3.

10. The antibody or antigen binding portion thereof according to (8), wherein the fragment of apolipoprotein E consists of the amino acid sequence of SEQ ID NO: 1.

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11. The antibody or antigen binding portion thereof according to (8), wherein the fragment of apolipoprotein E consists of the amino acid sequence of SEQ ID NO: 2.

25 12. The antibody or antigen binding portion thereof according to (11), wherein the antibody or antigen binding portion thereof binds to an epitope comprising amino acid residues 199-204 in full-length apolipoprotein E (AGQPLQ) or amino acid residues 199-205 in full-length apolipoprotein E (AGQPLQE).

30

13. The antibody or antigen binding portion thereof according to (8), wherein the fragment of apolipoprotein E consists of the amino acid sequence of SEQ ID NO: 3.

14. The antibody or antigen binding portion thereof according to (13), wherein the antibody or antigen binding portion thereof binds to an epitope comprising:

- 5 - amino acid residues 198-203 in full-length apolipoprotein E (LAGQPL);
- amino acid residues 198-204 in full-length apolipoprotein E (LAGQPLQ); or
- amino acid residues 198-205 in full-length apolipoprotein E
- 10 (LAGQPLQE).

- 15 15. A method of producing an antibody or an antigen binding portion thereof, comprising a step of immunizing a suitable host mammal with a peptide immunogen comprising an N-terminal amino acid sequence selected from the group consisting of LAGQPL (SEQ ID NO:4), AGQPLQ (SEQ ID NO:5), GQPLQE (SEQ ID NO:6), LAGQPLQ (SEQ ID NO:7), AGQPLQE (SEQ ID NO:8) and LAGQPLQE (SEQ ID NO:9).

- 20 16. The method according to (15), wherein said N-terminal amino acid sequence is GQPLQE (SEQ ID NO:6).

- 25 17. The method according to (15), wherein said N-terminal amino acid sequence is selected from LAGQPL (SEQ ID NO:4), LAGQPLQ (SEQ ID NO:7) and LAGQPLQE (SEQ ID NO:9).

18. The method according to (15), wherein said N-terminal amino acid sequence is selected from AGQPLQ (SEQ ID NO:5) and AGQPLQE (SEQ ID NO:8).

- 30 19. An antibody or antigen binding portion thereof, obtainable by a method according to any one of (15)-(18).

20. An antibody or antigen binding portion thereof according to any one of (1)-(14) or (19), wherein the antibody or antigen binding portion thereof comprises a variable heavy chain domain (VH) comprising three CDR sequences (CDR-H1, CDR-H2 and CDR-H3), wherein the three VH CDR
5 sequences are independently selected from:

- CDR-H1 selected from the group consisting of SEQ ID NO: 10, 15, 18 and 21;
- CDR-H2 selected from the group consisting of SEQ ID NO: 11, 13, 16, 19 and 22; and
- 10 - CDR-H3 selected from the group consisting of SEQ ID NO: 12, 14, 17, 20 and 23.

21. An antibody or antigen binding portion thereof according to any one of (1)-(14), (19) or (20), wherein the antibody or antigen binding portion
15 thereof comprises a variable light chain domain (VL) comprising three CDR sequences (CDR-L1, CDR-L2 and CDR-L3), wherein the three VL CDR sequences are independently selected from:

- CDR-L1 selected from the group consisting of SEQ ID NO: 24, 27, 29, 31 and 32;
- 20 - CDR-L2 being SEQ ID NO: 25; and
- CDR-L3 selected from the group consisting of SEQ ID NO: 26, 28, 30 and 33.

22. An antibody or antigen binding portion thereof that binds to
25 apolipoprotein E (ApoE), wherein the antibody or antigen binding portion thereof comprises a variable heavy chain domain (VH) comprising three CDR sequences (CDR-H1, CDR-H2 and CDR-H3), wherein the three VH CDR sequences are independently selected from:

- 30 - CDR-H1 selected from the group consisting of SEQ ID NO: 10, 15, 18 and 21;
- CDR-H2 selected from the group consisting of SEQ ID NO: 11, 13, 16, 19 and 22; and

- CDR-H3 selected from the group consisting of SEQ ID NO: 12, 14, 17, 20 and 23.

23. The antibody or antigen binding portion thereof according to (22),
5 wherein the antibody or antigen binding portion thereof additionally comprises a variable light chain domain (VL) comprising three CDR sequences (CDR-L1, CDR-L2 and CDR-L3), wherein the three VL CDR sequences are independently selected from:

- CDR-L1 selected from the group consisting of SEQ ID NO: 24, 27,
10 29, 31 and 32;
- CDR-L2 being SEQ ID NO: 25; and
- CDR-L3 selected from the group consisting of SEQ ID NO: 26, 28, 30 and 33.

15 24. The antibody or antigen binding portion thereof according to any one of (20)-(23), wherein the antibody or antigen binding portion thereof comprises the CDR sequences:

CDR-H1 comprising or consisting of SEQ ID NO: 10 (SYAMS);
CDR-H2 comprising or consisting of SEQ ID NO: 11
20 (EISGSGSRDHYTDSVTG);
CDR-H3 comprising or consisting of SEQ ID NO: 12 (QLTGTDYYGTDY);
CDR-L1 comprising or consisting of SEQ ID NO: 24 (RSSQSIVYSNGNTYLE);
25 CDR-L2 comprising or consisting of SEQ ID NO: 25 (KVSNRFS); and
CDR-L3 comprising or consisting of SEQ ID NO: 26 (FQGSHLPYT).

25. The antibody or antigen binding portion thereof according to any one of (20)-(23), wherein the antibody or antigen binding portion thereof
30 comprises the CDR sequences:

CDR-H1 comprising or consisting of SEQ ID NO: 10 (SYAMS);
CDR-H2 comprising or consisting of SEQ ID NO: 13
(EISSGGGSTNYLDTVGTG);

CDR-H3 comprising or consisting of SEQ ID NO: 14
(QLVGTDYYGTDY);

CDR-L1 comprising or consisting of SEQ ID NO: 27
(RSSQNIVYSNGNTYLE);

5 CDR-L2 comprising or consisting of SEQ ID NO: 25 (KVSNRFS); and
CDR-L3 comprising or consisting of SEQ ID NO: 28 (FQGSHVPYT).

26. The antibody or antigen binding portion thereof according to any
one of (20)-(23), wherein the antibody or antigen binding portion thereof
10 comprises the CDR sequences:

CDR-H1 comprising or consisting of SEQ ID NO: 15 (SFAMS);

CDR-H2 comprising or consisting of SEQ ID NO: 16
(EISRGGGYAFYSDTV TG);

CDR-H3 comprising or consisting of SEQ ID NO: 17
15 (QLTGTDYYAMDY);

CDR-L1 comprising or consisting of SEQ ID NO: 29
(RSSQSIVYTNGNTYLE);

CDR-L2 comprising or consisting of SEQ ID NO: 25 (KVSNRFS); and
CDR-L3 comprising or consisting of SEQ ID NO: 30 (FQGSQVPYT).

20

27. The antibody or antigen binding portion thereof according to any
one of (20)-(23), wherein the antibody or antigen binding portion thereof
comprises the CDR sequences:

CDR-H1 comprising or consisting of SEQ ID NO: 18 (RYAMS);

25 CDR-H2 comprising or consisting of SEQ ID NO: 19
(EINSGGSYSFYSDTV TG);

CDR-H3 comprising or consisting of SEQ ID NO: 12
(QLTGTDYYGTDY);

CDR-L1 comprising or consisting of SEQ ID NO: 31
30 (RSSQSLLYSNGNTYLE);

CDR-L2 comprising or consisting of SEQ ID NO: 25 (KVSNRFS); and
CDR-L3 comprising or consisting of SEQ ID NO: 28 (FQGSHVPYT).

28. The antibody or antigen binding portion thereof according to any one of (20)-(23), wherein the antibody or antigen binding portion thereof comprises the CDR sequences:

- CDR-H1 comprising or consisting of SEQ ID NO: 18 (RYAMS);
- 5 CDR-H2 comprising or consisting of SEQ ID NO: 19 (EINSGGSYSFYSDTVTG);
- CDR-H3 comprising or consisting of SEQ ID NO: 20 (QLSGTDYYGTDY);
- CDR-L1 comprising or consisting of SEQ ID NO: 31
- 10 (RSSQSLLYSNGNTYLE);
- CDR-L2 comprising or consisting of SEQ ID NO: 25 (KVSNRFS); and
- CDR-L3 comprising or consisting of SEQ ID NO: 28 (FQGSHVPYT).

29. The antibody or antigen binding portion thereof according to any one of (20)-(23), wherein the antibody or antigen binding portion thereof comprises the CDR sequences:

- CDR-H1 comprising or consisting of SEQ ID NO: 21 (NYAMH);
- CDR-H2 comprising or consisting of SEQ ID NO: 22 (WINTYTGEPTFADDFKG);
- 20 CDR-H3 comprising or consisting of SEQ ID NO: 23 (EGYYDRSHYFDY);
- CDR-L1 comprising or consisting of SEQ ID NO: 32 (RSSLSLVHGDGNTYLE);
- CDR-L2 comprising or consisting of SEQ ID NO: 25 (KVSNRFS); and
- 25 CDR-L3 comprising or consisting of SEQ ID NO: 33 (LQGSHIPFT).

30. An antibody or antigen binding portion thereof according to any one of (1)-(14) and (19)-(23), wherein the antibody or antigen binding portion thereof comprises a heavy chain variable domain (VH) comprising or

30 consisting of an amino acid sequence selected from:

- i) the group consisting of SEQ ID NOs: 34, 36, 38, 40, 42 and 43; and
- ii) a sequence having at least 70%, at least 80%, at least 90%, or at least 95% identity to any one of SEQ ID NOs: 34, 36, 38, 40, 42 and 43.

31. An antibody or antigen binding portion thereof according to any one of (1)-(14), (19)-(23) or (30), wherein the antibody or antigen binding portion thereof comprises a light chain variable domain (VL) comprising or
5 consisting of an amino acid sequence selected from:
i) the group consisting of SEQ ID NOs: 35, 37, 39, 41 and 44; and
ii) a sequence having at least 70%, at least 80%, at least 90%, or at least 95% identity to any one of SEQ ID NO: 35, 37, 39, 41 and 44.

10 32. The antibody or antigen binding portion thereof according to (30) or (31), comprising a variable heavy chain domain (VH) and a variable light chain domain (VL) selected from the group consisting of:

(i) a VH domain comprising or consisting of the amino acid sequence of SEQ ID NO: 34 or an amino acid sequence having at least 80%,
15 90%, 95%, 98%, or 99% identity thereto, and a VL domain comprising or consisting of the amino acid sequence of SEQ ID NO: 35 or an amino acid sequence having at least 80%, 90%, 95%, 98%, or 99% identity thereto;

(ii) a VH domain comprising or consisting of the amino acid sequence of SEQ ID NO: 36 or an amino acid sequence having at least 80%, 90%,
20 95%, 98%, or 99% identity thereto, and a VL domain comprising or consisting of the amino acid sequence of SEQ ID NO: 37 or an amino acid sequence having at least 80%, 90%, 95%, 98%, or 99% identity thereto;

(iii) a VH domain comprising or consisting of the amino acid sequence of SEQ ID NO: 38 or an amino acid sequence having at least 80%, 90%,
25 95%, 98%, or 99% identity thereto, and a VL domain comprising or consisting of the amino acid sequence of SEQ ID NO: 39 or an amino acid sequence having at least 80%, 90%, 95%, 98%, or 99% identity thereto;

(iv) a VH domain comprising or consisting of the amino acid sequence of SEQ ID NO: 40 or an amino acid sequence having at least 80%,
30 90%, 95%, 98%, or 99% identity thereto, and a VL domain comprising or consisting of the amino acid sequence of SEQ ID NO: 41 or an amino acid sequence having at least 80%, 90%, 95%, 98%, or 99% identity thereto;

(v) a VH domain comprising or consisting of the amino acid sequence of SEQ ID NO: 42 or an amino acid sequence having at least 80%, 90%, 95%, 98%, or 99% identity thereto, and a VL domain comprising or consisting of the amino acid sequence of SEQ ID NO: 41 or an amino acid sequence
5 having at least 80%, 90%, 95%, 98%, or 99% identity thereto; and

(vi) a VH domain comprising or consisting of the amino acid sequence of SEQ ID NO: 43 or an amino acid sequence having at least 80%, 90%, 95%, 98%, or 99% identity thereto, and a VL domain comprising or consisting of the amino acid sequence of SEQ ID NO: 44 or an amino acid
10 sequence having at least 80%, 90%, 95%, 98%, or 99% identity thereto.

33. The antibody or antigen binding portion thereof according to (32), comprising a variable heavy chain domain (VH) and a variable light chain domain (VL) selected from the group consisting of:

15 (i) a VH domain comprising or consisting of the amino acid sequence of SEQ ID NO: 34 and a VL domain comprising or consisting of the amino acid sequence of SEQ ID NO: 35;

(ii) a VH domain comprising or consisting of the amino acid sequence of SEQ ID NO: 36 and a VL domain comprising or consisting of the amino
20 acid sequence of SEQ ID NO: 37;

(iii) a VH domain comprising or consisting of the amino acid sequence of SEQ ID NO: 38 and a VL domain comprising or consisting of the amino acid sequence of SEQ ID NO: 39;

(iv) a VH domain comprising or consisting of the amino acid
25 sequence of SEQ ID NO: 40, and a VL domain comprising or consisting of the amino acid sequence of SEQ ID NO: 41;

(v) a VH domain comprising or consisting of the amino acid sequence of SEQ ID NO: 42, and a VL domain comprising or consisting of the amino acid sequence of SEQ ID NO: 41; and

30 (vi) a VH domain comprising or consisting of the amino acid sequence of SEQ ID NO: 43, and a VL domain comprising or consisting of the amino acid sequence of SEQ ID NO: 44.

34. An antibody or antigen binding portion thereof according to any one of (1)-(14) or (19), wherein the antibody or antigen binding portion thereof comprises a variable heavy chain domain (VH) comprising three CDR sequences (CDR-H1, CDR-H2 and CDR-H3), wherein the three VH CDR sequences are independently selected from:

- CDR-H1 selected from the group consisting of SEQ ID NO: 59, 62 and 65;
- CDR-H2 selected from the group consisting of SEQ ID NO: 60, 63, 66, 68 and 70; and
- CDR-H3 selected from the group consisting of SEQ ID NO: 61, 64, 67 and 69.

35. An antibody or antigen binding portion thereof according to any one of (1)-(14), (19) or (34), wherein the antibody or antigen binding portion thereof comprises a variable light chain domain (VL) comprising three CDR sequences (CDR-L1, CDR-L2 and CDR-L3), wherein the three VL CDR sequences are independently selected from:

- CDR-L1 selected from the group consisting of SEQ ID NO: 71, 74, 76, 79 and 80;
- CDR-L2 selected from the group consisting of SEQ ID NO: 72 and 77; and
- CDR-L3 selected from the group consisting of SEQ ID NO: 73, 75, 78 and 81.

36. An antibody or antigen binding portion thereof that binds to apolipoprotein E (ApoE), wherein the antibody or antigen binding portion thereof comprises a variable heavy chain domain (VH) comprising three CDR sequences (CDR-H1, CDR-H2 and CDR-H3), wherein the three VH CDR sequences are independently selected from:

- CDR-H1 selected from the group consisting of SEQ ID NO: 59, 62 and 65;
- CDR-H2 selected from the group consisting of SEQ ID NO: 60, 63, 66, 68 and 70; and

- CDR-H3 selected from the group consisting of SEQ ID NO: 61, 64, 67 and 69.

37. The antibody or antigen binding portion thereof according to (36),
5 wherein the antibody or antigen binding portion thereof additionally comprises a variable light chain domain (VL) comprising three CDR sequences (CDR-L1, CDR-L2 and CDR-L3), wherein the three VL CDR sequences are independently selected from:

- CDR-L1 selected from the group consisting of SEQ ID NO: 71, 74,
10 76, 79 and 80;

- CDR-L2 selected from the group consisting of SEQ ID NO: 72 and 77; and

- CDR-L3 selected from the group consisting of SEQ ID NO: 73, 75, 78 and 81.

15

38. The antibody or antigen binding portion thereof according to any one of (34)-(37), wherein the antibody or antigen binding portion thereof comprises the CDR sequences:

CDR-H1 comprising or consisting of SEQ ID NO: 59;
20 CDR-H2 comprising or consisting of SEQ ID NO: 60;
CDR-H3 comprising or consisting of SEQ ID NO: 61;
CDR-L1 comprising or consisting of SEQ ID NO: 71;
CDR-L2 comprising or consisting of SEQ ID NO: 72; and
CDR-L3 comprising or consisting of SEQ ID NO: 73.

25

39. The antibody or antigen binding portion thereof according to any one of (34)-(37), wherein the antibody or antigen binding portion thereof comprises the CDR sequences:

CDR-H1 comprising or consisting of SEQ ID NO: 62;
30 CDR-H2 comprising or consisting of SEQ ID NO: 63;
CDR-H3 comprising or consisting of SEQ ID NO: 64;
CDR-L1 comprising or consisting of SEQ ID NO: 74;
CDR-L2 comprising or consisting of SEQ ID NO: 72; and

CDR-L3 comprising or consisting of SEQ ID NO: 75.

40. The antibody or antigen binding portion thereof according to any one of (34)-(37), wherein the antibody or antigen binding portion thereof

5 comprises the CDR sequences:

CDR-H1 comprising or consisting of SEQ ID NO: 65;

CDR-H2 comprising or consisting of SEQ ID NO: 66;

CDR-H3 comprising or consisting of SEQ ID NO: 67;

CDR-L1 comprising or consisting of SEQ ID NO: 76;

10 CDR-L2 comprising or consisting of SEQ ID NO: 77; and

CDR-L3 comprising or consisting of SEQ ID NO: 78.

41. The antibody or antigen binding portion thereof according to any one of (34)-(37), wherein the antibody or antigen binding portion thereof

15 comprises the CDR sequences:

CDR-H1 comprising or consisting of SEQ ID NO: 62;

CDR-H2 comprising or consisting of SEQ ID NO: 68;

CDR-H3 comprising or consisting of SEQ ID NO: 69;

CDR-L1 comprising or consisting of SEQ ID NO: 79;

20 CDR-L2 comprising or consisting of SEQ ID NO: 72; and

CDR-L3 comprising or consisting of SEQ ID NO: 78.

42. The antibody or antigen binding portion thereof according to any one of (34)-(37), wherein the antibody or antigen binding portion thereof

25 comprises the CDR sequences:

CDR-H1 comprising or consisting of SEQ ID NO: 62;

CDR-H2 comprising or consisting of SEQ ID NO: 70;

CDR-H3 comprising or consisting of SEQ ID NO: 67;

CDR-L1 comprising or consisting of SEQ ID NO: 80;

30 CDR-L2 comprising or consisting of SEQ ID NO: 77; and

CDR-L3 comprising or consisting of SEQ ID NO: 81.

43. An antibody or antigen binding portion thereof according to any one of (1)-(14), (19) and (34)-(37), wherein the antibody or antigen binding portion thereof comprises a heavy chain variable domain (VH) comprising or consisting of an amino acid sequence selected from:

- 5 i) the group consisting of SEQ ID NOs: 82, 84, 86, 88 and 90; and
 ii) a sequence having at least 70%, at least 80%, at least 90%, or at least 95% identity to any one of SEQ ID NOs: 82, 84, 86, 88 and 90.

44. An antibody or antigen binding portion thereof according to any one of (1)-(14), (19), (34)-(37) and (43), wherein the antibody or antigen binding portion thereof comprises a light chain variable domain (VL) comprising or consisting of an amino acid sequence selected from:

- i) the group consisting of SEQ ID NOs: 83, 85, 87, 89 and 91; and
 ii) a sequence having at least 70%, at least 80%, at least 90%, or at least 95% identity to any one of SEQ ID NO: 83, 85, 87, 89 and 91.

45. The antibody or antigen binding portion thereof according to (43) or (44), comprising a variable heavy chain domain (VH) and a variable light chain domain (VL) selected from the group consisting of:

- 20 (i) a VH domain comprising or consisting of the amino acid sequence of SEQ ID NO: 82 or an amino acid sequence having at least 80%, 90%, 95%, 98%, or 99% identity thereto, and a VL domain comprising or consisting of the amino acid sequence of SEQ ID NO: 83 or an amino acid sequence having at least 80%, 90%, 95%, 98%, or 99% identity thereto;
- 25 (ii) a VH domain comprising or consisting of the amino acid sequence of SEQ ID NO: 84 or an amino acid sequence having at least 80%, 90%, 95%, 98%, or 99% identity thereto, and a VL domain comprising or consisting of the amino acid sequence of SEQ ID NO: 85 or an amino acid sequence having at least 80%, 90%, 95%, 98%, or 99% identity thereto;
- 30 (iii) a VH domain comprising or consisting of the amino acid sequence of SEQ ID NO: 86 or an amino acid sequence having at least 80%, 90%, 95%, 98%, or 99% identity thereto, and a VL domain comprising or consisting

of the amino acid sequence of SEQ ID NO: 87 or an amino acid sequence having at least 80%, 90%, 95%, 98%, or 99% identity thereto;

(iv) a VH domain comprising or consisting of the amino acid sequence of SEQ ID NO: 88 or an amino acid sequence having at least 80%,
5 90%, 95%, 98%, or 99% identity thereto, and a VL domain comprising or consisting of the amino acid sequence of SEQ ID NO: 89 or an amino acid sequence having at least 80%, 90%, 95%, 98%, or 99% identity thereto; and

(v) a VH domain comprising or consisting of the amino acid sequence of SEQ ID NO: 90 or an amino acid sequence having at least 80%, 90%,
10 95%, 98%, or 99% identity thereto, and a VL domain comprising or consisting of the amino acid sequence of SEQ ID NO: 91 or an amino acid sequence having at least 80%, 90%, 95%, 98%, or 99% identity thereto.

46. The antibody or antigen binding portion thereof according to (45),
15 comprising a variable heavy chain domain (VH) and a variable light chain domain (VL) selected from the group consisting of:

(i) a VH domain comprising or consisting of the amino acid sequence of SEQ ID NO: 82, and a VL domain comprising or consisting of the amino acid sequence of SEQ ID NO: 83;

20 (ii) a VH domain comprising or consisting of the amino acid sequence of SEQ ID NO: 84, and a VL domain comprising or consisting of the amino acid sequence of SEQ ID NO: 85;

(iii) a VH domain comprising or consisting of the amino acid sequence of SEQ ID NO: 86, and a VL domain comprising or consisting of the amino acid sequence of SEQ ID NO: 87;
25

(iv) a VH domain comprising or consisting of the amino acid sequence of SEQ ID NO: 88, and a VL domain comprising or consisting of the amino acid sequence of SEQ ID NO: 89; and

(v) a VH domain comprising or consisting of the amino acid sequence
30 of SEQ ID NO: 90, and a VL domain comprising or consisting of the amino acid sequence of SEQ ID NO: 91.

47. An antibody or antigen binding portion thereof according to any one of (1)-(14) or (19), wherein the antibody or antigen binding portion thereof comprises a variable heavy chain domain (VH) comprising three CDR sequences (CDR-H1, CDR-H2 and CDR-H3), wherein the three VH CDR sequences are independently selected from:

- CDR-H1 selected from the group consisting of SEQ ID NO: 62, 94 and 97;
- CDR-H2 selected from the group consisting of SEQ ID NO: 92, 95 and 98; and
- CDR-H3 selected from the group consisting of SEQ ID NO: 93, 96 and 99.

48. An antibody or antigen binding portion thereof according to any one of (1)-(14), (19) or (47), wherein the antibody or antigen binding portion thereof comprises a variable light chain domain (VL) comprising three CDR sequences (CDR-L1, CDR-L2 and CDR-L3), wherein the three VL CDR sequences are independently selected from:

- CDR-L1 selected from the group consisting of SEQ ID NO: 100, 103, 105 and 108;
- CDR-L2 selected from the group consisting of SEQ ID NO: 25, 101, 104 and 106; and
- CDR-L3 selected from the group consisting of SEQ ID NO: 28, 102 and 107.

49. An antibody or antigen binding portion thereof that binds to apolipoprotein E (ApoE), wherein the antibody or antigen binding portion thereof comprises a variable heavy chain domain (VH) comprising three CDR sequences (CDR-H1, CDR-H2 and CDR-H3), wherein the three VH CDR sequences are independently selected from:

- CDR-H1 selected from the group consisting of SEQ ID NO: 62, 94 and 97;
- CDR-H2 selected from the group consisting of SEQ ID NO: 92, 95 and 98; and

- CDR-H3 selected from the group consisting of SEQ ID NO: 93, 96 and 99.

50. The antibody or antigen binding portion thereof according to (49),
5 wherein the antibody or antigen binding portion thereof additionally comprises a variable light chain domain (VL) comprising three CDR sequences (CDR-L1, CDR-L2 and CDR-L3), wherein the three VL CDR sequences are independently selected from:

- CDR-L1 selected from the group consisting of SEQ ID NO: 100,
10 103, 105 and 108;
- CDR-L2 selected from the group consisting of SEQ ID NO: 25, 101, 104 and 106; and
- CDR-L3 selected from the group consisting of SEQ ID NO: 28, 102 and 107.

15

51. The antibody or antigen binding portion thereof according to any one of (47)-(50), wherein the antibody or antigen binding portion thereof comprises the CDR sequences:

- CDR-H1 comprising or consisting of SEQ ID NO: 62;
- 20 CDR-H2 comprising or consisting of SEQ ID NO: 92;
- CDR-H3 comprising or consisting of SEQ ID NO: 93;
- CDR-L1 comprising or consisting of SEQ ID NO: 100;
- CDR-L2 comprising or consisting of SEQ ID NO: 101; and
- CDR-L3 comprising or consisting of SEQ ID NO: 102.

25

52. The antibody or antigen binding portion thereof according to any one of (47)-(50), wherein the antibody or antigen binding portion thereof comprises the CDR sequences:

- CDR-H1 comprising or consisting of SEQ ID NO: 62;
- 30 CDR-H2 comprising or consisting of SEQ ID NO: 92;
- CDR-H3 comprising or consisting of SEQ ID NO: 93;
- CDR-L1 comprising or consisting of SEQ ID NO: 103;
- CDR-L2 comprising or consisting of SEQ ID NO: 104; and

CDR-L3 comprising or consisting of SEQ ID NO: 102.

53. The antibody or antigen binding portion thereof according to any one of (47)-(50), wherein the antibody or antigen binding portion thereof

5 comprises the CDR sequences:

CDR-H1 comprising or consisting of SEQ ID NO: 94;

CDR-H2 comprising or consisting of SEQ ID NO: 95;

CDR-H3 comprising or consisting of SEQ ID NO: 96;

CDR-L1 comprising or consisting of SEQ ID NO: 105;

10 CDR-L2 comprising or consisting of SEQ ID NO: 106; and

CDR-L3 comprising or consisting of SEQ ID NO: 107.

54. The antibody or antigen binding portion thereof according to any one of (47)-(50), wherein the antibody or antigen binding portion thereof

15 comprises the CDR sequences:

CDR-H1 comprising or consisting of SEQ ID NO: 97;

CDR-H2 comprising or consisting of SEQ ID NO: 98;

CDR-H3 comprising or consisting of SEQ ID NO: 99;

CDR-L1 comprising or consisting of SEQ ID NO: 108;

20 CDR-L2 comprising or consisting of SEQ ID NO: 25; and

CDR-L3 comprising or consisting of SEQ ID NO: 28.

55. An antibody or antigen binding portion thereof according to any one of (1)-(14), (19) and (47)-(50), wherein the antibody or antigen binding

25 portion thereof comprises a heavy chain variable domain (VH) comprising or consisting of an amino acid sequence selected from:

i) the group consisting of SEQ ID NOs: 109, 111, 113 and 115; and

ii) a sequence having at least 70%, at least 80%, at least 90%, or at least 95% identity to any one of SEQ ID NOs: 109, 111, 113 and 115.

30

56. An antibody or antigen binding portion thereof according to any one of (1)-(14), (19), (47)-(50) or (55), wherein the antibody or antigen binding

portion thereof comprises a light chain variable domain (VL) comprising or consisting of an amino acid sequence selected from:

- i) the group consisting of SEQ ID NOs: 110, 112, 114 and 116; and
- ii) a sequence having at least 70%, at least 80%, at least 90%, or at least 95% identity to any one of SEQ ID NO: 110, 112, 114 and 116.

57. The antibody or antigen binding portion thereof according to (55) or (56), comprising a variable heavy chain domain (VH) and a variable light chain domain (VL) selected from the group consisting of:

- (i) a VH domain comprising or consisting of the amino acid sequence of SEQ ID NO: 109 or an amino acid sequence having at least 80%, 90%, 95%, 98%, or 99% identity thereto, and a VL domain comprising or consisting of the amino acid sequence of SEQ ID NO: 110 or an amino acid sequence having at least 80%, 90%, 95%, 98%, or 99% identity thereto;
- (ii) a VH domain comprising or consisting of the amino acid sequence of SEQ ID NO: 111 or an amino acid sequence having at least 80%, 90%, 95%, 98%, or 99% identity thereto, and a VL domain comprising or consisting of the amino acid sequence of SEQ ID NO: 112 or an amino acid sequence having at least 80%, 90%, 95%, 98%, or 99% identity thereto;
- (iii) a VH domain comprising or consisting of the amino acid sequence of SEQ ID NO: 113 or an amino acid sequence having at least 80%, 90%, 95%, 98%, or 99% identity thereto, and a VL domain comprising or consisting of the amino acid sequence of SEQ ID NO: 114 or an amino acid sequence having at least 80%, 90%, 95%, 98%, or 99% identity thereto; and
- (iv) a VH domain comprising or consisting of the amino acid sequence of SEQ ID NO: 115 or an amino acid sequence having at least 80%, 90%, 95%, 98%, or 99% identity thereto, and a VL domain comprising or consisting of the amino acid sequence of SEQ ID NO: 116 or an amino acid sequence having at least 80%, 90%, 95%, 98%, or 99% identity thereto.

58. The antibody or antigen binding portion thereof according to (57), comprising a variable heavy chain domain (VH) and a variable light chain domain (VL) selected from the group consisting of:

(i) a VH domain comprising or consisting of the amino acid sequence of SEQ ID NO: 109 and a VL domain comprising or consisting of the amino acid sequence of SEQ ID NO: 110;

5 (ii) a VH domain comprising or consisting of the amino acid sequence of SEQ ID NO: 111 and a VL domain comprising or consisting of the amino acid sequence of SEQ ID NO: 112;

(iii) a VH domain comprising or consisting of the amino acid sequence of SEQ ID NO: 113 and a VL domain comprising or consisting of the amino acid sequence of SEQ ID NO: 114; and

10 (iv) a VH domain comprising or consisting of the amino acid sequence of SEQ ID NO: 115, and a VL domain comprising or consisting of the amino acid sequence of SEQ ID NO: 116.

59. A pharmaceutical composition, comprising an antibody or antigen
15 binding portion thereof according to any one of (1)-(14) or (19)-(58) and at least one pharmaceutically acceptable excipient or carrier.

60. An antibody or antigen binding portion thereof according to any one of (1)-(14) or (19)-(58), or pharmaceutical composition according to (59),
20 for use as a therapeutic, prognostic or diagnostic agent.

61. An antibody or antigen binding portion thereof according to any one of (1)-(14) or (19)-(58), or pharmaceutical composition according to (59),
for use as a therapeutic agent.

25

62. A method of preventing or treating a neurological disorder or a disorder characterized by a loss of cognitive memory capacity in a subject in need thereof, wherein the method comprises administering to the subject an antibody or antigen binding portion thereof according to any one of (1)-(14) or
30 (19)-(58), or pharmaceutical composition according to (59).

63. The method of (62), wherein the disorder is selected from Alzheimer's disease (AD), mild cognitive impairment (MCI), dementia with

Lewy body, Down's syndrome, hereditary cerebral hemorrhage with amyloidosis (Dutch type), cerebral amyloid angiopathy, Parkinson's disease, and cataract due to amyloid beta deposition.

5 64. The method of (63), wherein the disorder is Alzheimer's disease.

65. An antibody or antigen binding portion thereof according to any one of (1)-(14) or (19)-(58), or pharmaceutical composition according to (59) for use in the prevention or treatment of a neurological disorder or a disorder
10 characterized by a loss of cognitive memory capacity.

66. The antibody, antigen binding portion thereof or pharmaceutical composition for use according to (65), wherein the disorder is selected from Alzheimer's disease (AD), mild cognitive impairment (MCI), dementia with
15 Lewy body, Down's syndrome, hereditary cerebral hemorrhage with amyloidosis (Dutch type), cerebral amyloid angiopathy, Parkinson's disease, and cataract due to amyloid beta deposition.

67. The antibody, antigen binding portion thereof or pharmaceutical
20 composition for use according to (66), wherein the disorder is Alzheimer's disease.

68. An antibody or antigen binding portion thereof according to any one of (1)-(14) or (19)-(58), or pharmaceutical composition according to (59),
25 for use as a diagnostic agent.

69. A method of detecting or diagnosing a neurological disorder or a disorder characterized by a loss of cognitive memory capacity in a subject, the method comprising contacting a sample obtained from the subject with an
30 antibody or antigen binding portion thereof according to any one of (1)-(14) or (19)-(58).

70. The method of (69), wherein the disorder is selected from Alzheimer's disease (AD), mild cognitive impairment (MCI), dementia with Lewy body, Down's syndrome, hereditary cerebral hemorrhage with amyloidosis (Dutch type), cerebral amyloid angiopathy, Parkinson's disease,
5 and cataract due to amyloid beta deposition.

71. The method of (70), wherein the disorder is Alzheimer's disease.

72. An antibody or antigen binding portion thereof according to any
10 one of (1)-(14) or (19)-(58), or pharmaceutical composition according to (59) for use in the diagnosis of a neurological disorder or a disorder characterized by a loss of cognitive memory capacity.

73. The antibody, antigen binding portion thereof or pharmaceutical
15 composition for use according to (72), wherein the disorder is selected from Alzheimer's disease (AD), mild cognitive impairment (MCI), dementia with Lewy body, Down's syndrome, hereditary cerebral hemorrhage with amyloidosis (Dutch type), cerebral amyloid angiopathy, Parkinson's disease, and cataract due to amyloid beta deposition.

20

74. The antibody, antigen binding portion thereof or pharmaceutical composition for use according to (73), wherein the disorder is Alzheimer's disease.

25 Brief description of the figures

Figure 1 shows the results of Western blot analysis of human brain extracts as described in Example 1.

Figure 2 shows the results of Western blot analysis of human brain extract from AD brain of genotype APOE $\epsilon 4/\epsilon 4$ at sufficiently high resolution
30 to show individual low molecular weight ApoE fragments as described in Example 1.

Figure 3 is a diagram showing the ratio of 12 kDa ApoE fragment to full-length ApoE in AD (filled circles) and control (open squares), quantified as described in Example 1.

Figure 4 is a diagram showing the ratio of 12 kDa ApoE fragment to full-length ApoE in AD without (-E4; filled circles) or with (+E4; open squares) APOE E4 genotype, quantified as described in Example 1.

Figure 5 is a schematic overview of the workflow for the immunoprecipitation experiments described in Example 2.

Figure 6 shows the result of Western blot analysis of immunoprecipitated samples as described in Example 2.

Figure 7 shows the result of silver staining of immunoprecipitated samples as described in Example 2.

Figure 8 shows the result of LC-MS/MS analysis of tryptic digests of 12 kDa, 15 kDa and rhApoE4 gels as indicated, as described in Example 3.

Figure 9 shows the result of LysC cleavage site analysis of the ApoE sequence as described in Example 4.

Figure 10 shows the result of investigation by extracted-ion chromatograms (XIC) of theoretical ApoE cleavage sites as described in Example 5. Left side: Extracted ion chromatograms at theoretical values of three charge states of one of the possible peptides (200-233) with 5 ppm mass accuracy, with peaks observed at the same retention time for all three. Right side: The mass spectrum from each extracted peak.

Figure 11 shows the result of nanoLC-MS/MS with the shotgun proteomic method for detection of peptides around cleavage sites as described in Example 5. In replicate analyses of samples from the same donor (APOE $\epsilon 3/\epsilon 4$, A and B), peptides having an N terminus at L198, A199 or G200 and an intact C terminus of ApoE were detected.

Figure 12 is a diagram showing the MS intensity for peptides having an N terminus at L198, A199 or G200 in samples from APOE $\epsilon 4/\epsilon 4$, $\epsilon 2/\epsilon 3$ and $\epsilon 3/\epsilon 3$ carriers as indicated, as described in Example 6.

Figure 13 shows the mitochondrial damage induced by human ApoE4 and ApoE C-terminal fragments following the experiment described in Example 7, in (A) Neuro2A cells and (B) rat primary hippocampal neurons; as

well as (C) protein expression of human ApoE4 or ApoE C-terminal fragments as measured by Western blot analysis.

Figure 14 shows concentration-response curves for hybridoma antibodies generated using the G200 neo-epitope, as described in Example 10.

Figure 15 shows the binding of (A) hybridoma antibodies, generated using the G200 neo-epitope, to a brain extract from an Alzheimer's disease patient as described in Example 10, and (B) re-staining of the Western blot membrane with a polyclonal anti-ApoE antibody for staining of full-length ApoE.

Figure 16 shows concentration-response curves for recombinant antibodies against the G200 neo-epitope, as described in Example 12.

Figure 17 shows inhibition-response curves for recombinant antibodies against the G200 neo-epitope, as described in Example 12.

Figure 18 shows a sample of binding interactions for recombinant antibodies against the G200 neo-epitope, characterized via bio-layer interferometry as described in Example 12.

Figure 19 shows concentration-response curves for recombinant antibodies against the G200 neo-epitope, as described in Example 12.

Figure 20 shows inhibition-response curves for recombinant antibodies against the G200 neo-epitope, as described in Example 12.

Figure 21 shows a sample of binding interactions for recombinant antibodies against the G200 neo-epitope, characterized via surface plasmon resonance as described in Example 12.

Figure 22 shows the binding of recombinant antibodies against the G200 neo-epitope to a brain extract from an Alzheimer's disease patient either by (A) direct Western blot or by (C) IP/Western blot, as described in Example 12; (B) shows re-staining of the Western blot membrane with a polyclonal anti-ApoE antibody, demonstrating staining of full-length ApoE.

Figure 23 shows the binding of recombinant antibodies against the G200 neo-epitope to human brain by immunohistochemistry, as described in Example 12.

Figure 24 shows concentration-response curves for purified monoclonal antibodies against the L198 neo-epitope, as described in Example 16.

Figure 25 shows inhibition-response curves for purified monoclonal antibodies against the L198 neo-epitope, as described in Example 15.

Figure 26 shows a sample of binding interactions for purified monoclonal antibodies against the L198 neo-epitope, characterized via surface plasmon resonance as described in Example 15.

Figure 27 shows the binding of purified monoclonal antibodies against the L198 neo-epitope to a brain extract from an Alzheimer's disease patient either by (A) direct Western blot or by (C) IP/Western blot, as described in Example 15; (B) shows re-staining of the Western blot membrane with a polyclonal anti-ApoE antibody, demonstrating staining of full-length ApoE.

Figure 28 shows concentration-response curves for purified monoclonal antibodies against the A199 neo-epitope, as described in Example 18.

Figure 29 shows inhibition-response curves for purified monoclonal antibodies against the A199 neo-epitope, as described in Example 18.

Figure 30 shows a sample of binding interactions for purified monoclonal antibodies against the A199 neo-epitope, characterized via surface plasmon resonance as described in Example 18.

Figure 31 shows the binding of purified monoclonal antibodies against the A199 neo-epitope to a brain extract from an Alzheimer's disease patient by (A) direct Western blot, as described in Example 18; (B) shows re-staining of the Western blot membrane with a polyclonal anti-ApoE antibody, demonstrating staining of full-length ApoE.

Detailed Description

One object of the invention is to further elucidate the structure of ApoE fragments in the brains of AD patients.

Another object is to provide novel insights into the function that ApoE fragments have in the genesis of disease.

Another object of the invention is to enable therapeutic intervention through targeting of novel epitopes on such ApoE fragments.

Another object of the invention is to enable the diagnosis of AD and other neurodegenerative disorders via detection of ApoE fragments
5 implicated in disease formation and/or progression.

Another object of the invention is to provide antibodies, or antigen binding portions thereof, having a novel and useful binding specificity.

One or more of these and other objects that are apparent to the skilled person from reading the entire disclosure herein are met by the various
10 aspects disclosed.

Anti-ApoE antibodies and methods of production

Thus, in a first aspect, the disclosure provides an antibody or antigen binding portion that binds to a fragment of apolipoprotein E (ApoE), wherein
15 the fragment has

- an apparent molecular weight of 12 kDa as measured by SDS-PAGE, and

- an N-terminus corresponding to an amino acid in full-length apolipoprotein E which is selected from the group consisting of amino acids
20 L198, A199 and G200; and wherein the antibody or antigen binding portion thereof binds to an epitope comprising the N-terminus of the fragment. The full-length apolipoprotein E from which the fragment derives is typically human ApoE.

The invention is based on detailed insights into the structure of
25 putatively neurotoxic ApoE fragments that were isolated from brains of Alzheimer's disease patients, as detailed in Examples 1-7 which follow. The identification of the exact sequence of these fragments enables the generation of inventive antibodies, or antigen binding portions thereof, that are specific for the N-terminal neo-epitopes formed upon ApoE fragmentation.
30 Generation and characterization of exemplary such antibodies is detailed in Examples 8-18.

Without wishing to be bound by theory, it is contemplated that such novel antibodies, or antigen binding portions thereof, are useful in the

diagnosis, prognosis and/or treatment of neurodegenerative diseases such as Alzheimer's disease, through specific binding to the putatively neurotoxic ApoE fragments.

In certain embodiments, the antibodies and antigen binding portions thereof of the first aspect bind selectively to the ApoE fragments described herein. As used herein, the term "bind selectively" refers to the preferential binding of the antibody or antigen binding portion thereof to the ApoE fragment target. In certain embodiments, the antibodies and antigen binding portions thereof of the first aspect do not bind to full-length apolipoprotein E, particularly full-length human apolipoprotein E.

In one embodiment, the antibody or antigen binding portion thereof of the first aspect is capable of selective binding to an epitope comprising the N-terminus of a fragment of apolipoprotein E, which fragment has an N-terminus corresponding to amino acid G200 in full-length ApoE.

In another embodiment, the antibody or antigen binding portion thereof of the first aspect is capable of selective binding to an epitope comprising the N-terminus of a fragment of apolipoprotein E, which fragment has an N-terminus corresponding to amino acid A199 in full-length ApoE.

In another embodiment, the antibody or antigen binding portion thereof of the first aspect is capable of selective binding to an epitope comprising the N-terminus of a fragment of apolipoprotein E, which fragment has an N-terminus corresponding to amino acid L198 in full-length ApoE.

In another embodiment, the antibody or antigen binding portion thereof of the first aspect is capable of selective binding to an epitope comprising the N-terminus of a fragment of apolipoprotein E, which fragment is selected from

- i) the group consisting of SEQ ID NO:1-3; and
- ii) a sequence having at least 80 % identity to any one of SEQ ID NO:1-3.

In a more specific embodiment, i) in this definition is SEQ ID NO:1. In an alternative embodiment, i) in this definition is SEQ ID NO:2. In another embodiment, i) in this definition is SEQ ID NO:3.

As defined in this group of embodiments, the ApoE fragment with the epitope of interest may have a sequence that has at least 80 % sequence

identity to a sequence selected from SEQ ID NO:1-3. In one embodiment, said sequence may have at least 85 %, such as at least 90 %, such as at least 95 %, such as 100 %, identity to a sequence selected from SEQ ID NO:1-3. In one embodiment, such variation in the target sequence, to which
5 the antibody or antigen binding portion thereof binds, is subject to the condition that the fragment retains the first three, such as the first four, such as the first five, such as the first six, amino acids of the sequence selected from SEQ ID NO:1-3, starting from the N-terminal, so as to ensure that the N-terminal neo-epitope of the fragment is present.

10 In the embodiment wherein ii) has 100 % identity to i), the fragment of ApoE consists of an amino acid sequence selected from the group consisting of SEQ ID NO:1-3. In a more specific embodiment, the fragment consists of SEQ ID NO:1. In an alternative embodiment, the fragment consists of SEQ ID NO:2. In another alternative embodiment, the fragment consists of SEQ ID
15 NO:3.

The antibodies and antigen binding portions of the first aspect bind to neo-epitopes at the N-terminus of the ApoE fragments described herein. In one embodiment, the antibody or antigen binding portion thereof binds to an epitope comprising amino acid residues 200-205 in full-length apolipoprotein
20 E (GQPLQE). In one embodiment, the antibody or antigen binding portion thereof binds to an epitope comprising amino acid residues 199-204 in full-length apolipoprotein E (AGQPLQ). In one embodiment, the antibody or antigen binding portion thereof binds to an epitope comprising amino acid residues 199-205 in full-length apolipoprotein E (AGQPLQE). In one
25 embodiment, the antibody or antigen binding portion thereof binds to an epitope comprising amino acid residues 198-203 in full-length apolipoprotein E (LAGQPL). In one embodiment, the antibody or antigen binding portion thereof binds to an epitope comprising amino acid residues 198-204 in full-length apolipoprotein E (LAGQPLQ). In one embodiment, the antibody or
30 antigen binding portion thereof binds to an epitope comprising amino acid residues 198-205 in full-length apolipoprotein E (LAGQPLQE).

In a second aspect of the disclosure, there is provided a method of production of an antibody or an antigen binding portion thereof, comprising a

step of immunizing a suitable host mammal with an immunogen that comprises one of the identified neo-epitopes of the putatively neurotoxic ApoE fragments disclosed herein. As such, this method comprises immunizing the host with a peptide immunogen comprising an N-terminal
5 amino acid sequence selected from the group consisting of LAGQPL (SEQ ID NO:4), AGQPLQ (SEQ ID NO:5), GQPLQE (SEQ ID NO:6), LAGQPLQ (SEQ ID NO:7), AGQPLQE (SEQ ID NO:8) and LAGQPLQE (SEQ ID NO:9). The host mammal is preferably a non-human mammal.

In one embodiment of the second aspect, the peptide immunogen
10 comprises an N-terminal amino acid sequence which is GQPLQE (SEQ ID NO:6). In another embodiment, the N-terminal amino acid sequence of the peptide immunogen is selected from LAGQPL (SEQ ID NO:4), LAGQPLQ (SEQ ID NO:7) and LAGQPLQE (SEQ ID NO:9). In another embodiment, the N-terminal amino acid sequence is selected from AGQPLQ (SEQ ID NO:5)
15 and AGQPLQE (SEQ ID NO:8).

In addition to the step of immunizing a suitable host, the method of the second aspect of the disclosure may suitably comprise additional steps of a standard nature for the generation of antibodies from the immunized animals, such as plasma screening for reactive antibodies, isolation of spleen cells,
20 generation of hybridomas, and other measures known to the person of skill in the art of antibody generation.

In a third aspect of the disclosure, there is provided an antibody or antigen binding portion thereof, which is obtainable by a method according to the second aspect. Such an antibody or antigen binding portion thereof is
25 likely to exhibit the desired selectivity shown by the antibody, or antigen binding portion thereof, of the first aspect, and to be useful in the same contexts of diagnosis, prognosis and treatment of neurodegenerative disease.

In some embodiments of the antibody or antigen binding portion thereof according to the first and third aspects, the antibody or antigen
30 binding portion thereof is selected from the group consisting of full-length antibodies, Fab fragments, Fab' fragments, F(ab')₂ fragments, Fc fragments, Fv fragments, single chain Fv fragments, (scFv)₂ and domain antibodies. In one embodiment, said at least one antibody or antigen binding portion thereof

is selected from full-length antibodies, Fab fragments and scFv fragments. In one particular embodiment, the antibody is a full length antibody. In one embodiment, the antibody or antigen binding portion thereof is selected from the group consisting of monoclonal antibodies, human antibodies, humanized antibodies, and antigen binding portions thereof. In another embodiment, it is a monoclonal antibody or antigen binding portion thereof.

In a more specific such embodiment, the antibody or antigen binding portion thereof comprises at least three complementarity determining regions (CDRs).

As the skilled person will realize, embodiments of the antibody or antigen binding portion thereof of the first and third aspects of the disclosure may be characterized by specific amino acid sequences in the regions determining its binding capability, such as the CDRs of the variable light and variable heavy chains, or indeed the entire variable light and/or heavy chain domains or regions. Non-limiting examples of such specific amino acid sequences are provided herein for the specific antibodies generated as described in Examples 9-18. It is contemplated that the specific sequence information provided for the generated antibodies enables the skilled person to define combinations and variations of these sequences within the scope of the invention.

Thus, in one embodiment, the antibody or antigen binding portion thereof comprises the following three CDRs in any combination of CDR-H1 / CDR-H2 / CDR-H3, e.g. in a heavy chain variable region when present:

- CDR-H1 selected from the group consisting of SEQ ID NO:10, 15, 18 and 21;
- CDR-H2 selected from the group consisting of SEQ ID NO:11, 13, 16, 19 and 22; and
- CDR-H3 selected from the group consisting of SEQ ID NO:12, 14, 17, 20 and 23.

In another embodiment, the antibody or antigen binding portion thereof comprises the following three CDRs in any combination of CDR-L1 / CDR-L2 / CDR-L3, e.g. in a light chain variable region when present:

- CDR-L1 selected from the group consisting of SEQ ID NO:24, 27, 29, 31 and 32;
- CDR-L2 being SEQ ID NO:25; and
- CDR-L3 selected from the group consisting of SEQ ID NO:26, 28, 30 and 33.

In another embodiment, the antibody or antigen binding portion thereof comprises the six CDRs CDR-H1 / CDR-H2 / CDR-H3 / CDR-L1 / CDR-L2 / CDR-L3 selected from the sequences listed above, in any combination thereof.

- 10 In specific embodiments of said first moiety, the combinations of CDRs are those present in the antibodies exemplified in Examples 9-12 (see Table 4).

- 15 In another more specific embodiment of the antibody or an antigen binding portion thereof according to the first or third aspect of the disclosure, the antibody or antigen binding portion thereof comprises a heavy chain variable region (VH) sequence selected from the group consisting of SEQ ID NO:34, 36, 38, 40, 42 and 43, and sequences having at least 70 % identity thereto.

- 20 In another more specific embodiment of the antibody or an antigen binding portion thereof according to the first or third aspect of the disclosure, the antibody or antigen binding portion thereof comprises a light chain variable region (VL) sequence selected from the group consisting of SEQ ID NO:35, 37, 39, 41 and 44, and sequences having at least 70 % identity thereto.

- 25 In another embodiment, the antibody or antigen binding portion thereof comprises the following three CDRs in any combination of CDR-H1 / CDR-H2 / CDR-H3, e.g. in a heavy chain variable region when present:

- CDR-H1 selected from the group consisting of SEQ ID NO: 59, 62 and 65;
- 30 - CDR-H2 selected from the group consisting of SEQ ID NO: 60, 63, 66, 68 and 70; and
- CDR-H3 selected from the group consisting of SEQ ID NO: 61, 64, 67 and 69.

In another embodiment, the antibody or antigen binding portion thereof comprises the following three CDRs in any combination of CDR-L1 / CDR-L2 / CDR-L3, e.g. in a light chain variable region when present:

- CDR-L1 selected from the group consisting of SEQ ID NO: 71, 74,
5 76, 79 and 80;
- CDR-L2 selected from the group consisting of SEQ ID NO: 72 and 77; and
- CDR-L3 selected from the group consisting of SEQ ID NO: 73, 75, 78 and 81.

10 In another embodiment, the antibody or antigen binding portion thereof comprises the six CDRs CDR-H1 / CDR-H2 / CDR-H3 / CDR-L1 / CDR-L2 / CDR-L3 selected from the sequences listed above, in any combination thereof.

In specific embodiments of said first moiety, the combinations of CDRs
15 are those present in the antibodies exemplified in Examples 13-15 (see Table 8).

In another more specific embodiment of the antibody or an antigen binding portion thereof according to the first or third aspect of the disclosure, the antibody or antigen binding portion thereof comprises a heavy chain
20 variable region (VH) sequence selected from the group consisting of SEQ ID NOs: 82, 84, 86, 88 and 90, and sequences having at least 70 % identity thereto.

In another more specific embodiment of the antibody or an antigen binding portion thereof according to the first or third aspect of the disclosure,
25 the antibody or antigen binding portion thereof comprises a light chain variable region (VL) sequence selected from the group consisting of SEQ ID NOs: 83, 85, 87, 89 and 91, and sequences having at least 70 % identity thereto.

In another embodiment, the antibody or antigen binding portion thereof
30 comprises the following three CDRs in any combination of CDR-H1 / CDR-H2 / CDR-H3, e.g. in a heavy chain variable region when present:

- CDR-H1 selected from the group consisting of SEQ ID NO: 62, 94 and 97;

- CDR-H2 selected from the group consisting of SEQ ID NO: 92, 95 and 98; and

- CDR-H3 selected from the group consisting of SEQ ID NO: 93, 96 and 99.

5 In another embodiment, the antibody or antigen binding portion thereof comprises the following three CDRs in any combination of CDR-L1 / CDR-L2 / CDR-L3, e.g. in a light chain variable region when present:

- CDR-L1 selected from the group consisting of SEQ ID NO: 100, 103, 105 and 108;

10 - CDR-L2 selected from the group consisting of SEQ ID NO: 25, 101, 104 and 106; and

- CDR-L3 selected from the group consisting of SEQ ID NO: 28, 102 and 107.

In another embodiment, the antibody or antigen binding portion thereof
15 comprises the six CDRs CDR-H1 / CDR-H2 / CDR-H3 / CDR-L1 / CDR-L2 / CDR-L3 selected from the sequences listed above, in any combination thereof.

In specific embodiments of said first moiety, the combinations of CDRs are those present in the antibodies exemplified in Examples 16-18 (see Table
20 13).

In another more specific embodiment of the antibody or an antigen binding portion thereof according to the first or third aspect of the disclosure, the antibody or antigen binding portion thereof comprises a heavy chain variable region (VH) sequence selected from the group consisting of SEQ ID
25 NOs: 109, 111, 113 and 115, and sequences having at least 70 % identity thereto.

In another more specific embodiment of the antibody or an antigen binding portion thereof according to the first or third aspect of the disclosure, the antibody or antigen binding portion thereof comprises a light chain
30 variable region (VL) sequence selected from the group consisting of SEQ ID NOs: 110, 112, 114 and 116, and sequences having at least 70 % identity thereto.

In one embodiment, the definitions of VH and VL sequences of the antibody or antigen binding portion thereof is limited to any one of the listed sequences and sequences having at least 75 %, such as at least 80 %, such as at least 85 %, such as at least 90 %, such as at least 95 %, such as at least 98 %, such as at least 100 % identity thereto.

In specific embodiments, the combinations of VH/VL are those present in the antibodies exemplified in Examples 9-18 (see Tables 3, 7 and 12 in particular).

For embodiments wherein the variable domains of the antibodies or antigen binding fragments are defined by a particular percentage sequence identity to a reference sequence, the VH and/or VL domains may retain identical CDR sequences to those present in the reference sequence such that the variation is present only within the framework regions.

As used herein, the terms "selective binding to X" and "affinity for X", wherein X is an antigen or an epitope, refer to a property of an antibody or antigen binding portion thereof which may be tested for example by ELISA, by surface plasmon resonance (SPR), by Kinetic Exclusion Assay (KinExA®) or by bio-layer interferometry (BLI). The skilled person is aware of these methods and others.

For example, binding affinity for antigen or epitope X may be tested in an experiment in which an antibody or antigen binding portion thereof to be tested is captured on ELISA plates coated with antigen X or an antigen exhibiting the epitope X, and a biotinylated detector antibody is added, followed by streptavidin-conjugated HRP. Alternatively, said detector antibody may be directly conjugated with HRP. TMB substrate is added and the absorbance at 450 nm is measured using an ELISA multi-well plate reader. The skilled person may then interpret the results obtained by such experiments to establish at least a qualitative measure of the binding affinity of the antibody or antigen binding portion thereof for X. If a quantitative measure is desired, for example to determine the EC50 value (the half maximal effective concentration) for the interaction, ELISA may also be used. The response of the antibody or antigen binding portion thereof against a dilution series of X may be measured using ELISA as described above. The

skilled person may then interpret the results obtained by such experiments and EC50 values may be calculated from the results, using for example GraphPad Prism 8 and non-linear regression.

As used herein, the term “EC50” refers to the half maximal effective
5 concentration of an antibody or antigen binding portion thereof which induces a response halfway between the baseline and maximum after a specified exposure time.

Additionally, inhibition ELISA may be used to obtain a quantitative measure of interaction by determination of the “IC50” (the half maximal
10 inhibitory concentration). In an inhibition ELISA, the concentration of an antigen or epitope X in a fluid sample is measured by detecting interference in an expected signal output. In principle, a known antigen or epitope-bearing substance is used to coat a multiwell plate. In parallel, an antibody or antigen binding portion thereof with putative affinity for the antigen or epitope is added
15 and incubated with a solution containing antigen at varied concentrations. Following standard blocking and washing steps, samples containing the mixture of said antibody or antigen binding portion thereof and the antigen or epitope are added to the well. Labeled detection antibody with affinity for the antigen- or epitope-binding antibody or antigen binding portion thereof is then
20 applied for detection using relevant substrates (for example TMB). In principle, if there is a high concentration of antigen or epitope in the fluid sample, a significant reduction in signal output will be observed. In contrast, if there is very little antigen or epitope in the fluid sample, there will be very little reduction in the expected signal output. The skilled person appreciates that
25 the signal output is also dependent on the affinity of the antibody or antigen binding portion thereof for said antigen or epitope.

As used herein, the term “IC50” refers to the half maximal inhibitory concentration of an antibody or antigen binding portion thereof which induces a response halfway between the baseline and maximum inhibition after a
30 specified exposure time. Herein, a lower IC50 value indicates that a lower concentration of antigen or epitope is required to interfere with the binding of the detection antibody to the known antigen or epitope coated on the plate, as

compared to a higher IC₅₀ value. Thus, a lower IC₅₀ value typically corresponds to a higher affinity.

The binding affinity of an antibody or antigen binding portion thereof may also be tested by surface plasmon resonance (SPR). For example, said
5 binding affinity may be tested in an experiment in which antigen or epitope X is immobilized on a sensor chip of the instrument, and the sample containing the antibody or antigen binding portion thereof to be tested is passed over the chip. Alternatively, the antibody or antigen binding portion thereof to be tested may be immobilized on a sensor chip of the instrument, and a sample
10 containing X is passed over the chip. The skilled person may then interpret the results obtained by such experiments to establish at least a qualitative measure of the binding affinity of the moiety for X. If a quantitative measure is desired, for example to determine a K_D value for the interaction, SPR may also be used. Binding values may for example be defined in a Biacore (GE
15 Healthcare) or ProteOn XPR 36 (Bio-Rad) instrument. The antigen or epitope is suitably immobilized on a sensor chip of the instrument, and samples of the antibody or antigen binding portion thereof whose affinity is to be determined are prepared by serial dilution and injected. K_D values may then be calculated from the results using for example the 1:1 Langmuir binding model of the
20 Biacore Insight Evaluation Software 2.0 or other suitable software, typically provided by the instrument manufacturer.

Another method for determining binding affinity of an antibody or antigen binding portion thereof to antigen or epitope X is the Kinetic Exclusion Assay (KinExA; Sapidyne Instruments Inc; Darling and Brault, Assay and
25 Drug Dev Tech (2004) 2(6):647-657) for measurements of the equilibrium binding affinity and kinetics between unmodified molecules in solution. A KinExA K_D analysis requires immobilization of one interaction partner (e.g. the titrated binding partner) to a solid phase, which is then used as a probe to capture the other interaction partner (e.g. the constant binding partner) free in
30 solution once an equilibrium is reached.

The binding affinity may also be measured by bio-layer interferometry (BLI), a label-free technology for measuring biomolecular interactions within the interactome. It is an optical analytical technique that analyzes the

interference pattern of white light reflected from two surfaces: a layer of immobilized protein on the biosensor tip, and an internal reference layer. The binding between a ligand (antigen or epitope X) immobilized on the biosensor tip surface and an analyte (such as an antibody or antigen binding portion thereof with affinity for X) in solution produces an increase in optical thickness at the biosensor tip resulting in a wavelength shift, $\Delta\lambda$, which is a direct measure of the change in thickness of the biological layer. Interactions are measured in real time, providing the ability to monitor binding specificity, rates of association and dissociation, or concentration, with precision and accuracy.

10 The skilled person is aware of the above mentioned and other methods for measuring the affinity of an antibody or antigen binding portion thereof for antigen or epitope X, either qualitatively or quantitatively or both.

As used herein, the term "antibody or antigen binding portion thereof" encompasses not only full-length or intact polyclonal or monoclonal antibodies, but also antigen binding portions thereof, such as Fab, Fab', F(ab')₂, Fab₃, Fv and variants thereof, fusion proteins comprising one or more antibody portions, humanized antibodies, chimeric antibodies, minibodies, diabodies, triabodies, tetrabodies, linear antibodies, single chain antibodies, multispecific antibodies (e.g. bispecific antibodies) and any other modified configuration of the immunoglobulin molecule that comprises an antigen recognition site of the required specificity, including glycosylation variants of antibodies, amino acid sequence variants of antibodies and covalently modified antibodies. Further examples of modified antibodies and antigen binding portions thereof include nanobodies, AlbudAbs, DARTs (dual affinity re-targeting), BiTEs (bispecific T-cell engager), TandAbs (tandem diabodies), DAFs (dual acting Fab), two-in-one antibodies, SMIPs (small modular immunopharmaceuticals), FynomAbs (fynomers fused to antibodies), DVD-IgG (dual variable domain immunoglobulin), CovX-bodies (peptide modified antibodies), duobodies and triomAbs. This listing of variants of antibodies and antigen binding portions thereof is not to be seen as limiting, and the skilled person is aware of other suitable variants.

A full-length antibody comprises two heavy chains and two light chains. Each heavy chain contains a heavy chain variable region (VH) and first,

second and third constant regions (CH1, CH2 and CH3). Each light chain contains a light chain variable region (VL) and a light chain constant region (CL). Depending on the amino acid sequence of the constant domain of its heavy chains, antibodies are assigned to different classes. There are six
5 major classes of antibodies: IgA, IgD, IgE, IgG, IgM and IgY, and several of these may be further divided into subclasses, e.g., IgG1, IgG2, IgG3, IgG4, IgA1 and IgA2. The term "full-length antibody" as used herein, refers to an antibody of any class, such as IgD, IgE, IgG, IgA, IgM or IgY (or any sub-class thereof). The subunit structures and three-dimensional configurations of
10 different classes of antibodies are well known.

The term "antigen binding portion" refers to a portion or region of an antibody molecule, or a derivative thereof, that retains all or a significant part of the antigen binding of the corresponding full-length antibody. An antigen binding portion may comprise the heavy chain variable region (VH), the light
15 chain variable region (VL), or both. Each of the VH and VL regions or domains typically contains three complementarity determining regions CDR1, CDR2 and CDR3, denoted CDR-H1, CDR-H2 and CDR-H3 for the CDRs from the VH domain and CDR-L1, CDR-L2 and CDR-L3 for the CDRs from the VL domain. The three CDRs in VH or VL are flanked by framework
20 regions (FR1, FR2, FR3 and FR4). As briefly listed above, examples of antigen binding portions include, but are not limited to: (1) a Fab fragment, which is a monovalent fragment having a VL-CL chain and a VH-CH1 chain; (2) a Fab' fragment, which is a Fab fragment with the heavy chain hinge region, (3) a F(ab')₂ fragment, which is a dimer of Fab' fragments joined by
25 the heavy chain hinge region, for example linked by a disulfide bridge at the hinge region; (4) an Fc fragment; (5) an Fv fragment, which is the minimum antibody fragment having the VL and VH domains of a single arm of an antibody; (6) a single chain Fv (scFv) fragment, which is a single polypeptide chain in which the VH and VL domains of an scFv are linked by a peptide
30 linker; (7) an (scFv)₂, which comprises two VH domains and two VL domains, which are associated through the two VH domains via disulfide bridges and (8) a domain antibody, which may be an antibody single variable domain (VH or VL) polypeptide that specifically bind antigen. Antigen binding portions can

be prepared via routine methods. For example, F(ab')₂ fragments can be produced by pepsin digestion of a full-length antibody molecule, and Fab fragments can be generated by reducing the disulfide bridges of F(ab')₂ fragments. Alternatively, portions can be prepared via recombinant technology by expressing the heavy and light chain portions in suitable host cells (e.g., *E. coli*, yeast, mammalian, plant or insect cells) and having them assembled to form the desired antigen binding portions either *in vivo* or *in vitro*. A single-chain antibody can be prepared via recombinant technology by linking a nucleotide sequence coding for a heavy chain variable region and a nucleotide sequence coding for a light chain variable region. For example, a flexible linker may be incorporated between the two variable regions.

Furthermore, the skilled person is aware of the meaning of the terms polyclonal antibodies and monoclonal antibodies. Polyclonal antibodies are normally generated by administering an antigen to an animal. Said antigen will evoke an immune response giving rise to polyclonal antibodies. Monoclonal antibodies are made by immunizing an animal, usually a mouse, with an antigen and the subsequent isolation of the spleen from said animal. Isolated spleen cells are immortalized by fusion with myeloma cells to give rise to hybridoma cells. Each hybridoma cell produces a unique monoclonal antibody. The term "human antibody" as used herein, refers to antibodies having variable and constant regions corresponding to, or derived from, antibodies obtained from human subjects. The term "chimeric antibodies" as used herein, refers to recombinant or genetically engineered antibodies, such as for example antibodies with variable regions (VH and VL) of mouse origin and human constant region (Fc), to reduce the antibodies' immunogenicity. The term "humanized antibodies" refers to antibodies from non-human species whose protein sequences have been modified to increase their similarity to antibody variants produced naturally in humans, in order to reduce immunogenicity of the full antibody itself.

In yet another embodiment, the antibody or antigen binding portion thereof of the first and third aspects of the disclosure is selected from the group consisting of human antibodies, humanized antibodies and antigen binding portions thereof. In one particular embodiment, said antibody or

antigen binding portion thereof is a humanized antibody or an antigen binding portion thereof.

Pharmaceutical compositions

5 In a fourth aspect, there is provided a pharmaceutical composition comprising an antibody or antigen binding portion thereof as described herein and at least one pharmaceutically acceptable excipient or carrier.

Techniques for formulating antibodies for human therapeutic use are well known in the art and are reviewed, for example, in Wang et al., Journal of
10 Pharmaceutical Sciences, Vol.96, pp1-26, 2007, the contents of which are incorporated herein in their entirety.

Pharmaceutically acceptable excipients that may be used to formulate the compositions include, but are not limited to, ion exchangers, alumina, aluminum stearate, lecithin, serum proteins, such as human serum albumin,
15 buffer substances such as phosphates, glycine, sorbic acid, potassium sorbate, partial glyceride mixtures of saturated vegetable fatty acids, water, salts or electrolytes, such as protamine sulfate, disodium hydrogen phosphate, potassium hydrogen phosphate, sodium chloride, zinc salts, colloidal silica, magnesium trisilicate, polyvinyl pyrrolidone, cellulose-based
20 substances (for example sodium carboxymethylcellulose), polyethylene glycol, polyacrylates, waxes, polyethylene- polyoxypropylene- block polymers, polyethylene glycol and wool fat.

In certain embodiments, the pharmaceutical compositions are formulated for administration to a subject via any suitable route of
25 administration including but not limited to intramuscular, intravenous, intradermal, intraperitoneal injection, subcutaneous, epidural, nasal, oral, rectal, topical, inhalational, buccal (e.g., sublingual), and transdermal administration. In preferred embodiments, the composition is formulated for intravenous or subcutaneous administration.

30

Methods of prevention, treatment, detection and diagnosis

The antibodies or antigen binding portions thereof according to the present disclosure may be useful as therapeutic and/or diagnostic agents.

Hence, in a fifth aspect of the present disclosure, there is provided an antibody or antigen binding portion thereof according to the first or third aspect, or a pharmaceutical composition according to the fourth aspect, for use as a medicament.

- 5 In a sixth aspect of the disclosure, there is provided an antibody or antigen binding portion thereof according to the first or third aspect, or a pharmaceutical composition according to the fourth aspect, for use as a diagnostic agent.

10 Also provided are methods of preventing, treating or diagnosing disease or assessing disease prognosis, wherein an antibody or antigen binding portion thereof as disclosed herein is administered to a subject, typically a human subject.

15 Also provided is the use of the disclosed antibodies or antigen binding portions thereof for the manufacture of compositions (such as medicaments) for use in the prevention, treatment, diagnosis and/or prognosis of any one of the listed diseases.

20 Also provided are methods of detecting or diagnosing a disease in a subject, wherein the methods comprise contacting a sample obtained from the subject with an antibody or antigen binding portion thereof as described herein. These methods are typically *in vitro* methods.

 Thus, said antibody or antigen binding portion thereof, or pharmaceutical composition comprising it, is useful in the treatment, prevention and/or diagnosis of a condition selected from neurological disorders or conditions characterized by a loss of cognitive memory capacity.

25 Such diseases or conditions include but are not limited to Alzheimer's disease (AD), mild cognitive impairment (MCI), dementia with Lewy body, Down's syndrome, hereditary cerebral hemorrhage with amyloidosis (Dutch type); as well as other diseases which are based on or associated with amylogenic proteins, such as cerebral amyloid angiopathy, Parkinson's disease, and

30 cataract due to amyloid beta deposition.

 Thus, in one embodiment, there is provided an antibody or antigen binding portion thereof, or pharmaceutical composition comprising it, for use in the treatment, prevention and/or diagnosis of an A β peptide-associated

condition, such as amyloidosis. In one embodiment, there is provided an antibody or antigen binding portion thereof, or pharmaceutical composition comprising it, for use in the treatment, prevention and/or diagnosis of an A β peptide-associated condition, selected from the group consisting of

5 Alzheimer's disease (AD), mild cognitive impairment (MCI), dementia with Lewy bodies, Down's syndrome, hereditary cerebral hemorrhage with amyloidosis (Dutch type), cerebral amyloid angiopathy, Parkinson's disease and cataract due to amyloid beta deposition. In one specific embodiment, said antibody or antigen binding portion thereof, or pharmaceutical composition

10 comprising it, is provided for use in the treatment, prevention and/or diagnosis of Alzheimer's disease.

In a seventh aspect, there is provided a method of treatment, prevention and/or diagnosis of an A β peptide-associated condition in a mammal having, or being at risk of developing, said disorder, comprising

15 administering to said mammal an amount, such as a therapeutically effective amount, of an antibody or antigen binding portion thereof, or pharmaceutical composition comprising it.

In one embodiment, said A β peptide-associated condition is, for example selected from the group consisting of amyloidosis, which refers to a

20 group of diseases and disorders associated with amyloid plaque formation including secondary amyloidosis and age-related amyloidosis including, but not limited to, neurological disorders or conditions characterized by a loss of cognitive memory capacity such as, for example, Alzheimer's disease (AD), mild cognitive impairment (MCI), dementia with Lewy body, Down's

25 syndrome, hereditary cerebral hemorrhage with amyloidosis (Dutch type); as well as other diseases which are based on or associated with amylogenic proteins, such as cerebral amyloid angiopathy, Parkinson's disease, and cataract due to amyloid beta deposition.

In a more specific embodiment, there is provided a method for

30 treatment, prevention and/or diagnosis of an A β peptide-associated condition as disclosed herein, wherein said A β peptide-associated condition is selected from the group consisting of Alzheimer's disease (AD), mild cognitive impairment (MCI), dementia with Lewy bodies, Down's syndrome, hereditary

cerebral hemorrhage with amyloidosis (Dutch type), cerebral amyloid angiopathy, Parkinson's disease and cataract due to amyloid beta deposition. In one particular embodiment, there is provided a method for treatment, prevention and/or diagnosis as disclosed herein, wherein said A β peptide-associated condition is Alzheimer's disease.

With regard to therapeutic or preventive use of the disclosed antibody, or antigen binding portion thereof, for the treatment of neurodegenerative diseases, there are several putative mechanisms of action. Without wishing to be bound by theory, non-limiting and independently possible mechanisms of action are for example i) the neutralization of putatively neurotoxic ApoE fragments and aggregated forms thereof; ii) Restoration of A β metabolism by removal of C-terminal ApoE fragments which have disrupted normal cholesterol/lipid transportation and thereby affected the processing of amyloid precursor protein (APP) so as to cause an increased production of A β ; iii) reduction of A β aggregation caused by C-terminal ApoE fragments through binding of A β via the lipid binding region contained in these fragments; and iv) increased A β clearance in a situation wherein the C-terminal ApoE fragments form part of amyloid aggregates as such.

With regard to diagnostic or prognostic use of the disclosed antibody, or antigen binding portion thereof, in neurodegenerative diseases, the putatively neurotoxic ApoE fragments can be detected and measured in patients at risk of disease or showing signs of incipient disease. One such method is PET scan using a radio-labelled antibody of the disclosure. Another method for diagnosis and prognosis is biochemical analysis analyzing the levels of neurotoxic ApoE fragments in blood/plasma using ELISA/MSD.

While the invention has been described with reference to various exemplary aspects and embodiments, it will be understood by those skilled in the art that various changes may be made and equivalents may be substituted for elements thereof without departing from the scope of the invention. In addition, many modifications may be made to adapt a particular situation or molecule to the teachings of the invention without departing from the essential scope thereof. Therefore, it is intended that the invention not be limited to any particular embodiment, but that the invention will include all

embodiments falling within the scope of the appended claims. The invention will be further illustrated by the following non-limiting Examples.

5 Incorporation by Reference

Various publications are cited in the present application, each of which is incorporated by reference herein in its entirety.

10 Examples

Example 1

Analysis of ApoE fragments in human brain extracts from Alzheimer's disease patients and controls

This example describes the homogenization of human brain tissues
15 and the following Western blot analysis of ApoE fragments from brain extracts in Radio-Immunoprecipitation Assay (RIPA) buffer with 2% sodium dodecyl sulfate (SDS).

Materials and methods

20 Brain tissue homogenization and sample preparation: Fresh frozen human brain tissue from Alzheimer's disease (AD) patients (n=24) and controls (n=14), with various APOE genotypes, were homogenized by 1:5 weight:volume in RIPA 2% SDS extraction buffer followed by a 16000 x g centrifugation for 1 h. The resulting supernatant was frozen at -80°C until
25 analysis.

Analysis of ApoE fragments in human brain extracts: RIPA 2% SDS brain extract containing 10 µg total protein was mixed with 2x Laemmli sample buffer, boiled for 5 min at 95°C and loaded onto SDS-PAGE gels (Bolt™ 12% Bis-Tris Plus 10 well, Thermo Fisher). Gels were run for 30-40
30 min at 180 V, after which proteins were transferred from the gels to nitrocellulose membranes using the Trans-Blot® Turbo™ system (BioRad). Membranes were blocked in Odyssey® blocking buffer for 1 h and then incubated over night at room temperature with a polyclonal anti-ApoE antibody (Calbiochem, cat. No. 178479) diluted 1:2000 in Odyssey® blocking
35 buffer with 0.1% Tween® 20. Membranes were washed and incubated for 1 h

at room temperature with detection antibody anti-goat-800CW (LI-COR, cat. No 925-32214) diluted 1:25000 in Odyssey® blocking buffer with 0.1% Tween® 20. Membranes were washed and images acquired using Odyssey® FC (LI-COR). Image Studio Software (version 5.2) was used to quantify the
5 relative amount of ApoE fragments in ratio to the amount of full-length ApoE in the acquired Western blot images.

Results

Full-length ApoE as well as several low molecular weight (LMW) ApoE
10 fragments were identified by Western blot analysis of human brain RIPA 2% SDS extracts (n=38). Figure 1 shows a representative membrane from Western blot analysis. The LMW ApoE fragments were estimated to be 10, 12, 14-15 and 17 kDa in size (Figure 2).

Analysis of ApoE fragments in ratio to full-length (FL) ApoE,
15 demonstrated that the 12 kDa ApoE fragment was significantly increased in the AD group (n=24) as compared to the control group (n=14) (Figure 3). In addition, a significant increase of the 12 kDa ApoE fragment was observed in APOE ε4 carriers in the AD group (Figure 4).

20

Example 2

Extraction and isolation of ApoE fragments from human brain extracts from Alzheimer's disease patients

This example describes a procedure for isolation and concentration of
25 full-length ApoE and 12 and 15 kDa ApoE fragments from human brain extracts, in order to prepare pure samples of ApoE with a protein concentration sufficient for amino acid sequence analysis.

Materials and methods

30 Isolation of ApoE from human brain extracts from AD patients with various APOE genotypes: A protocol for immunoprecipitation (IP) of ApoE from human brain extracts was established. Protocol optimization resulted in pure samples of ApoE with a protein concentration sufficient for amino acid

sequence analysis. For a schematic overview of the workflow, see Figure 5. Human brain RIPA 2% SDS extracts, with a total protein content of 1.5 mg, were mixed with IP buffer (1xPBS, 0.05% Tween® 20, 0.1% Triton X-100, protease inhibitor cocktail) and ApoE was immunoprecipitated by adding 200
5 µg of an anti-ApoE C-terminal antibody, with a binding epitope within amino acids 237-299 (Thermo Scientific, cat. No PA5-27088). Complexes between IP antibody and ApoE in the brain extract were allowed to form during an incubation for 2 h at room temperature with head-over-tail rotation. 500 µl Protein A Dynabeads (Dyna, Thermo Scientific, cat. No 10002D) were added
10 to the IP mixture and incubated for 1 h at room temperature with head-over-tail rotation, after which the Protein A Dynabeads were washed to remove unspecific binding to the beads. ApoE proteins bound to the Protein A Dynabeads (via the IP antibody) were eluted in 250 µl elution buffer (1.25 mM Tris pH 6.8, 0.005% SDS) and incubated for 5 min at 95°C with shaking at
15 900 rpm. After a quick spin, the samples were placed on the DynaMag™-2 magnet and the liquid was transferred to a new tube.

Concentration of isolated ApoE followed by analysis by SDS-PAGE: In order to concentrate the ApoE protein, the eluted IP sample was centrifuged in a rotational vacuum concentrator at 1300 rpm at 40°C for approximately
20 2 h, to reduce the volume from 250 µl to approximately 15 µl. 2x Laemmli buffer was added to the concentrated samples and the samples were incubated for 5 min at 95°C with 900 rpm. After a quick spin, the samples were loaded onto SDS-PAGE gels (Bolt™ 12% Bis-Tris Plus 10 well, Thermo Fisher, cat. No NW04120BOX). Gels were run for 30-40 min at 180 V, after
25 which one gel was used for confirmation of ApoE fragments by Western blot analysis and one gel was silver stained and used for excision of ApoE.

Western blot analysis of SDS-PAGE gels: Proteins were transferred from the gels to nitrocellulose membranes using the Trans-Blot® Turbo™ system (BioRad). Membranes were blocked in Odyssey® blocking buffer for
30 1 h and then incubated over night at room temperature with the anti-ApoE C-terminal antibody (Thermo Scientific, cat. No PA5-27088) diluted 1:2000 in Odyssey® blocking buffer with 0.1% Tween® 20. Membranes were washed and incubated for 1 h at room temperature with detection antibody anti-rabbit-

800CW (LI-COR, cat. No 925-32211) diluted 1:25000 in Odyssey® blocking buffer with 0.1% Tween® 20. Membranes were washed and images acquired using Odyssey® FC (LI-COR).

5 Silver staining of SDS-PAGE gels: Gels were fixated and stained with silver staining according to manufacturer's instructions (Pierce Silver Stain for Mass Spectrometry, Thermo Scientific, cat. No 24600). After the silver staining was complete, the stop buffer was exchanged to Milli-Q H₂O and rinsed 2x 10 min. Full-length ApoE, and the 12 and 15 kDa ApoE bands were excised from the gel and placed in Milli-Q H₂O in clean Eppendorf tubes.

10

Results

Using the established IP protocol (Figure 5), ApoE was isolated from human AD brains with various APOE genotypes ($\epsilon 2/\epsilon 3$, $\epsilon 3/\epsilon 3$, $\epsilon 3/\epsilon 4$ and $\epsilon 4/\epsilon 4$), and the eluted proteins were run on SDS-PAGE.

15 Extraction of ApoE was confirmed by Western blot analysis. Figure 6 shows a representative Western blot membrane demonstrating several bands with ApoE fragments, as well as full-length ApoE. In addition, isolated and concentrated ApoE proteins were stained by silver staining of the SDS-PAGE gels as shown in Figure 7. ApoE fragments of approximately 12 and 15 kDa
20 in size were visualized and excised from the silver stained gels. As reference samples, recombinant full-length ApoE protein and full-length ApoE from the human brain IP sample were also excised from the silver stained gels.

25

Example 3

Identification of trypsin cleavage sites in 12 kDa ApoE fragment

Sample preparation

30 Silver-stained strips of gels from Example 2 in 1.5 ml PP-tubes, including a band of recombinant human full-length ApoE4 (rhApoE4) and/or 34 kDa from immunoprecipitation, band of 15 kDa from immunoprecipitation, and band of 12 kDa from immunoprecipitation, were washed with enough water and followed by dehydration using 500 μ l acetonitrile (ACN; Wako).

After turning each gel white, any solvent was removed and followed by adding 500 µl of water to get each gel swelling. After removal of water, 500 µl of Silver Quest Destainer (Invitrogen) was added to each gel and incubated for 15 min at room temperature. After removal of any destainer solvent, 1000 µl
5 of water was added, then incubated for 10 min at room temperature. After the removal of water, 1000 µl of water was added again to wash each gel, then any solvent was removed from tubes. 500 µl ACN was added to each gel, then excess ACN was removed after turning each gel white.

500 µl of 10 mM dithiothreitol (DTT; Wako) was added into gels,
10 followed by incubation at 56°C for 30 min. After removal of DTT solution, 500 µl ACN was added to shrink each gel with gentle mixing incubation at room temperature for 10 min. After removal of ACN, 55 mM iodoacetoamide (IAA; Wako) was added into each tube, then incubated at room temperature in the dark for 30 min. After removal of IAA solution, 500 µl ACN was added
15 into each tube again, with occasional vortex mixing for 10 min, in order to obtain shrunk gels. After removal of ACN, 300 µl of 13 µg/ml trypsin in 10 mM ammonium bicarbonate with 10% ACN was added into the gels, then incubated at 5°C for 6 hours. Then, gels were placed in a 37°C chamber to promote digestion of proteins in each gel, followed by incubation over night.

20 600 µl of 5% formic acid in water / ACN in a 1/2 (v/v) solution was added to each tube and mixed well with vortex. Then, incubation at 37°C with gentle rotating was conducted to obtain a solution including tryptic peptides from each gel. The obtained solution was dried by SpeedVac system (Thermo Fisher Scientific), followed by reconstitution using 300 µl of 5% methanol in
25 0.1% TFA-water. The solution was desalted by Monospin C18 solid extraction column (GL Sciences) according to the vendor's instruction manual, after which the eluent was dried by SpeedVac system. 30 µl of 5% methanol in 0.1% TFA-water was added into each tube to obtain the final reconstituted solution. The solution was subjected to LC-MS analysis.

30

LC/MS analysis

The obtained samples were analyzed in a nano-flow LC-MS/MS system using a Q Exactive HF mass spectrometer (Thermo Fisher Scientific)

coupled with an online UltiMate 3000 Rapid Separation LC (Dionex) and an HTC PAL sample injector (CTC Analytics) fitted with a microcapillary column (360 nm outer diameter (OD) × 100 µm ID), which was packed with < 20 cm of ReproSil C18-AQ 3 µm beads (Dr. Maisch GmbH) and equipped with an
5 integrated electrospray emitter tip (P-2000 laser-based puller, Sutter Instruments). Each sample was loaded onto the capillary column by 4 µl full-loop mode injection. For LC separation, a mobile phase A of 4% ACN and 0.5% acetic acid (Wako) and a mobile phase B of 80% acetonitrile and 0.5% acetic acid were used for multiple linear gradient elution from 1-40% of B over
10 60 min, 40-70% of B over 10 min, and 70-99% of B over 5 min, and then held at 99% of B for 10 min at 500 nl/min. The total analysis time for each sample was 120 min.

Each sample was analyzed using data dependent analysis (DDA) mode, which used higher energy collision dissociation (HCD) MS/MS scans
15 (resolution 30000) for the top 15 most abundant ions of each full-scan MS from m/z 300 to 3000 (resolution 60000) with a full-scan MS ion target of 3×10^6 ions and an MS/MS ion target of 2×10^5 ions. The maximum ion injection time for the MS/MS scans was 100 ms. The HCD normalized collision energy was set to 27, the dynamic exclusion time was set to 20 s,
20 and the peptide match and isotope exclusion functions were enabled.

Data analysis

All DDA mass spectra were analyzed with Proteome Discoverer ver. 2.1 (Thermo Fisher Scientific) using a human ApoE4 FASTA file (SEQ ID
25 NO:45). SEQUEST-HT algorithm was used for MS/MS searching of the data sets with the following parameters: oxidation of methionine as variable modifications, carbamidomethylation of cysteine as a fixed modification, and trypsin as the digestion enzyme. Two missed cleavages per peptide were allowed. The mass tolerance for precursor ions was set to 10 ppm, and the
30 mass tolerance for product ions was set to 20 mDa. A maximum false discovery rate (FDR) of 1% was applied for peptide identification. Protein identification required more than two peptides per protein. Then, a detailed

analysis focusing only on ApoE4 was conducted to identify the cleavage sites of the 12 kDa band (ApoE4 fragment).

Results

5 The 12 kDa ApoE fragment was subjected to tryptic digestion to survey the cleavage sites of ApoE on a peptide basis. rhApoE4 and 15 kDa bands were analyzed as references. The results (Figure 8) showed there was an “abundance cliff” in the tryptic peptides from the 12 kDa band between a peptide corresponding to amino acid residues 192-206 of ApoE and a peptide
10 corresponding to amino acid residues 207-213. This means that there is at least one cleavage site in the region from amino acid residue 190 to amino acid residue 206, because the “207-213 peptide” was clearly detected with high MS intensity. Short peptides (less than 5 residues of amino acids) were eliminated from the analysis, so e.g. the VR dipeptide at positions 190-191
15 was not observed.

Example 4

Identification of LysC cleavage sites in 12 kDa ApoE fragment

20

Materials and methods

Sample preparation, LC/MS analysis and data analysis were performed as described above for Example 3.

Results

25 To narrow down the cleavage site of 12 kDa ApoE fragment on an amino acid basis, digestion by another enzyme, lysyl endopeptidase (LysC), was carried out. As a result of standard LysC proteomic analysis of the 12 kDa band (fixed cleavage at lysine C-terminal), the only peptide detected was
30 a peptide corresponding to amino acid residues 234-299 of ApoE (Figure 9). This confirms the result of Example 3, to the effect that there is at least one cleavage site between positions 190-206. Notably, a peptide corresponding to amino acid residues 158-233 of ApoE was detected upon cleavage of

rhApoE4 (not shown), but was not detected when cleaving the 12 kDa band, further supporting the existence of at least one cleavage site between positions 190-206.

5

Example 5

Further characterization of LysC cleavage sites in 12 kDa ApoE fragment

Materials and methods

Sample preparation and LC/MS analysis were performed as described
10 above for Example 4. Data analysis was performed as described above for
Example 4, except that target analysis (describing peaks and the integration)
from extracted-ion chromatograms (XIC) was performed for the specific
peptides cleaved at unexpected regions. This peak qualification analysis was
conducted by Qual Browser in Xcalibur 4.0 software (Thermo Fisher
15 Scientific).

Results

Prior to the detailed analysis of possible cleavage sites that give rise to
the identified 12 kDa fragment, it was investigated whether the peptide
20 corresponding to amino acid residues 158-233 of ApoE (SEQ ID NO:46)
obtained by LysC digestion was detected in any of the rhApoE4 band, the 34
kDa band from immunoprecipitation, and the 12 kDa band from
immunoprecipitation. This was done by describing each XIC with the
theoretical m/z ($z=10-15$, 5 ppm mass tolerance). The results showed that the
25 158-233 peptide was clearly detected in the solution from rhApoE4 and the 34
kDa band, which means that there is no artifact cleavage in the sample
preparation step. On the other hand, the 158-233 peptide was not observed in
the sample solution from the 12 kDa band. That indicated that there is at least
one cleavage site between aa 158 and aa 233 in the 12 kDa ApoE4 fragment.
30 In summary, the LC/MS results from the tryptic process described in Example
3 elucidated the preliminary cleavage site between positions 190-205, then
the site was confirmed by the LysC process as described in Example 4 and
above. To narrow down the possible cleavage sites between 190-205 on an

amino acid basis, all theoretical “non-conventional” peptides provided by LysC digestion of the 12 kDa band (i.e. 190-233, 191-233, 192-233, 193-233, 194-233, 195-233, 196-233, 197-233, 198-233, 199-233, 200-233, 201-233, 202-233, 203-233, 204-233, 205-233, and 206-233) were searched by

5 describing each XIC to check whether the fragment peak was detected or not. Figure 10 shows an example of the results, when looking for “non-conventional LysC peptide” corresponding to amino acid residues 200-233 of ApoE (SEQ ID NO:47; [M]= 4054.04490). The theoretical monoisotopic m/z values (charges 6, 7 and 8) for the 200-233 peptide are 676.68143,

10 580.15655 and 507.76289, respectively. The extracted chromatogram for each m/z value provides a single peak at the same retention time, and the observed masses agree with the theoretical in each case with a mass accuracy of less than 2 ppm. These results strongly reinforced that non-conventional LysC peptides had been identified, leading to a positive

15 identification of the specific cleavage sites that yield the 12 kDa ApoE fragment (Figure 11A). A duplicate experiment on another sample (ApoE $\epsilon 3/\epsilon 4$ allele) showed reproducible results (Figure 11B), confirming the determination of the cleavage sites.

In conclusion, nanoLC-MS/MS analysis of brain samples from three

20 individual donors (ApoE $\epsilon 3/\epsilon 4$) demonstrated that the major cleavage sites that yield the 12 kDa ApoE fragment were at the N-terminus of L198, A199 and G200 (Figure 11).

25

Example 6

Identification of cleavage sites in 12 kDa ApoE fragment in human brains with $\epsilon 4/\epsilon 4$, $\epsilon 2/\epsilon 3$ and $\epsilon 3/\epsilon 3$ alleles

Materials and methods

30

Sample preparation, LC/MS analysis and data analysis were performed as described above for Examples 3-5.

Results

The N-termini L198, A199 and G200 were identified as the main cleavage sites to yield the 12 kDa ApoE fragment from ApoE $\epsilon 3/\epsilon 4$. To clarify if these cleavage sites are specific only to the $\epsilon 4$ allele and not to the $\epsilon 2$ or $\epsilon 3$ alleles, 12 kDa bands from the brains of ApoE $\epsilon 4/\epsilon 4$, $\epsilon 2/\epsilon 3$ and $\epsilon 3/\epsilon 3$ carriers were analyzed by means of the same manner as the previous section.

The results are presented in Figure 12 and showed that $\epsilon 4/\epsilon 4$ carriers exhibited the expected cleavages at the N-terminus of L198, A199 and G200 (mainly A199 and G200), whereas $\epsilon 2/\epsilon 3$ and $\epsilon 3/\epsilon 3$ carriers showed considerably lower signal of the sites cleavages than $\epsilon 4/\epsilon 4$ carriers. The results indicated that cleavage at the N-terminus of L198, A199 and G200 is more abundant in $\epsilon 3/\epsilon 4$ and $\epsilon 4/\epsilon 4$ allele carriers.

15

Example 7

Neuronal toxicity of identified ApoE fragments

Materials and methods

Cell culture: Neuro2A cells (ATCC) were seeded at 5.0×10^4 cells/well in a 24 well plate (Falcon) and cultured in D-MEM High Glucose (WAKO) containing 10% fetal bovine serum. Transfection of pAAV-CMV vectors encoding human ApoE4 (full-length, SEQ ID NO:45) or the identified ApoE fragments (aa 198-299, SEQ ID NO:3; aa 199-299, SEQ ID NO:2; aa 200-299, SEQ ID NO:1) was done using Lipofectamine LTX and Plus Reagent (Invitrogen) on 1 day after seeding. 2 days later, vector-transfected cells were collected for Western blot analysis or seeded again at 2.0×10^4 cells/well in a Seahorse XF96 cell culture microplate (Agilent Technologies) 4 hours before mitochondrial respiration measurement.

For assays using rat hippocampal neurons, the dissected hippocampi from fetuses obtained on embryonic day (E) 18 from timed pregnant Wistar rats (Charles River Laboratories) were digested using trypsinization and mechanical dissociation. The dissociated neurons were seeded at 1.5×10^4 cells/well in Seahorse XF96 cell culture microplate (Agilent Technologies) for

mitochondrial respiration measurement or 1.0×10^5 cells/well in 24-well plate (Falcon) for Western blot analysis. Infection of AAV6 with full-length human ApoE4 or identified ApoE fragments (198-299, 199-299, 200-299) was performed at 7 days *in vitro* (DIV). Measurement of mitochondrial respiration or sample collection for Western blot analysis was performed at 7 days after infection (14 DIV).

Western blot analysis: Cells were lysed by RIPA buffer (50 mM Tris-HCl pH 7.6, 5 mM EDTA, 1 mM EGTA, 1% NP40, 0.25% sodium deoxycholate, 0.1 M NaCl, 0.5 mM PMSF) containing complete (EDTA-free) protease inhibitor cocktail (Roche) and PhosSTOP protein phosphatase inhibitor (Sigma), and sonicated. Sample Buffer Solution with Reducing Reagent (6x) (Nacalai Tesque) was added before SDS-PAGE. For SDS-PAGE, XV PANTERA MP Gel (DRC) 15% was used. For transfer, Trans-Blot Turbo (BIO-RAD) was used. For immunoblotting, iBind Western Systems (ThermoFisher Scientific) was used together with the following antibodies: anti-ApoE PA5-27088 (ThermoFisher Scientific); 178479 (Calbiochem).

Mitochondrial respiration measurement: Real-time measurement of oxygen consumption rates (OCR) was performed using an Extracellular Flux Analyzer XFe96 (Agilent Technologies). Before measurement, the culture medium was replaced by 37 °C pre-warmed XF Base Medium (Agilent Technologies) containing 10 mM sodium pyruvate (Sigma), 10 mM D-glucose (Sigma), 2 mM glutamine (Sigma). The pH of the measurement medium was adjusted to 7.4. The culture plates were incubated at 37 °C for 60 min prior to the assay. For analysis of mitochondrial function, XF Cell Mito Stress Test Kit (Agilent Technologies) was used. Following measurement of basal OCR, mitochondrial complex inhibitors were injected sequentially into each cell. The inhibitors were used at the following concentrations: oligomycin 1 μ M; carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP) 0.25 μ M for Neuro2A cells, 2 μ M for rat hippocampal neurons; rotenone/antimycin A 0.5 μ M. OCR values were automatically calculated, recorded and plotted by

the XFe96 software. Spare respiratory capacity was measured as (FCCP respiration – basal respiration).

Results

5 In both Neuro2A cells and rat primary hippocampal neurons, the groups expressing either one of the identified ApoE fragments (198-299, 199-299, 200-299) showed a reduction in spare respiratory capacity (Figure 13A and B), indicating that these fragments inflict mitochondrial damage. In addition, the fragments caused mitochondrial dysfunction at much lower
10 expression levels than did full-length ApoE4 (Figure 13A-C). The results show that the C-terminal fragments of ApoE identified from human brain are neurotoxic.

15

Example 8

Plasmid preparation and antigen production

This example describes plasmid preparation and production by transient transfection of Expi293 cells and subsequent purification, to produce
20 the antigens listed in Table 1 below.

Antigen (short)	Info/Tags	Purification	SEQ ID NO:
ApoE4-1-272-HIS	10xHIS-ApoE4(1-272)	IMAC, desalting	56
L198-HIS	ApoE-L198-H299-10xHIS	IMAC, desalting	57
A199-HIS	ApoE-A199-H299-10xHIS	IMAC, desalting	58
G200-HIS	ApoE-G200-H299-10xHIS	IMAC, desalting	48
ApoE4-FL-HIS	10xHIS-ApoE4(1-299)	IMAC, desalting	49

Table 1. Produced antigens

Plasmid preparation: pcDNA3.4-TOPO plasmids with sub-cloned
25 custom inserts of interest were ordered from GeneArt (Thermo Fisher Scientific). Plasmids were transformed into DH5α *E. coli* to produce enough material for cell transfections, unless preparative amounts of plasmid DNA were ordered alongside the cloning services.

Transformations and plasmid preparations were performed using established protocols. Briefly, 50 µl of freshly thawed competent DH5α° *E. coli* were transformed by adding 10-100 ng plasmid, incubated for 30 min on ice, followed by a 45 s heat shock at 42 °C and a recovery on ice for 5 min, after which 250 µl SOC medium (Invitrogen) were added and the bacteria were cultured for 1 h at 37 °C. 25-100 µl of culture were used for streaking out on ampicillin-containing agar plates, from which colonies were selected the following day after incubation at 37 °C. Clones were selected from colony re-streaks.

Preparative cultures of LB medium with ampicillin were inoculated with clones and expanded at 37 °C over night before pelleting the bacteria. Bacterial pellets were used to extract plasmid DNA using HiSpeed Maxi kits (Qiagen), and the supplier's instructions were followed throughout the procedure to obtain plasmid DNA.

Expression of antigen by transient transfection: The desired antigens were transiently expressed using the Expi293 system (Expi293 cells and ExpiFectamine™ 293 reagent; Thermo Fisher Scientific) and designed to be secreted into the supernatant.

Briefly, Expi293 cells were maintained in Expi293™ expression medium (37 °C, 8 % CO₂, 85 % humidity and 125 rpm). Cells seeded at 2 x 10⁶ /ml one day prior to transfection were transfected with 1 µg/ml of plasmid DNA complexes, prepared using the ExpiFectamine™ 293 reagent kit, as per the supplier's instructions (typically >95 % viable and counts 3-4 x 10⁶ /ml at Day 0). Enhancers I+II were added on Day 1 after transfection according to protocol. Cell viability was monitored regularly (AO/PI staining, Nexcelom K2 cellometer) and cultures harvested once viability dropped below 50 %. For harvesting, the supernatants were cleared by centrifugation (2000 g, 15 min, 4 °C), and then sterile filtered using 0.22 µm bottle top filters (Millipore). Filtered supernatant not immediately used for purification was stored at -80 °C for later processing.

Purification of HIS-tagged antigen by immobilized metal affinity chromatography (IMAC): The polyhistidine-tagged recombinant target

proteins listed in Table 1 were purified by nickel-based IMAC, according to adapted protocols.

Briefly, input supernatants were re-filtered before being loaded onto pre-equilibrated (Buffer A: 20 mM Tris + 0.5 M NaCl, pH 8) IMAC columns (HisTrap Excel, GE Healthcare, cat. no. 17-3712-06). Unbound protein was washed out before applying elution gradients optimized for sample concentration, purity and collection (Buffer B: 20 mM Tris + 0.5 M NaCl + 500 mM imidazole, pH 8). Fractions were analyzed, and relevant ones pooled prior to buffer exchange.

Purified samples were buffer exchanged to sterile PBS (pH 7.4) using HiPrep™ 26/10 desalting columns (GE Healthcare, cat. no. 17-5087-01) and concentrated using Amicon® Ultra Centrifugal Filters (Millipore). As a standard, final products were sterile-filtered using 0.2 µm syringe filters (Pall).

15

Example 9

Generation and screening of antibodies to the N-terminal ApoE fragment neo-epitope G200

This example describes the immunization of BALB/c mice and subsequent generation and screening of hybridoma cell lines.

Materials and methods

Peptide synthesis: The immunogen used in this experiment was designed to incorporate one of the N-terminal neo-epitopes of the neurotoxic ApoE fragment identified in the previous Examples. As its N-terminal sequence, the immunogen comprised the amino acid residues corresponding to amino acid residues 200-205 in full-length ApoE. This N-terminal sequence was coupled C-terminally to a 6-aminocaproic acid linker (Acp; also denoted aminohexanoic acid linker (Ahx)), followed by a cysteine residue for the purposes of conjugation to for example keyhole limpet hemocyanin (KLH) or bovine serum albumin (BSA) as indicated. The entire immunogen sequence

30

used was GQPLQE-Acp-C (SEQ ID NO:50). SEQ ID NO:50 was prepared by Innovagen AB and delivered at 95.5 % purity.

Additionally, ApoE-derived peptides were prepared which incorporated the other two identified putative N-terminal neo-epitopes of the neurotoxic ApoE fragment, as well as a negative control peptide without any of the identified neo-epitopes. These peptides were likewise coupled to 6-aminocaproic acid and a cysteine residue. The first neo-epitope peptide comprised as its N-terminal sequence the amino acid residues corresponding to amino acid residues 199-204 in full-length ApoE. Thus, the entire sequence was AGQPLQ-Acp-C (SEQ ID NO:51). SEQ ID NO:51 was prepared by Innovagen AB and delivered at 96.7 % purity. The second neo-epitope peptide comprised as its N-terminal sequence the amino acid residues corresponding to amino acid residues 198-203 in full-length ApoE. Thus, the entire sequence was LAGQPL-Acp-C (SEQ ID NO:52). SEQ ID NO:52 was prepared by Innovagen AB and delivered at 95.2 % purity. The entire sequence of the negative control peptide was AATVGSLAGQPLQER-Acp-C (SEQ ID NO:53). SEQ ID NO:53 was prepared by Innovagen AB and delivered at 97.1 % purity.

Immunization: 10-12 weeks old Balb/c mice were immunized with SEQ ID NO:50 conjugated to KLH. In the first injection (subcutaneous, s.c.), the immunogen was given together with Freund's complete adjuvant. In subsequent injections (s.c.) except the last one, the immunogen was given in Freund's incomplete adjuvant. Plasma samples were collected three weeks after each immunization. Every mouse can receive up to 10 injections, but fewer immunizations were used for all of the mice. The final immunizations (booster dose) were administered intraperitoneally (i.p.) without adjuvant.

Plasma screening by direct ELISA: Plasma samples were analyzed by ELISA for reactivity against the target peptide SEQ ID NO:50 conjugated to bovine serum albumin, BSA, and against the recombinant ApoE fragment G200-HIS (SEQ ID NO:48) to determine when to stop immunizations and initiate hybridoma generation. Briefly, 96-well half area plates (Corning) were coated with 1 µg/ml antigen, i.e. G200 N-terminal neo-epitope peptide (ApoE sequence 200-205 incorporated in SEQ ID NO:50) coupled to BSA or purified

recombinant C-terminal ApoE fragment G200-HIS (SEQ ID NO:48), in PBS at 50 µl/well over night at 4 °C. The plates were blocked with 150 µl/well of protein-free blocking solution (Pierce) for 1 h at room temperature with shake (600-900 rpm). The plates were washed four times with washing buffer
5 containing 0.28 mM NaH₂PO₄, 2.5 mM Na₂HPO₄, 150 mM NaCl, 0.1 %
TWEEN®-20 and 0.0075 % Kathon CG. Plasma samples 3-fold serially
diluted with a starting dilution of 1/450 were added to the plates (dilution
buffer: PBS with 0.1 % BSA and 0.05 % TWEEN®-20) and incubated for 2 h
at room temperature. The plates were washed four times as before. Detection
10 antibody (HRP-conjugated anti-mouse IgG, Southern Biotech, cat. no. 1030-
05, diluted 1/10000 in dilution buffer) was added 50 µl/well, and the plates
were incubated for 1 h at room temperature. After another wash (as above),
50 µl/well of K-Blue® aqueous substrate (Neogen) was added, and the
reaction was stopped after 10-15 min with 50 µl/well of 0.5-2 M H₂SO₄. The
15 optical density at 450 nm was read using an ELISA reader (Tecan). The end
point titers were defined as the dilution above the average of the blank wells
(background) plus 3 standard deviations of the blank wells. An end point titer
of 1/100,000 was considered high enough, and after this had been reached,
no more immunizations were done until generation of hybridomas.

20 Generation of hybridomas: The mice received a final booster dose of
immunogen i.p. (no adjuvant) 3 days before generation of hybridomas.
Isolated splenocytes from sacrificed mice were fused with Sp2/0 cells at a 3:1
ratio and plated in 96-well plates in the presence of ClonaCell™-HY
hybridoma selection medium D (StemCell Technologies), containing HAT for
25 selection.

Wells were preferably screened twice during the next two weeks for
reactivity to the G200-HIS recombinant ApoE fragment, and positive wells
containing visible clones were processed further. Identified clones were
expanded and subjected to at least two rounds of limiting dilution assay to
30 assure monoclonality. Clones of interest were cryopreserved, expanded for
production of antibody, and sequenced (Absolute Antibodies, UK).

Hybridoma screening by direct ELISA: ELISA experiments to identify
hybridoma clones producing antibodies with reactivity against the target

epitope were performed according to standard ELISA protocols as described above in the section "Plasma screening by direct ELISA". During screening of hybridomas, and to reach monoclonality, three different antigens were used at a concentration of 1 µg/ml antigen. These were the G200 N-terminal neo-
5 epitope peptide (ApoE sequence 200-205 incorporated in SEQ ID NO:50) coupled to BSA, purified recombinant C-terminal ApoE fragment G200-HIS (SEQ ID NO:48) and recombinant full-length ApoE4 (SEQ ID NO:45). Hybridoma supernatants were diluted 2-fold (dilution buffer: PBS with 0.1 % BSA and 0.05 % TWEEN®-20) and screened against binding to G200-HIS
10 fragment. For the hybridoma screening ELISAs, "positive" wells were selected based on OD-values of >2 and the presence of clone/s. The identified positive clones were then subjected to positive and negative screenings using the same ELISA protocol, with G200-HIS fragment (SEQ ID NO:48), G200-peptide (SEQ ID NO:50) coupled to BSA, and ApoE4 full-length protein (SEQ
15 ID NO:45) as coat for the plates. Clones that showed no substantial binding in the negative screening were subjected to 2 rounds of limiting dilution assay to ensure monoclonality and screened for binding to the G200-HIS fragment. Monoclonal clones that still bound G200-HIS fragment but not full-length ApoE4 and continued to grow were considered especially interesting for
20 further characterization.

Results

Generation of monoclonal antibodies by hybridoma technology:

Antibodies that bind selectively to the N-terminal neo-epitope starting at
25 amino acid G200 of the ApoE protein were generated by immunizations using an ApoE specific sequence consisting of the six first amino acids following the N-terminal in the 200-299 ApoE fragment. The shortness of the immunization peptide was considered necessary in order to enable generation of antibodies that bind selectively to the N-terminal neo-epitope starting at amino acid G200
30 of the ApoE protein, without any binding to the linear epitope found in full-length ApoE protein. The ApoE-specific sequence peptide 200-205, conjugated to keyhole limpet haemocyanin (KLH) via an Acp linker and a cysteine residue, was used in the immunizations. The plasma samples were

analyzed by ELISA for reactivity against the corresponding peptide conjugated to BSA (to avoid detecting reactivity generated towards KLH), and against recombinant ApoE fragment. When titers were at least $>1/100,000$ the mice were sacrificed and the spleens were collected and used for hybridoma
5 generation.

ELISA screening for antibodies that bind selectively to the N-terminal neo-epitope of ApoE fragment starting at G200: Generated hybridoma clones were screened for reactivity towards the recombinant ApoE fragment G200-HIS, as well as reactivity towards the target peptide SEQ ID NO:50
10 conjugated to BSA. In addition, lack of reactivity towards recombinant full-length ApoE4 was evaluated. Seven clones were identified as antibodies selective for the N-terminal neo-epitope of ApoE fragments starting at amino acid G200, and were denoted 4E6, 7B10, 7C7, 17G4, 21C3, 23D5 and 28F2.

15

Example 10

Characterization of unpurified hybridoma supernatants containing monoclonal antibodies to the N-terminal ApoE fragment neo-epitope G200

20 This example describes the characterization, by direct ELISA, of monoclonal antibodies with affinity for the N-terminal neo-epitope of C-terminal ApoE fragments resulting from cleavage in the hinge region (starting at amino acid G200). In addition, the monoclonal antibodies were evaluated for their ability to selectively bind to ApoE fragments (≤ 12 kDa) with the N-
25 terminal neo-epitope starting at amino acid G200 in Alzheimer's disease brain extracts, without any binding to full-length ApoE, using Western blot.

Materials and methods

Selectivity evaluation by direct ELISA: The direct ELISA described
30 below was used to evaluate the binding selectivity of generated monoclonal anti-ApoE antibodies with affinity for the N-terminal neo-epitope of C-terminal ApoE fragments starting at amino acid G200. The ability to selectively bind to N-terminal neo-epitope peptide (ApoE sequence aa 200-205, SEQ ID NO:6,

incorporated in SEQ ID NO:50 for experimental purposes) and to the recombinant C-terminal ApoE fragment resulting from cleavage in the hinge region (ApoE sequence aa 200-299, SEQ ID NO:1, His-tagged for experimental purposes as SEQ ID NO:48) was compared with binding to
5 recombinant full-length ApoE (ApoE4 sequence aa 1-299, SEQ ID NO:45).

The screening was performed according to standard ELISA protocols. Briefly, 1 µg/ml solutions of N-terminal neo-epitope peptide (SEQ ID NO:50) conjugated to BSA, the recombinant C-terminal ApoE fragment G200-HIS (SEQ ID NO:48) and recombinant full-length ApoE4 (SEQ ID NO:45; Abcam;
10 cat. no. ab50243) were prepared by dilution in PBS. 50 µl/well were added to an ELISA half-area 96 well microtiter plate. Then, the plate was sealed with adhesive sealer and incubated over night at 4 °C. The plates were blocked with 150 µl/well of protein-free blocking solution (Pierce) for 1 h at room temperature with shake (600-900 rpm). The plates were washed four times
15 with washing buffer containing 0.28 mM NaH₂PO₄, 2.5 mM Na₂HPO₄, 150 mM NaCl, 0.1 % TWEEN®-20 and 0.0075 % Kathon CG. Antibodies of interest were serially diluted 3-fold with a starting dilution at 1 µg/ml (mixed 1:1 with dilution buffer: PBS with 0.1 % BSA and 0.05 % TWEEN®-20) and incubated for 2 h at room temperature. The plates were washed four times as
20 before. Detection antibody (HRP-conjugated anti-mouse IgG, Southern Biotech, cat. no. 1030-05, diluted 1/10000 in dilution buffer) was added 50 µl/well, and the plates were incubated for 1 h at room temperature. After another wash (as above), 50 µl/well of K-Blue® aqueous substrate (Neogen) were added, and the reaction was stopped after 5-15 min with 50 µl/well of
25 0.5 M H₂SO₄. The optical density at 450 nm was read using an ELISA reader (Tecan). The optical density was plotted against the antibody concentration to generate concentration-response curves (Figure 14).

Isotyping: The isotype of each antibody clone was determined using Mouse Monoclonal Antibody Isotyping kit (Roche) according to the
30 manufacturer's instructions.

Selectivity evaluation and human target binding in Alzheimer's disease brain extracts by Western blot analysis: The Western blot analysis described below was used to evaluate selective binding of monoclonal antibodies with

affinity for the N-terminal neo-epitope of ApoE fragments ≤ 12 kDa, starting at G200, without binding to ApoE full-length protein, in human brain extracts from an Alzheimer's disease patient. Fresh frozen brain tissue from the Alzheimer's disease patient was homogenized in RIPA 2 % SDS extraction
5 buffer, followed by centrifugation at 16000 x g for 1 h. The subsequent supernatant was subjected to standard protein concentration determination.

RIPA 2 % SDS brain extract containing 80 μ g total protein was mixed with 2x Laemmli sample buffer, boiled for 5 min at 95 °C and loaded onto SDS-PAGE gels (Bolt™ 12 % Bis-Tris Plus 10 well, Thermo Fisher, cat. no.
10 NW00120BOX). Gels were run for 30-40 min at 180 V, after which proteins were transferred from the gels to nitrocellulose membranes using the Trans-Blot® Turbo™ system (Bio-Rad). Membranes were blocked in Odyssey® blocking buffer for 1 h and incubated over night at room temperature with hybridoma supernatant diluted 1:1 in Odyssey® blocking buffer with 0.1 %
15 TWEEN®-20. Membranes were washed and incubated for 1 h at room temperature with the detection antibody anti-mouse-800CW (LI-COR, cat. no. 925-32210) diluted 1:25000 in Odyssey® blocking buffer with 0.1 % TWEEN®-20. Membranes were washed and images acquired using Odyssey® FC (LI-COR).

20 To confirm that the obtained bands on the Western blot membranes were of ApoE origin, the membranes were re-stained over night with a polyclonal anti-ApoE antibody (Calbiochem, cat. no. 178479; immunogen ApoE aa 1-299), diluted 1:2000 in Odyssey® blocking buffer with 0.1 % TWEEN®-20. Membranes were washed and incubated for 1 h at room
25 temperature with detection antibody anti-goat-680RD (LI-COR, cat. no. 925-68074) diluted 1:25000 in Odyssey® blocking buffer with 0.1 % TWEEN®-20. Membranes were washed and images acquired using Odyssey® FC (LI-COR).

30 *Results*

Selectivity evaluation: The seven hybridoma clones (4E6, 7B10, 7C7, 17G4, 21C3, 23D5 and 28F2) that were selective for the N-terminal neo-epitope of ApoE fragments starting at amino acid G200 demonstrated binding

to the G200-BSA peptide (SEQ ID NO:50 conjugated to BSA) and to the recombinant G200-HIS fragment (SEQ ID NO:48), whereas no binding to recombinant ApoE4 full-length protein (aa 1-299; SEQ ID NO:45) was shown (Figure 14). The reference antibody ApoE Ab (Santa Cruz, cat. no. SC-393302, epitope ApoE aa 274-299) binds equally well to the recombinant G200-HIS fragment and to recombinant ApoE4 full-length protein. However, since the epitope for the reference antibody is ApoE aa 274-299, no binding to the G200-BSA peptide was shown, as expected. The experiment shown in Figure 14 was performed using unpurified cell supernatants from the monoclonal hybridomas 4E6, 7B10, 7C7, 17G4, 21C3, 23D5 and 28F2.

Isotyping: The isotype of the antibody clones, as determined using the Mouse Monoclonal Antibody Isotyping kit (Roche), is shown in Table 2.

Clone	Sub-class	Light chain
4E6	IgG1	κ
7B10	IgG1	κ
7C7	IgG2b	κ
17G4	IgG1	κ
21C3	IgG2b	κ
23D5	IgG1	κ
28F2	IgG1	κ

Table 2. Isotyping results

15

Selectivity evaluation and human target binding in Alzheimer's disease brain extracts by Western blot analysis: The hybridoma clones (4E6, 7B10, 7C7, 17G4, 21C3, 23D5 and 28F2) selective for the N-terminal neo-epitope of ApoE fragments starting at amino acid G200 were tested for their ability to bind selectively to ApoE fragments ≤12 kDa in human brain extracts from an Alzheimer's disease patient, and without any binding to full-length ApoE. Western blot analysis demonstrated binding of the monoclonal hybridomas to two ApoE fragments approximately 12 kDa and 10 kDa in size, without any visual binding to full-length ApoE (Figure 15A; data not shown for 21C3 because its sequence turned out to be identical to that of 4E6, see further below). Re-staining of the Western blot membranes with a polyclonal anti-ApoE antibody showed staining of full-length ApoE, high molecular weight

25

(HMW) ApoE fragments (~20-25 kDa) and a distinct 12 kDa ApoE fragment (Figure 15B).

Example 11

5 Hybridoma sequencing and production of recombinant antibody

Materials and methods

Hybridoma sequencing: Hybridoma clones producing monoclonal antibodies as generated and characterized in Examples 8-10, having a
10 demonstrated selectivity for the N-terminal neo-epitope of ApoE fragment starting at amino acid G200, and a proven human target binding in brain extracts from Alzheimer's disease, were sent to Absolute Antibody for sequencing. Briefly, hybridoma sequencing was performed by whole transcriptome shotgun sequencing. The DNA and protein sequences of the
15 mature VH and VL regions were identified.

Expression, production and purification of recombinant antibodies at Absolute Antibody: The variable domains were designed and optimized for expression in mammalian cells (HEK293) prior to being synthesized. The sequences were then subcloned into an Absolute Antibody cloning and
20 expression vector for the appropriate isotype and subtype of immunoglobulin heavy and light chains.

HEK293 cells were passaged to the optimum stage for transient transfection. Cells were transiently transfected with heavy and light chain expression vectors and cultured for a further 6-14 days. An appropriate
25 volume of cells were transfected with the aim of obtaining 2 mg of purified antibody.

Cultures were harvested and a one-step purification performed using affinity chromatography, after which the purified antibodies were buffer exchanged into PBS. Antibodies were analyzed for purity by SDS-PAGE and
30 the concentration was determined by UV spectroscopy.

Results

Hybridoma sequencing and recombinant antibody production:

Hybridoma clones with a demonstrated selectivity for the N-terminal neo-epitope of ApoE fragment starting at amino acid G200, in addition to a demonstrated binding to human target in brain extracts from Alzheimer's disease, were sequenced.

The following hybridoma clones were sequenced: 4E6, 7B10, 7C7, 17G4, 21C3, 23D5 and 28F2. Sequencing revealed that antibodies 4E6 and 21C3 have the same sequence in both the primary VH and VL. The amino acid sequences of the entire antibodies were obtained. Amino acid sequences obtained for the respective variable heavy (VH) and variable light (VL) chains are given in Table 3 below.

Region	Antibody	SEQ ID NO:
	4E6 / 21C3	
VH	EVQLVESGGDLVKPGGSLKLSCAASGFTFSSYAMSWVRQSPPEKRLE WVAEISGSGSRDHYTDSVTGRFTVSRDPAKNTLYEMSSLRSEDTAIY YCARQLTGTDYYGTDYWGQGTSTVTVSS	34
VL	DVLTMTQIPLSLPVSLGDQASISCRSSQSIVYSNGNTYLEWYLQRPQGQS PKLLIYKVSNNRFGVDPDRFSGSGSGTDFTLKISRVEAEDLGVVYCFQG SHLPYTFGGGTKEIK	35
	7B10	
VH	AVQLVESGGGLVKPGRSLKLSCAASGFTFSSYAMSWVRQFPDKRLD WVAEISSGGGSTNYLDTVTGRFTISRDNKNTLYEMNSLRSEDTAMY YCARQLVGTDDYYGTDYWGQGTSTVTVSS	36
VL	DVLTMTQTPLSLPVSLGDQASISCRSSQNIVYSNGNTYLEWYLQKPGQGS PKLLIYKVSNNRFGVDPDRFSGSGSGTDFTLKISRVEAEDLGVVYCFQG SHVPYTFGGGTKEIK	37
	7C7	
VH	EVQLVESGGGLVKPGGSLRLSCAASGFTFSSFAMSWVRQSPERRLE WVAEISRGGGYAFYSDTVTGRFTISRDNARNTLFLEMGLRSEDTAMY YCSRQLTGTDYYAMDYWGQGTSTVTVSS	38
VL	DVLLTQTPLSLFVSLGDQASISCRSSQSIVYTNGNTYLEWYLQKPGQGS PKLLIYKVSNNRFGVDPDRFSGSGSGTDFTLKISRVEAEDLGVVYCFQG SQVPYTFGGGTKELEK	39
	17G4	
VH	EVQLVESGGGLVKPGGSLKLSCSASGFTFSRYAMSWVRQSPPEKRLE WVAEINSGGSYSFYSDTVTGRFTISRDNKNTLFLEMSSLRSEDTAIYY CARQLTGTDYYGTDYWGQGTSTVTVSS	40
VL	DVLTMTQTPLSLPVSHGDQASISCRSSQSLLYSNGNTYLEWYLQKPGQ SPKLLIYKVSNNRFGVDPDRFSGSGSGTDFTLKISRVEAEDLGVVYCFQ GSHVPYTFGGGTKEIK	41
	23D5	
VH	EVQLVESGGGLVKPGGSLKLSCSASGFTFSRYAMSWVRQSPPEKRLE WVAEINSGGSYSFYSDTVTGRFTISRDNKNTLFLEMSSLRSEDTAIYY	42

	CARQLSGTDYYGTDYWGGQTSVTVSS	
VL	DVLMTQTPLSLPVSHGDQASISCRSSQSLLYSNGNTYLEWYLQKPGQ SPKLLIYKVSNRFSGVDPDRFSGSGSGTDFTLKISRVEAEDLGVYYCFQ GSHVPYTFGGGKLEIK	41
	28F2	
VH	QIQLVQSGPDLKKPGETVKISCKASGYTFTNYAMHWMKQAPGKALKW MGWINTYTGEPTFADDFKGRFAFSLETSASTAYLQINSLKNEDMATYF CAREGYDRSHYFDYWGGQTTLTVSS	43
VL	DVLMTQTPLSLPVSLGDQASISCRSSLSLVHGDGNTYLEWYLQKPGQ SPKLLIYKVSNRFSGVDPDRFSGSGSGTDFTLKISRVEAEDLGVYYCLQ GSHIPFTFGSGTKLEIK	44

Table 3. Variable region sequences

The complementarity determining regions (CDRs) were identified using the Kabat definition for the primary VH and VL sequences and are given in

5 Table 4 below.

Antibody	CDR H1	CDR H2	CDR H3
4E6/21C3	SYAMS (SEQ ID NO:10)	EISGSGSRDHYTDSVTG (SEQ ID NO:11)	QLTGTDYYGTDY (SEQ ID NO:12)
7B10	SYAMS (SEQ ID NO:10)	EISSGGGSTNYLDTVTVG (SEQ ID NO:13)	QLVGTDDYYGTDY (SEQ ID NO:14)
7C7	SFAMS (SEQ ID NO:15)	EISRGGGYAFYSDTVTVG (SEQ ID NO:16)	QLTGTDYYAMDY (SEQ ID NO:17)
17G4	RYAMS (SEQ ID NO:18)	EINSGGSYSFYSDTVTVG (SEQ ID NO:19)	QLTGTDYYGTDY (SEQ ID NO:12)
23D5	RYAMS (SEQ ID NO:18)	EINSGGSYSFYSDTVTVG (SEQ ID NO:19)	QLSGTDYYGTDY (SEQ ID NO:20)
28F2	NYAMH (SEQ ID NO:21)	WINTYTGEPTFADDFKG (SEQ ID NO:22)	EGYYDRSHYFDY (SEQ ID NO:23)
	CDR L1	CDR L2	CDR L3
4E6/21C3	RSSQSIVYSNGNTYLE (SEQ ID NO:24)	KVSNRFS (SEQ ID NO:25)	FQGSHLPYT (SEQ ID NO:26)
7B10	RSSQNIVYSNGNTYLE (SEQ ID NO:27)	KVSNRFS (SEQ ID NO:25)	FQGSHVPYT (SEQ ID NO:28)
7C7	RSSQSIVYTNGNTYLE (SEQ ID NO:29)	KVSNRFS (SEQ ID NO:25)	FQGSQVPYT (SEQ ID NO:30)
17G4	RSSQSLLYSNGNTYLE (SEQ ID NO:31)	KVSNRFS (SEQ ID NO:25)	FQGSHVPYT (SEQ ID NO:28)
23D5	RSSQSLLYSNGNTYLE (SEQ ID NO:31)	KVSNRFS (SEQ ID NO:25)	FQGSHVPYT (SEQ ID NO:28)
28F2	RSSLSLVHGDGNTYLE (SEQ ID NO:32)	KVSNRFS (SEQ ID NO:25)	LQGSHIPFT (SEQ ID NO:33)

Table 4. CDR region sequences

The monoclonal antibodies 4E6, 7B10, 7C7, 17G4, 23D5 and 28F2

10 were selected for production as recombinant IgG2c antibodies, whereas 21C3 was not produced (because of the sequence redundancy with 4E6). All

recombinant antibodies were successfully produced and purified to a final concentration of 1 mg/ml. Antibody purity, as defined by SDS-PAGE, was >98 % for all antibodies.

5

Example 12

Characterization of recombinant antibodies

This example describes the characterization of the recombinant antibodies produced in Example 11 by various methods, including direct
10 ELISA, inhibition ELISA, biolayer interferometry, surface plasmon resonance, immunoprecipitation on human brain extract and immunohistochemistry on human brain sections.

Materials and methods

15 Selectivity evaluation of recombinant antibodies by direct ELISA: The direct ELISA described below was used to evaluate the binding selectivity of the recombinant antibodies produced in Example 11. The ability to selectively bind to the G200 N-terminal neo-epitope ApoE peptide (BSA-conjugated SEQ ID NO:50) and to the recombinant G200-HIS C-terminal ApoE fragment
20 resulting from cleavage in the hinge region (SEQ ID NO:48) was compared with binding to the L198, A199 and negative control peptides (BSA-conjugated SEQ ID NO:51, 52 and 53, respectively), and to His-tagged, recombinant full-length ApoE4 (ApoE sequence aa 1-299; SEQ ID NO:49).

The screening was performed according to standard ELISA protocols.
25 Briefly, 1 µg/ml solutions of BSA-conjugated neo-epitope peptides, recombinant C-terminal ApoE fragment, negative control peptide and full-length ApoE were prepared by dilution in PBS. 50 µl/well were added to an ELISA half-area 96 well microtiter plate, the plate was sealed with adhesive sealer and incubated over night at 4 °C. After discarding the solution, the
30 plates were blocked with 150 µl/well of protein-free blocking solution (Pierce) for 1 h at room temperature with shake (900 rpm). The plates were washed four times with washing buffer containing 0.28 mM NaH₂PO₄, 2.5 mM Na₂HPO₄, 150 mM NaCl, 0.1 % TWEEN®-20 and 0.0075 % Kathon CG.

Generated recombinant antibodies of interest were serially diluted 3-fold with a starting dilution at 0.5 µg/ml (mixed 1:1 with dilution buffer: PBS with 0.1 % BSA and 0.05 % TWEEN®-20). An anti-ApoE C-terminal antibody was used as a reference antibody (ApoE E-8 mouse monoclonal, cat. no. sc-393302; Santa Cruz Biotechnology). 50 µl/well were added into the ELISA plates and incubated for 2 h at room temperature with shake (900 rpm). The plates were washed four times as described previously. Detection antibody (HRP-conjugated anti-mouse IgG, Southern Biotech, cat. no. 1030-05, diluted 1:10000 in dilution buffer) was added 50 µl/well, and the plates were incubated for 1 h at room temperature with shake (900 rpm). After another wash (as described previously), 50 µl/well of K-Blue® aqueous substrate (Neogen) were added, and the reaction was stopped after 5-15 min with 50 µl/well of 0.5 M H₂SO₄. The optical density at 450 nm was read using an ELISA reader (Tecan). The optical density was plotted against the antibody concentration to generate concentration-response curves (Figure 16).

In addition, a second ELISA experiment was run, in which the plate was coated with both i) the recombinant G200-HIS C-terminal ApoE fragment resulting from cleavage in the hinge region (SEQ ID NO:48) and ii) recombinant full-length ApoE4 (ApoE sequence aa 1-299; SEQ ID NO:45; Abcam; cat. no. ab50243). These were added in the same molarity (0.1 µM) to provide an equal number of molecules available for binding by the antibodies (BSA-coupled peptides were coated at 1 µg/ml as before). The antibody concentration was increased to 3 µg/ml, and EC₅₀ values were determined from a log agonist concentration response curve (Figure 19).

Selectivity evaluation and IC₅₀ determination of recombinant antibodies by inhibition ELISA: The inhibition ELISA described below was used to evaluate the binding strength and selectivity for the recombinant antibodies produced in Example 11. The ability of the recombinant antibodies to bind to the N-terminal neo-epitope of synthetic ApoE peptides starting at amino acid G200 and to recombinant C-terminal ApoE fragment G200-HIS was evaluated in comparison with their ability to bind to synthetic ApoE peptides starting at amino acids L198 and A199, as well as to full-length ApoE4 (aa 1-299) in solution.

In brief, the recombinant antibody to be tested was allowed to interact with the N-terminal neo-epitope of synthetic ApoE peptides conjugated to BSA and starting at amino acid L198 (SEQ ID NO:52), A199 (SEQ ID NO:51) or G200 (SEQ ID NO:50), or to BSA-conjugated negative control peptide (SEQ ID NO:53), or to His-tagged recombinant C-terminal ApoE fragment G200-HIS (SEQ ID NO:48), or to His-tagged recombinant full-length ApoE4 in solution (SEQ ID NO:49). Thereafter, the mix was added to a microtiter plate coated with the BSA-coupled G200 synthetic ApoE peptide. If the recombinant antibody binds to any of the antigens in the pre-incubation step (the synthetic ApoE peptides, recombinant C-terminal ApoE fragment or full-length ApoE4), the antibody is prevented from binding to the synthetic G200 ApoE peptide immobilized on the microtiter plate. This leads to inhibition of the ELISA detection signal.

0.5 µg/ml solution of N-terminal neo-epitope peptide G200 (SEQ ID NO:50) conjugated to BSA was prepared by dilution in PBS. 50 µl/well were added to an ELISA half-area 96 well microtiter plate, the plate was sealed with adhesive sealer and incubated over night at 4 °C. After discarding the above solution, the plates were blocked with PBS-Tween 20 (0.05 %) (150 µl/well) at room temperature for at least 1 h with shaking (900 rpm). The blocked plate was washed four times with washing buffer containing 0.28 mM NaH₂PO₄, 2.5 mM Na₂HPO₄, 150 mM NaCl, 0.1 % TWEEN®-20 and 0.0075 % Kathon CG.

3-fold serial dilutions of each synthetic ApoE peptide, recombinant C-terminal ApoE fragment G200-HIS and full-length ApoE4 were prepared by 4-fold dilution starting from 1000 ng/ml in 96 well storage plates (30 µl final volume). Each recombinant antibody to be tested was added (30 µl) to the serial dilutions of each of the synthetic ApoE peptides, G200-HIS or full-length ApoE4 at a final concentration of 0.05 µg/ml per well. Samples were pre-incubated for 45 min at room temperature with shaking (900 rpm).

The pre-incubated samples were transferred (50 µl/well) to the blocked ELISA plates, and plates were incubated for 25 min at room temperature without shaking. Plates were washed as described above. Alkaline phosphatase conjugated anti-mouse IgG detection antibody (Mabtech, cat.

no. 3310-4) was diluted 1:1000 and added to each plate (50 µl/well). The plate was sealed and incubated with shaking (900 rpm) for 45 min at room temperature and subsequently washed as described above. Alkaline phosphatase substrate (50 µl/well) was added to the plate and the optical density was read every 15 min at a wavelength of 405 nm for up to 120 min. The IC₅₀ values were determined from a log inhibitor concentration response curve (Figure 17).

In addition, a second inhibition ELISA with the ApoE peptides conjugated to BSA was run, in which the starting concentration of the antigens in solution was increased 10-fold. i.e. the starting concentration of the N-terminal neo-epitope of synthetic ApoE peptides conjugated to BSA and starting at amino acid L198 (SEQ ID NO:52), A199 (SEQ ID NO:51) or G200 (SEQ ID NO:50), or to BSA-conjugated negative control peptide (SEQ ID NO:53), was 10000 ng/ml. Furthermore, a 4-fold serial dilution of antigen was used, the plates were blocked with protein-free blocking solution (Pierce) for 90 min, the pre-incubated samples were incubated on the coated plates for 10 min (instead of 25 min), and the optical density was read every 10 min (instead of every 15 min). IC₅₀ values were determined from a log inhibitor concentration response curve (Figure 20).

Selectivity evaluation and K_D determination of recombinant antibodies by bio-layer interferometry: Binding interactions between the N-terminal neo-epitope peptide G200 (SEQ ID NO:50) conjugated to BSA (ligand) and the recombinant antibodies produced in Example 11 (analyte) were evaluated using an Octet RED384 instrument (ForteBio). All analyzed proteins were diluted in 1x Kinetics Buffer (ForteBio).

To analyze the antibody-target interaction and to determine the binding affinity of the recombinant antibodies for recombinant C-terminal ApoE fragment G200-HIS (SEQ ID NO:48), anti-HIS capture biosensors (HIS1K) were used. In the first step (loading step), HIS-tagged recombinant C-terminal ApoE fragment was captured onto the surface of the biosensor. Next, a concentration gradient of pure antibodies was prepared ranging from 150 nM to 2.5 nM in 2-fold serial dilutions. Subsequently, the HIS1K biosensors with ligand were dipped in wells containing diluted antibodies, and the association

phase was monitored for 600 s. For monitoring the dissociation phase, the HIS1K biosensors with ligand were moved to a well containing 1x Kinetics Buffer and the dissociation was monitored for 1000 s. For evaluation of the kinetic experiments, the Octet Data Analysis software was used. All values
5 collected from interaction analysis were blank subtracted, and a 1:2 binding kinetics global fit model (bivalent analyte) was used for the evaluation.

Selectivity evaluation and K_D determination of recombinant antibodies by surface plasmon resonance: Binding interactions between the antigens and antibodies were evaluated by surface plasmon resonance (SPR) using a
10 Biacore 8K instrument (GE Healthcare) according to standard procedures.

To determine the binding affinity of the monoclonal antibodies for N-terminal neo-epitopes compared to full-length ApoE, single-cycle kinetics experiments were conducted. The recombinant fragments were immobilized onto a CM5 Sensor Chip (GE Healthcare, cat. no. 29104988) using an amine
15 coupling kit (GE Healthcare, cat. no. BR100050) according to the manufacturer's instructions. Reference (no immobilized antigen) and active surfaces were treated with the same conditions using the amine coupling reagents on flow cell 1 (Fc1) and flow cell 2 (Fc2), respectively. The immobilization level for the active surfaces was kept at approximately 150-
20 200 response units (RU). The same protocol setup was used to immobilize full-length ApoE onto the CM5 chip surface.

Purified antibodies were prepared in 2-fold serial dilution from 14 to 0.3 nM (in 5-7 steps). Next, the prepared serial dilution of purified antibodies was injected (30 μ l/min, contact time 360 s, dissociation time 2500 s) over
25 both flow cells of the sensor chip. The interaction series was done in triplets. Values were blank subtracted, and a bivalent analyte binding kinetics fit model was used for the evaluation.

In all SPR experiments, 1xPBS-P+ (GE Healthcare, cat. no. 28995084) was used to dilute antibodies and target antigens. Experiments were
30 performed at 25 °C.

Selectivity evaluation and human target binding in Alzheimer's disease brain extracts by Western blot analysis: The Western blot analysis described below was used to evaluate selective binding of recombinant antibodies with

affinity for the N-terminal neo-epitope of ApoE fragments ≤ 12 kDa starting at G200, without binding to ApoE full-length protein, in human brain extracts from an Alzheimer's disease patient. Fresh frozen brain tissue from the Alzheimer's disease patient was homogenized in RIPA 2 % SDS extraction
5 buffer, followed by centrifugation at 16000 x g for 1 h. The subsequent supernatant was subjected to standard protein concentration determination.

RIPA 2 % SDS brain extract containing approximately 60 μ g total protein was mixed with 2x Laemmli sample buffer, boiled for 5 min at 95 °C and loaded onto SDS-PAGE gels (Bolt™ 12 % Bis-Tris Plus 10 well, Thermo
10 Fisher, cat. no. NW00120BOX). Gels were run for 30-40 min at 180 V, after which proteins were transferred from the gels onto nitrocellulose membranes using the Trans-Blot® Turbo™ system (Bio-Rad). Membranes were blocked in Intercept® PBS Blocking Buffer (LI-COR) for 1 h and incubated over night at room temperature with recombinant antibodies with affinity for the N-terminal
15 neo-epitope of ApoE fragments ≤ 12 kDa starting at G200 (2 μ g/ml in Intercept® PBS Blocking Buffer (LI-COR) with 0.1 % TWEEN®-20). Membranes were washed and incubated for 1 h at room temperature with the detection antibody anti-mouse-800CW (LI-COR, cat. no. 925-32210) diluted 1:25000 in Intercept® PBS Blocking Buffer (LI-COR) with 0.1 % TWEEN®-20.
20 Membranes were washed and images acquired using Odyssey® FC (LI-COR).

To confirm that the obtained bands on the Western blot membranes were of ApoE origin, the membranes were re-stained over night with a polyclonal anti-ApoE antibody (Calbiochem, cat. no. 178479; immunogen
25 ApoE aa 1-299), diluted 1:2000 in Intercept® PBS Blocking Buffer (LI-COR) with 0.1 % TWEEN®-20. Membranes were washed and incubated for 1 h at room temperature with detection antibody anti-goat-680RD (LI-COR, cat. no. 925-68074) diluted 1:25000 in Intercept® PBS Blocking Buffer (LI-COR) with 0.1 % TWEEN®-20. Membranes were washed and images acquired using
30 Odyssey® FC (LI-COR).

Selectivity evaluation and human target binding in Alzheimer's disease brain extracts by immunoprecipitation and Western blot analysis: The immunoprecipitation/Western blot analysis described below was used to

evaluate selective binding of recombinant antibodies with affinity for the N-terminal neo-epitope of ApoE fragments ≤ 12 kDa starting at G200, without binding to ApoE full-length protein, in human brain extracts from Alzheimer's disease patients. Fresh frozen brain tissue from Alzheimer's disease patients
5 was homogenized in RIPA 2 % SDS extraction buffer, followed by centrifugation at 16000 x g for 1 h. The subsequent supernatants were subjected to standard protein concentration determination.

RIPA 2 % SDS brain extract containing approximately 75 μ g total protein was mixed with recombinant antibody with affinity for the N-terminal
10 neo-epitope of ApoE fragments ≤ 12 kDa starting at G200, in IP buffer (PBS, 0.05% TWEEN®-20, 1% Triton X-100 and cOmplete™ Protease Inhibitor Cocktail) and incubated for 2 h (room temperature with head-to-tail rotation). Dynabeads™ Protein A beads (Invitrogen) were added and the extract-antibody-dynabeads mixture was incubated for 1 h (room temperature with
15 head-to-tail rotation). The supernatant was removed and the magnetic beads were washed. The immunoprecipitate was eluted from the beads by addition of 2x Laemmli sample buffer and boiling for 5 min at 95 °C. The eluate was loaded onto a SDS-PAGE gel (Bolt™ 12 % Bis-Tris Plus 10 well, Thermo Fisher, cat. no. NW00120BOX) and run for 30-40 min at 180 V, after which
20 proteins were transferred from the gels to nitrocellulose membranes using the Trans-Blot® Turbo™ system (Bio-Rad). Membrane was blocked in Intercept® PBS Blocking Buffer (LI-COR) for 1 h and incubated over night at room temperature with anti-ApoE C-terminal antibody (Sigma, cat. no. sab2701946) diluted 1:1000 in Intercept® PBS Blocking Buffer (LI-COR) with 0.1 %
25 TWEEN®-20. Membrane was washed and incubated for 1 h at room temperature with the detection antibody anti-rabbit-800CW (LI-COR, cat. no. 925-32211) diluted 1:25000 in Intercept® PBS Blocking Buffer (LI-COR) with 0.1 % TWEEN®-20. Membrane was washed and image acquired using Odyssey® FC (LI-COR).

30 Human target engagement in Alzheimer's disease brain by immunohistochemistry (IHC): Paraffin embedded tissues from individuals with Alzheimer's disease (APOE $\epsilon 3/\epsilon 3$ and APOE $\epsilon 4/\epsilon 4$) were obtained from Netherlands Brain Bank. Sections were cut at 4 μ m.

IHC staining was performed with the recombinant antibody 7C7 (see Example 11) and with anti-ApoE C-terminus antibody (Sigma, cat. no. sab2701946; immunogen ApoE aa 237-299) and anti-ApoE antibody (Calbiochem, cat. no. 178479; immunogen ApoE aa 1-299), using a
5 Discovery XT Robot (Ventana Medical Systems). In addition, brain sections were stained using the anti-A β antibodies 6E10 (Covance, cat. no. SIG-39320) and 4G8 (Covance, cat. no. SIG-39220) for staining of amyloid plaques.

Briefly, for single fluorescent or brightfield IHC staining of amyloid beta
10 or ApoE, an automated staining robot and HQ hapten technology were used. The detection system Discovery XT and kits from Ventana Medical Systems (F. Hoffmann-La Roche, Ltd) were used. The Amp HQ kit, in conjunction with 1) an Anti-HQ HRP, DISCOVERY (Roche, cat. no. 760-48202), Anti-Rabbit HQ, DISCOVERY (Roche, cat. no. 760-4815); 2) an OmniMap or UltraMap
15 HRP Multimer with a monoclonal anti mouse IgG bridge antibody (Abcam, cat. no. [M204-3] ab133469); and 3) a chromogenic DISCOVERY ChromoMap DAB Kit (Roche, cat. no. 760-159) or fluorescent detection kit Cy5 Kit, DISCOVERY (Roche, cat. no. 760-238), enables the enhancement of the chromogenic or fluorescent signal in standard IHC. The paraffin sections
20 were incubated at 60 °C for 30 min, deparaffinized in 2x xylene for 15 min, followed by gradient ethanol wash before rehydration in 1x PBS. Before application of antibodies, the sections were exposed to antigen retrieval solutions during an automated heat process (standard CC1 protocol from Ventana at 95 °C, or a pre-treatment by protease (Ventana Protease 2, cat.
25 no. 780-4148) applied to slides for 24 min.

Stained slides were scanned in brightfield or fluorescence channels Dapi (nuclear counterstain), 3,3'-diaminobenzidine (DAB) or CY5 (primary antibodies) and autofluorescence channel 44DCC using a Pannoramic 250
FLASH II slide scanner (3D Histech). The resulting image files were uploaded
30 into viewer software (Pannoramic CaseViewer) and adjusted for optimal brightness and contrast, for manual assessment of the staining result.

Results

Selectivity evaluation of recombinant antibodies by direct ELISA: The six produced recombinant antibodies 4E6, 7B10, 7C7, 17G4, 23D5 and 28F2 were screened for reactivity towards the BSA-conjugated peptides 198-203, 199-204 or 200-205 and the ApoE recombinant fragment aa 200-299. In addition, absence of reactivity towards recombinant full-length ApoE (ApoE4 1-299) and the negative control peptide (SEQ ID NO:53) conjugated to BSA was studied.

All recombinant antibodies demonstrated strong binding to the N-terminal neo-epitope peptide G200 (SEQ ID NO:50) and to the recombinant C-terminal ApoE fragment G200-HIS (aa 200-299; SEQ ID NO:48), whereas no binding to recombinant ApoE4 full-length protein (SEQ ID NO:49) or to the negative control peptide (SEQ ID NO:53) was shown (Figure 16). In addition, no binding to the 198-203 or 199-204 BSA-conjugated peptides (SEQ ID NOs:51 and 52) was observed using the recombinant anti-G200 antibodies. On the other hand, the reference antibody directed to the ApoE C-terminal could detect and bind the recombinant G200 fragment (aa 200-299) and the recombinant ApoE4 full-length protein (aa 1-299) equally well. Because the epitope for the reference antibody is ApoE aa 274-299, no binding to the G200-BSA peptide was shown, as expected (Figure 16).

No difference was found in the second direct ELISA, in which the coat consisted of recombinant G200-HIS C-terminal ApoE fragment (SEQ ID NO:48) and recombinant full-length ApoE4 (SEQ ID NO:45) added at equal molarities (0.1 μ M) (Figure 19). The calculated EC50 values are shown in Table 5 below.

Selectivity evaluation and IC50 determination of recombinant antibodies by inhibition ELISA: The six produced recombinant antibodies 4E6, 7B10, 7C7, 17G4, 23D5 and 28F2 were screened for binding strength and binding selectivity towards the N-terminal neo-epitope peptide G200-BSA (aa 200-205) and the recombinant C-terminal ApoE fragment G200-HIS (aa 200-299). In addition, antibodies were screened for lack of reactivity towards BSA-conjugated peptides 198-203 and 199-204, and recombinant full-length ApoE4.

All recombinant antibodies demonstrated strong binding and selectivity for the G200-BSA peptide (aa 200-205) and the recombinant G200 fragment (aa 200-299), whereas no binding to recombinant ApoE4 full-length protein (aa 1-299) was shown (Figure 17). In addition, no binding was shown to 198-203 or 199-204 BSA-conjugated peptides. Increasing the concentration of the antigen 10-fold to a starting concentration of 10000 ng/ml did not show any binding to the 198-203, 199-204, and 192-206 BSA-conjugated peptides either (Figure 20). The calculated IC₅₀ values (ng/ml and nM values) are shown in Table 5 below.

10

Clone	EC ₅₀ (ng/ml)		IC ₅₀		
	G200-BSA peptide	G200-HIS fragment	G200-BSA peptide (ng/ml)	G200-HIS fragment (nM)	G200-BSA peptide (ng/ml) 2 nd protocol
4E6	6.9	9.5	2.9	4.0	11.5
7B10	7.3	10.7	2.2	2.8	5.8
7C7	13.0	18.1	3.1	2.8	6.3
17G4	5.1	7.9	2.9	3.0	5.8
23D5	11.6	10.1	2.8	2.4	3.8
28F2	7.2	21.5	3.2	2.4	3.7

Table 5. Summary of results from direct and inhibition ELISA analysis of recombinant anti-G200 antibodies

Selectivity evaluation and K_D determination of recombinant antibodies by bio-layer interferometry: Binding interactions between the recombinant G200 C-terminal ApoE fragment (ligand) and recombinant antibodies (analyte) were evaluated by using an Octet RED384 instrument (ForteBio). The results from the Octet experiment are outlined in Figure 18 and summarized in Table 6 below.

Selectivity evaluation and K_D determination of recombinant antibodies by surface plasmon resonance: The six recombinant antibodies 4E6, 7B10, 7C7, 17G4, 23D5, and 28F2 were evaluated for selectivity and their K_D values determined, by surface plasmon resonance.

All recombinant antibodies demonstrated binding to the G200-HIS fragment (Figure 21), whereas no binding was shown to recombinant full-length ApoE. The calculated k_{a1}, k_{d1} and K_{D1} values are shown in Table 6 below.

Clone	Octet: G200-HIS fragment on biosensor K_D (nM)	Biacore: G200-HIS fragment		
		Bivalent analyte k_a ($\times 10^5$ 1/Ms)	k_d ($\times 10^{-3}$ 1/s)	K_D (nM)
4E6	0.9	815	6.0	1030
7B10	0.8	12.0	3.8	330
7C7	1.9	28.8	2.9	417
17G4	0.8	87.3	2.4	141
23D5	5.2	2.6	10.5	299
28F2	9.7	49.8	3.5	9830

Table 6. Summary of results from biolayer interferometry analysis and surface plasmon resonance of recombinant anti-G200 antibodies

- 5 Selectivity evaluation and human target binding in Alzheimer's disease brain extracts by Western blot analysis: The recombinant antibodies 4E6, 7B10, 7C7, 17G4, 23D5 and 28F2 were tested for their ability to bind selectively to ApoE fragments ≤ 12 kDa in human brain extracts from an Alzheimer's disease patient, while not binding to full-length ApoE. Western
- 10 blot analysis demonstrated binding of the recombinant antibodies to two ApoE fragments of approximately 12 kDa and 10 kDa in size, without any visual binding to full-length ApoE (Figure 22A). Re-staining of the Western blot membranes with a polyclonal anti-ApoE antibody showed staining of full-length ApoE, high molecular weight (HMW) ApoE fragments (~ 20 -25 kDa)
- 15 and distinct 12 and 10 kDa ApoE fragments (Figure 22B).

- Selectivity evaluation and human target binding in Alzheimer's disease brain extracts by immunoprecipitation/Western blot analysis: The recombinant antibodies 4E6, 7B10, 7C7, 17G4, 23D5 and 28F2 were tested for their ability to find and bind selectively to ApoE fragments ≤ 12 kDa in solution, in human
- 20 brain extracts from Alzheimer's disease patients. Immunoprecipitation (IP) of brain extracts from Alzheimer's disease patients using the recombinant anti-G200 antibodies, followed by Western blot using an anti-ApoE C-terminal antibody, demonstrated immunoprecipitation of a 12 kDa ApoE fragment by four of the recombinant anti-G200 antibodies, namely 4B10, 7C7, 17G4 and
- 25 28F2. No full-length ApoE was immunoprecipitated from the brain extracts.

Results from the Western blot also show intense bands for the IP antibody heavy and light chain (Figure 22C).

- Human target engagement in Alzheimer's disease brain by immunohistochemistry: Immunohistochemical staining of brain sections from
- 5 Alzheimer's disease (APOE $\epsilon 3/\epsilon 3$ and $\epsilon 4/\epsilon 4$) individuals demonstrates weak binding of 7C7 to amyloid plaques when compared to total A β IHC and ApoE reference antibodies (Figure 23).

Example 13

- 10 Generation and screening of antibodies to the N-terminal ApoE fragment neo-epitope L198

This Example describes the immunization of BALB/c and C57Bl/6 mice and subsequent generation and screening of hydridoma cell lines.

15

Materials and methods

- Peptide synthesis: The immunogen used in this experiment was designed to incorporate one of the N-terminal neo-epitopes of the neurotoxic ApoE fragment identified in Examples 1-7. As its N-terminal sequence, the
- 20 immunogen comprised the amino acid residues corresponding to amino acid residues 198-205 in full-length ApoE. This N-terminal sequence was coupled C-terminally to a 6-aminocaproic acid linker (Acp; also denoted aminohexanoic acid linker (Ahx)), followed by a cysteine residue for the
- 25 purposes of conjugation to for example keyhole limpet hemocyanin (KLH) or bovine serum albumin (BSA) as indicated. The entire immunogen sequence used was LAGQPLQE-Acp-C (SEQ ID NO:54). SEQ ID NO:54 was prepared by Innovagen AB and delivered at 95.9 % purity. In addition, the shorter
- 30 peptide LAGQPL-Acp-C (SEQ ID NO:52), corresponding to amino acids 198-203, was used for screening for positive clones against the neo-epitope. SEQ ID NO:52 was prepared by Innovagen AB and delivered at 95.2 % purity.

Additionally, as in Example 9, ApoE-derived peptides were prepared which incorporated the other two identified putative N-terminal neo-epitopes of the neurotoxic ApoE fragment, as well as a negative control peptide without

any of the identified neo-epitopes. These peptides were AGQPLQ-Acp-C (SEQ ID NO:51, prepared by Innovagen AB and delivered at 96.7 % purity), GQPLQE-Acp-C (SEQ ID NO:50, prepared by Innovagen AB and delivered at 95.5 % purity) and the negative control peptide AATVGSLAGQPLQER-Acp-C
5 (SEQ ID NO:53, prepared by Innovagen AB and delivered at 97.8 % purity).

Immunization: 6-8 weeks old BALB/c or C57Bl/6 mice were immunized with SEQ ID NO:54 conjugated to KLH. In the first injection (s.c.), the immunogen was given together with Freund's complete adjuvant. In subsequent injections (s.c.) except the last one, the immunogen was given in
10 Freund's incomplete adjuvant. Plasma samples were collected three weeks and five weeks after the first immunization. Every mouse received between 2-3 injections. The final immunization (booster dose) was administered intraperitoneally (i.p.) without adjuvant.

Plasma screening by direct ELISA: Plasma samples were analyzed by
15 ELISA for reactivity against the target peptide SEQ ID NO:54 conjugated to bovine serum albumin, BSA, and against the recombinant ApoE fragment L198-HIS (SEQ ID NO:57) to determine when to stop immunizations and initiate hybridoma generation. In addition, negative control peptide (SEQ ID NO:53) and ApoE 1-272 (SEQ ID NO:56) were tested for reactivity. Briefly,
20 96-well full area plates were coated with 1 µg/ml antigen, i.e. L198 N-terminal neo-epitope peptide (ApoE sequence 198-205 incorporated in SEQ ID NO:54) coupled to BSA, negative control peptide (ApoE sequence 192-206 incorporated in SEQ ID NO:53) coupled to BSA, or purified recombinant C-terminal ApoE fragment L198-HIS (SEQ ID NO:57) or ApoE 1-272-HIS (SEQ
25 ID NO:56). ELISA was run according to a standard protocol and a peroxidase-conjugated goat anti-mouse IgG antibody was used as detection antibody. Only mice that responded well to immunizations and gave positive responses towards the target peptide SEQ ID NO:54 conjugated to BSA and the recombinant ApoE fragment L198-HIS (SEQ ID NO:57), and a negative
30 response to ApoE 1-272 (SEQ ID NO:56) were considered interesting for fusion to hybridomas.

Generation of hybridomas: A selected mouse received a final booster dose of immunogen i.p. (no adjuvant) 4 days before generation of

hybridomas. Generation of hybridomas was performed by fusion with Sp2/0 cells and the fused cells were plated in 96-well plates. Primary screening was performed by ELISA using BSA-conjugated peptide and ApoE fragment, and positive clones were screened against the negative control peptide and ApoE
5 1-272 fragment. Supernatant from the best responders (20 clones) were characterized further for selection of clones to be processed for limiting dilution assay to ensure monoclonality. After a limiting dilution assay, unpurified supernatant was subjected to further characterization. The clones of interest were cryopreserved and expanded for production and purification
10 of antibody (Innovagen AB) and subjected to sequencing (Absolute Antibodies).

Hybridoma screening by direct ELISA: ELISA experiments to identify hybridoma clones that produced antibodies with reactivity against the target epitope were performed according to standard protocols as described above
15 under "Plasma screening by direct ELISA" and the corresponding section of Example 9. During screening of hybridomas, and to reach monoclonality, six different antigens were used at a concentration of 1 µg/ml. These were the two L198 N-terminal neo-epitope peptides (ApoE sequence 198-205 incorporated in SEQ ID NO:54 and ApoE sequence 198-203 incorporated in
20 SEQ ID NO:52) coupled to BSA, negative control peptide (SEQ ID NO:53) coupled to BSA, recombinant C-terminal ApoE fragment L198-HIS (SEQ ID NO:57), recombinant ApoE4 1-272 fragment (SEQ ID NO:56), and recombinant full-length ApoE4 (SEQ ID NO:45, Abcam, cat. no. ab50243).

For the hybridoma screening ELISAs, "positive" wells were selected
25 based on positive response towards the target peptide SEQ ID NO:54 conjugated to BSA and the recombinant ApoE fragment L198-HIS (SEQ ID NO:57) and the presence of clone(s). The identified positive clones were then subjected to negative screenings using the same ELISA protocol, with negative control peptide (SEQ ID NO:53) coupled to BSA, and ApoE fragment
30 1-272 (SEQ ID NO:56) as coat for the plates. Supernatants from the top 20 clones that showed no binding in the negative screening were further characterized by determination of IgG concentration, by establishing concentration-response curves towards the L198 N-terminal neo-epitope

peptide coupled to BSA, the recombinant ApoE fragment L198-HIS and ApoE4 full-length protein (performed as in Example 9), and by studying human target engagement by Western blot.

Determination of IgG concentration by direct ELISA: A standard ELISA
5 protocol was used to determine the IgG concentration in the hybridoma supernatants. To be able to measure IgG concentration, plates were coated with a 0.5 µg/ml solution of an α-mouse IgG, F(ab')₂ specific antibody, and a reference antibody with a known IgG concentration was used as standard. The optical density at 450 nm was plotted against the antibody concentration
10 to generate concentration-response curves. IgG concentrations for the hybridoma supernatants were calculated from the curve fit.

Selectivity evaluation and human target binding in Alzheimer's disease brain extracts by Western blot analysis: Western blot analysis was used to evaluate selective binding of monoclonal antibodies with affinity for the N-
15 terminal neo-epitope of ApoE fragments ≤12 kDa, starting at L198, without binding to ApoE full-length protein, in a human brain extract from an Alzheimer's disease patient. The same protocol as in Example 10 was used, except that a total protein content of 60 µg in the RIPA 2 % SDS brain extract was mixed with 2x Laemmli sample buffer.

20 Based on the result from IgG concentration, ELISA and Western blot, the top five candidate clones were subjected to at least two rounds of limiting dilution assay to ensure monoclonality, and screened for binding to the L198-HIS fragment (SEQ ID NO:57) and the target peptide SEQ ID NO:54 conjugated to BSA. Furthermore, IgG concentrations were determined and
25 concentration-response curves towards the recombinant ApoE fragment L198-HIS, the target peptide SEQ ID NO:54 conjugated to BSA, and ApoE4 full-length protein were performed as described in Example 10. Clones that still bound L198-HIS fragment but not full-length ApoE4 and continued to grow were considered especially interesting for further characterization.

30 Antibodies were produced from these clones and purified to generate purified monoclonal antibodies (Innovagen AB), and the clones were sent for sequencing (Absolute Antibodies).

Results

Generation of monoclonal antibodies by hybridoma technology:

Antibodies that bind selectively to the N-terminal neo-epitope starting at amino acid L198 of the ApoE protein were generated by immunizations using
5 an ApoE specific sequence consisting of the eight first amino acids following the N-terminal in the 198-299 ApoE fragment. The shortness of the immunization peptide was considered necessary in order to enable generation of antibodies that bind selectively to the N-terminal neo-epitope starting at amino acid L198 of the ApoE protein, without any binding to the
10 linear epitope found in full-length ApoE protein. The ApoE-specific sequence peptide 198-205, conjugated to keyhole limpet haemocyanin (KLH) via an Acp linker and a cysteine residue, was used in the immunizations. The plasma samples were analyzed by ELISA for reactivity against the corresponding peptide conjugated to BSA (to avoid detecting reactivity
15 towards KLH), and against recombinant ApoE fragment. The best responder of the immunized mice (a C57BL/6 mouse) was chosen for hybridoma generation.

ELISA screening for antibodies that bind selectively to the N-terminal neo-epitope of ApoE fragment starting at L198: Generated hybridoma clones
20 were screened for reactivity towards the recombinant ApoE fragment L198-HIS, as well as reactivity towards the target peptide SEQ ID NO:54 conjugated to BSA. In addition, lack of reactivity towards recombinant full-length ApoE4, recombinant ApoE fragment 1-272-HIS, and negative control peptide SEQ ID NO:53 conjugated to BSA was evaluated. Twenty clones
25 were identified as antibodies selective for the N-terminal neo-epitope of ApoE fragments starting at amino acid L198 and were evaluated further.

Determination of IgG concentration: The range of IgG concentration was between 0.3-11.3 µg/ml. Antibody concentration was normalized in cell medium, so that the starting concentration in ELISA experiments would be the
30 same.

ELISA screening of top twenty antibodies that bind selectively to the N-terminal neo-epitope of ApoE fragment starting at L198: All antibodies were reactive towards the target peptide SEQ ID NO:52 conjugated to BSA and the

recombinant ApoE fragment L198-HIS, but not towards recombinant full-length ApoE4. The top ten responders were further evaluated by Western blot.

Selectivity evaluation and human target binding in Alzheimer's disease

- 5 brain extracts by Western blot analysis: All antibodies were able to bind to the recombinant ApoE fragment L198-HIS, but only 9 of 10 bound to fragments in a human brain extract.

Of the twenty top responders, five were chosen based on IgG concentration, direct ELISA, and binding to human target. These clones were
10 subjected to limiting dilution assay to ensure monoclonality and continued reactivity towards the recombinant ApoE fragment L198-HIS, and the target peptides SEQ ID NO:54 and SEQ ID NO:52 conjugated to BSA, but not towards recombinant full-length ApoE4. All five clones were identified as
15 antibodies selective for the N-terminal neo-epitope of ApoE fragments starting at amino acid L198, and denoted 6F4, 8B3, 12B1, 15F8 and 15H11. All were produced and purified as monoclonal antibodies and clones were sent for sequencing.

Example 14

- 20 Hybridoma sequencing and isotype determination of antibodies to the N-terminal ApoE fragment neo-epitope L198

Materials and methods

- Hybridoma sequencing: Hybridoma clones producing the monoclonal
25 antibodies generated and characterized in Example 13, having a demonstrated selectivity for the N-terminal neo-epitope of ApoE fragment starting at amino acid L198, and a proven human target binding in brain extracts from Alzheimer's disease, were sent to Absolute Antibody for sequencing. Briefly, hybridoma sequencing was performed by whole
30 transcriptome shotgun sequencing. The DNA and protein sequences of the mature VH and VL regions were identified.

Results

Hybridoma sequencing: Hybridoma clones with a demonstrated selectivity for the N-terminal neo-epitope of ApoE fragment starting at amino acid L198, in addition to a demonstrated binding to human target in brain extracts from Alzheimer's disease, were sequenced.

The following hybridoma clones were sequenced: 6F4, 8B3, 12B1, 15F8 and 15H11. The amino acid sequences of the entire antibodies were obtained. Amino acid sequences obtained for the respective variable heavy (VH) and variable light (VL) chains are given in Table 7 below.

10

Region	Antibody	SEQ ID NO:
	6F4	
VH	EVNLEESGGDLVQPGRSLKLSVASGFTFSDYWMNWVRQSPEKGLE WVAQIRLKSDNYVTHYVESLKGRFTISRDDSRSCVYLQMNNLRAEDTG IYYCTGHILLRRRYFDVWGTGTTVTSS	82
VL	DIVMTQSQKFMSTSVGDRVSVTCEASQNVGTNVAWYQQKPGQSPKG LIYSASYRYSQVPDRFTGSGSGTDFTLTISTVQSEDLAEYFCHQFNTYP YTFGGGTLEII	83
	8B3	
VH	EVKVEESGGGVVQPGGSMKLSCLASGFTFRNYWMNWVRQSPEKGL EWVAQIRLKSDNYATRYAESVKGRFTISRDDSKSSVYLQMNNLRAEDT GTYCSCGHILLRYYALDFWQGQTSVTSS	84
VL	DIVLTQSQKFMSTSVGDRVSVTCKASQNVYTDVAWYQQKPGQSPKG MIYSASYRYSQVPDRFTGSGSGTDFTLISNVQSEDLAEYFCQQYNSF PYTFGGGKLEIK	85
	12B1	
VH	EVKLEESGGGLVQPGGSMKLSVASGITFSKYWMNWVRQCPEKGLE WVAQIRLRSDNYATHYAESVKGRFTISRDDSKSSVYLQMNLTLEEDTG IYYCTHHYGGSGYVDVWGTGTTVTSS	86
VL	DIVMTQSQKFMSTSVGDRVSVTCKASQNVGTNVAWYQQKPGQCPKG MILSASQRYSGVPDRFTGSGFGTEFTLTISNVQSEDLAEYFCQQYNSY PYTFGGGKLEIK	87
	15F8	
VH	EVKLEESGGGLVHPGGSMKLSVSGSFTFSNYWMNWVRQSPEKGLE WVAQIKLRSDNYATHYAESVKGKFTISRDDAKSSVYLQMDNLRAEDTG IYYCSHHYLDGGYFDVWGTGTSVTSS	88
VL	DIVMTQSQKFMSTSVGDRVSVTCKASQNVGINVAWYQDKPGQSPKGL ILSASYRYSQVPDRFTGSGSGTDFTLTISNVQSEDLAEYFCQQYNSYP YTFGGGKLEIK	89

	15H11	
VH	EVKLEESGGGLVQPGGAMKLSKVASGITFSNYWMNWVRQCPEKGLE WVAQIRLKSDNYATHYAESVKGRFTISRDDSKSSVYLQMNTLRAEDTG IYYCTHHYYGSSGYVDVWGTGTTVTVSS	90
VL	DIVMTQSQKFMSTSVGDRVSVTCKASQNVGNNVAWYQKKPGQCPKG MILSASQRYSGVPDRFTGSGFGTEFTLIISNVQSEDLAEYFCHQYNSYP YTFGGGKLERK	91

Table 7. Variable region sequences

The complementarity determining regions (CDRs) were identified using the Kabat definition for the primary VH and VL sequences and are given in Table 8 below.

5

Antibody	CDR H1	CDR H2	CDR H3
6F4	DYWMN (SEQ ID NO:59)	QIRLKSDNYVTHYVESLKG (SEQ ID NO:60)	HILLRRRYFDV (SEQ ID NO:61)
8B3	NYWMN (SEQ ID NO:62)	QIRLKSDNYATRYAESVKG (SEQ ID NO:63)	HILLRYALDF (SEQ ID NO:64)
12B1	KYWMN (SEQ ID NO:65)	QIRLRSDNYATHYAESVKG (SEQ ID NO:66)	HYYGSSGYVDV (SEQ ID NO:67)
15F8	NYWMN (SEQ ID NO:62)	QIKLRSDNYATHYAESVKG (SEQ ID NO:68)	HYLDSSGGYFDV (SEQ ID NO:69)
15H11	NYWMN (SEQ ID NO:62)	QIRLKSDNYATHYAESVKG (SEQ ID NO:70)	HYYGSSGYVDV (SEQ ID NO:67)
	CDR L1	CDR L2	CDR L3
6F4	EASQNVGTNVA (SEQ ID NO:71)	SASYRYS (SEQ ID NO:72)	HQFNTYPYT (SEQ ID NO:73)
8B3	KASQNVYTDVA (SEQ ID NO:74)	SASYRYS (SEQ ID NO:72)	QQYNSFPYT (SEQ ID NO:75)
12B1	KASQNVGTNVA (SEQ ID NO:76)	SASQRYYS (SEQ ID NO:77)	QQYNSYPYT (SEQ ID NO:78)
15F8	KASQNVGINVA (SEQ ID NO:79)	SASYRYS (SEQ ID NO:72)	QQYNSYPYT (SEQ ID NO:78)
15H11	KASQNVGNNVA (SEQ ID NO:80)	SASQRYYS (SEQ ID NO:77)	HQYNSYPYT (SEQ ID NO:81)

Table 8. CDR region sequences

The antibodies were isotyped based on their sequences, and their respective sub-class and light chain are summarized in Table 9 below.

Clone	Sub-class	Light chain
6F4	IgG1	K
8B3	IgG2b	K
12B1	IgG2c	K
15F8	IgG2c	K
15H11	IgG1	K

10 Table 9. Isotyping results

Example 15

Characterization of purified monoclonal antibodies to the N-terminal ApoE fragment neo-epitope L198

5 This example describes the characterization of the purified monoclonal antibodies described in Examples 13 and 14 by various methods, including direct ELISA, inhibition ELISA, surface plasmon resonance, Western blot on human brain extract and immunoprecipitation on human brain extract.

10 *Materials and methods*

Selectivity evaluation of purified monoclonal antibodies by direct ELISA: The direct ELISA described below was used to evaluate the binding selectivity of the purified monoclonal antibodies produced in Example 14. The ability to selectively bind to the L198 N-terminal neo-epitope ApoE peptide
15 (BSA-conjugated SEQ ID NO:52) and to the recombinant L198-HIS C-terminal ApoE fragment resulting from cleavage in the hinge region (SEQ ID NO:57) was compared with binding to the A199, G200 and negative control peptides (BSA-conjugated SEQ ID NO:51, 50 and 53, respectively), and to recombinant full-length ApoE4 (ApoE sequence aa 1-299; SEQ ID NO:45).

20 The screening was performed according to standard ELISA protocols. Briefly, 1 µg/ml solutions of BSA-conjugated neo-epitope peptides and negative control peptide, and 0.1 µM recombinant C-terminal ApoE fragment and full-length ApoE (Abcam, cat. no. ab50243) were prepared by dilution in PBS. 50 µl/well were added to an ELISA half-area 96 well microtiter plate,
25 and the plate was sealed with adhesive sealer and incubated over night at 4 °C. After discarding the solution, the plates were blocked with 150 µl/well of protein-free blocking solution (Pierce) for 1 h at room temperature with shake (600-900 rpm). The plates were washed four times with washing buffer containing 0.28 mM NaH₂PO₄, 2.5 mM Na₂HPO₄, 150 mM NaCl, 0.1 %
30 TWEEN®-20 and 0.0075 % Kathon CG. Purified monoclonal antibodies of interest were 3-fold serially diluted with a starting dilution at 3 µg/ml (mixed 1:2 with dilution buffer (PBS with 0.1 % BSA and 0.05 % TWEEN®-20)). An anti-ApoE C-terminal antibody was used as a reference antibody (ApoE E-8

mouse monoclonal, Santa Cruz Biotechnology, cat. no. sc-393302). 50 µl/well were added into the ELISA plates and incubated for 2 h at room temperature with shake (600-900 rpm). The plates were washed four times as described previously. Detection antibody (HRP-conjugated anti-mouse IgG, Southern Biotech, cat. no. 1030-05, diluted 1:10000 in dilution buffer) was added 50 µl/well, and the plates were incubated for 1 h at room temperature with shake (600-900 rpm). After another wash (as described previously), 50 µl/well of K-Blue® aqueous substrate (Neogen) were added, and the reaction was stopped after 5-15 min with 50 µl/well of 0.5 M H₂SO₄. The optical density at 450 nm was read using an ELISA reader (Tecan). The optical density was plotted against the antibody concentration to generate concentration-response curves (Figure 24) and the EC₅₀ values were determined from the log agonist concentration response curve.

Selectivity evaluation and IC₅₀ determination of purified monoclonal antibodies by inhibition ELISA: The inhibition ELISA described below was used to evaluate the binding strength and selectivity for the purified monoclonal antibodies described in Examples 13 and 14. The ability of the purified monoclonal antibodies to bind to the N-terminal neo-epitope of synthetic ApoE peptides starting at amino acid L198 was evaluated in comparison with their ability to bind to synthetic ApoE peptides starting at amino acids A199 and G200, as well as to the negative control peptide (aa 192-206) in solution.

In brief, the purified monoclonal antibody to be tested was allowed to interact with the N-terminal neo-epitope of synthetic ApoE peptides conjugated to BSA and starting at amino acid L198, A199 or G200, or to BSA-conjugated negative control peptide (SEQ ID NO:52, 51, 50 and 53, respectively). Thereafter, the mix was added to a microtiter plate coated with the BSA-coupled L198 synthetic ApoE peptide. If the purified monoclonal antibody binds to any of the antigens in the pre-incubation step (the synthetic ApoE peptides), the antibody is prevented from binding to the synthetic L198 ApoE peptide immobilized on the microtiter plate. This leads to inhibition of the ELISA detection signal.

0.5 µg/ml solution of N-terminal neo-epitope peptide L198 (SEQ ID NO:52) conjugated to BSA was prepared by dilution in PBS. 50 µl/well were added to an ELISA half-area 96 well microtiter plate, the plate was sealed with adhesive sealer and incubated over night at 4 °C. After discarding the solution, the plates were blocked with protein-free blocking solution (Pierce) (150 µl/well) at room temperature for 90 min with shaking (900 rpm). The blocked plate was washed four times with washing buffer containing 0.28 mM NaH₂PO₄, 2.5 mM Na₂HPO₄, 150 mM NaCl, 0.1 % TWEEN®-20 and 0.0075 % Kathon CG.

Serial dilutions of each synthetic ApoE peptide were prepared by 4-fold dilution starting from 10000 ng/ml in 96 well storage plates (30 µl final volume). Each recombinant antibody to be tested was added (30 µl) to the serial dilutions of each of the synthetic ApoE peptides at a final concentration of 0.05 µg/ml per well. Samples were pre-incubated for 45 min at room temperature with shaking (900 rpm).

The pre-incubated samples were transferred (50 µl/well) to the blocked ELISA plates, and plates were incubated for 10 min at room temperature without shaking. Plates were washed as described above. Alkaline phosphatase conjugated anti-mouse IgG detection antibody (Mabtech, cat. no. 3310-4) was diluted 1:1000 and added to each plate (50 µl/well). The plate was sealed and incubated with shaking (900 rpm) for 45 min at room temperature and subsequently washed as described above. Alkaline phosphatase substrate (50 µl/well) was added to the plate and the optical density was read every 10 min at a wavelength of 405 nm for up to 120 min. The IC₅₀ values were determined from a log inhibitor concentration response curve (Figure 25).

Selectivity evaluation and K_D determination of purified monoclonal antibodies by surface plasmon resonance: Binding interactions between the antigens and antibodies were evaluated by surface plasmon resonance (SPR) using a Biacore 8K instrument (GE Healthcare) according to standard procedures.

To determine the binding affinity of the monoclonal antibodies for N-terminal neo-epitopes compared to full-length ApoE, single-cycle kinetics

experiments were conducted. The recombinant fragments were immobilized onto a CM5 Sensor Chip (GE Healthcare, cat. no. 29104988) using an amine coupling kit (GE Healthcare, cat. no. BR100050) according to the manufacturer's instructions. Reference (no immobilized antigen) and active surfaces were treated with the same conditions using the amine coupling reagents on flow cell 1 (Fc1) and flow cell 2 (Fc2) respectively. The immobilization level for the active surfaces was kept at approximately 150-200 response units (RU). The same protocol setup was used to immobilize full-length ApoE on the CM5 chip surface.

10 Purified antibodies were prepared in 2-fold serial dilution from 14 to 0.3 nM (in 5-7 steps). Next, the prepared serial dilution of purified antibodies was injected (30 μ l/min, contact time 360 s, dissociation time 2500 s) over both flow cells of the sensor chip. The interaction series was done in triplets. Values were blank subtracted, and a bivalent analyte binding kinetics fit
15 model was used for the evaluation.

In all SPR experiments, 1xPBS-P+ (GE Healthcare, cat. no. 28995084) was used to dilute antibodies and target antigens. Experiments were performed at 25 °C.

Selectivity evaluation and human target binding in Alzheimer's disease
20 brain extracts by Western blot analysis: The Western blot analysis described below was used to evaluate selective binding of monoclonal antibodies with affinity for the N-terminal neo-epitope of ApoE fragments ≤ 12 kDa, starting at L198, without binding to ApoE full-length protein, in human brain extracts from an Alzheimer's disease patient. Fresh frozen brain tissue from the
25 Alzheimer's disease patient was homogenized in RIPA 2 % SDS extraction buffer, followed by centrifugation at 16000 x g for 1 h. The subsequent supernatant was subjected to standard protein concentration determination.

RIPA 2 % SDS brain extract containing approximately 60 μ g total protein was mixed with 2x Laemmli sample buffer, boiled for 5 min at 95 °C
30 and loaded onto SDS-PAGE gels (Bolt™ 12 % Bis-Tris Plus 10 well, Thermo Fisher, cat. no. NW00120BOX). Gels were run for 30-40 min at 180 V, after which proteins were transferred from the gels onto nitrocellulose membranes using the Trans-Blot® Turbo™ system (Bio-Rad). Membranes were blocked in

Intercept® PBS Blocking Buffer (LI-COR) for 1 h and incubated over night at room temperature with monoclonal antibodies with affinity for the N-terminal neo-epitope of ApoE fragments ≤ 12 kDa starting at L198 (2 $\mu\text{g/ml}$ in Intercept® PBS Blocking Buffer (LI-COR) with 0.1 % TWEEN®-20).

- 5 Membranes were washed and incubated for 1 h at room temperature with the detection antibody anti-mouse-800CW (LI-COR, cat. no. 925-32210) diluted 1:25000 in Intercept® PBS Blocking Buffer (LI-COR) with 0.1 % TWEEN®-20. Membranes were washed and images acquired using Odyssey® FC (LI-COR).

- 10 To confirm that the obtained bands on the Western blot membranes were of ApoE origin, the membranes were re-stained over night with a polyclonal anti-ApoE antibody (Calbiochem, cat. no. 178479; immunogen ApoE aa 1-299), diluted 1:2000 in Intercept® PBS Blocking Buffer (LI-COR) with 0.1 % TWEEN®-20. Membranes were washed and incubated for 1 h at
15 room temperature with detection antibody anti-goat-680RD (LI-COR, cat. no. 925-68074) diluted 1:25000 in Intercept® PBS Blocking Buffer (LI-COR) with 0.1 % TWEEN®-20. Membranes were washed and images acquired using Odyssey® FC (LI-COR).

Selectivity evaluation and human target binding in Alzheimer's disease

- 20 brain extracts by immunoprecipitation and Western blot analysis: The immunoprecipitation/Western blot analysis described below was used to evaluate selective binding of monoclonal antibodies with affinity for the N-terminal neo-epitope of ApoE fragments ≤ 12 kDa starting at L198, without binding to ApoE full-length protein, in human brain extracts from Alzheimer's
25 disease patients. Fresh frozen brain tissue from Alzheimer's disease patients was homogenized in RIPA 2 % SDS extraction buffer, followed by centrifugation at 16000 x g for 1 h. The subsequent supernatants were subjected to standard protein concentration determination.

- RIPA 2 % SDS brain extract containing approximately 75 μg total
30 protein was mixed with monoclonal antibody with affinity for the N-terminal neo-epitope of ApoE fragments ≤ 12 kDa starting at L198, in IP buffer (PBS, 0.05% TWEEN®-20, 1% TritonX-100 and cOmplete™ Protease Inhibitor Cocktail) and incubated for 2 h (room temperature with head-to-tail rotation).

Dynabeads™ Protein A beads (Invitrogen) were added and the extract-antibody-dynabeads mixture was incubated for 1 h (room temperature with head-to-tail rotation). The supernatant was removed and the magnetic beads were washed. The immunoprecipitate was eluted from the beads by addition
5 of 2x Laemmli sample buffer and boiling for 5 min at 95 °C. The eluate was loaded onto a SDS-PAGE gel (Bolt™ 12 % Bis-Tris Plus 10 well, Thermo Fisher, cat. no. NW00120BOX) and run for 30-40 min at 180 V, after which proteins were transferred from the gels to nitrocellulose membranes using the Trans-Blot® Turbo™ system (Bio-Rad). Membrane was blocked in Intercept®
10 PBS Blocking Buffer (LI-COR) for 1 h and incubated over night at room temperature with anti-ApoE C-terminal antibody (Sigma, cat. no. sab2701946) diluted 1:1000 in Intercept® PBS Blocking Buffer (LI-COR) with 0.1 % TWEEN®-20. Membrane was washed and incubated for 1 h at room temperature with the detection antibody anti-rabbit-800CW (LI-COR, cat. no.
15 925-32211) diluted 1:25000 in Intercept® PBS Blocking Buffer (LI-COR) with 0.1 % TWEEN®-20. Membrane was washed and image acquired using Odyssey® FC (LI-COR).

Results

20 Selectivity evaluation of purified monoclonal antibodies by direct ELISA: The five purified monoclonal antibodies 6F4, 8B3, 12B1, 15F8 and 15H11 were screened for reactivity towards the BSA-conjugated peptides 198-203, 199-204 or 200-205 and the ApoE recombinant fragment aa 198-299. In addition, absence of reactivity towards recombinant full-length ApoE
25 (ApoE4 1-299) and the negative control peptide (SEQ ID NO:53) conjugated to BSA was studied.

 All purified monoclonal antibodies demonstrated strong binding to the N-terminal neo-epitope peptide L198 (SEQ ID NO:52) and to the recombinant C-terminal ApoE fragment L198-HIS (aa 198-299; SEQ ID NO:57). At the
30 highest concentration of antibody, four of the five antibodies (all except 6F4) showed some binding to recombinant ApoE4 full-length protein (SEQ ID NO:49), the negative control peptide (SEQ ID NO:53), and the 198-203 or

199-204 BSA-conjugated peptides (SEQ ID NO:51 and 52, respectively) (Figure 24). The calculated EC₅₀ values are shown in Table 10 below.

As expected, the reference antibody directed to the ApoE C-terminal could detect and bind both the recombinant L198 fragment (aa 198-299) and the recombinant ApoE4 full-length protein (aa 1-299). Also as expected, because the epitope for the reference antibody is ApoE aa 274-299, no binding to the L198-BSA peptide was detected (Figure 24).

Selectivity evaluation and IC₅₀ determination of purified monoclonal antibodies by inhibition ELISA: The five purified monoclonal antibodies 6F4, 8B3, 12B1, 15F8 and 15H11 were screened for binding strength and binding selectivity towards the N-terminal neo-epitope peptide L198-BSA (aa 198-203). In addition, antibodies were screened for lack of reactivity towards BSA-conjugated peptides 199-204, 200-205 and the negative control peptide 192-206.

All purified monoclonal antibodies demonstrated strong binding and selectivity for the L198-BSA peptide (aa 198-203), whereas there was no binding to 199-204, 200-205 or negative control BSA-conjugated peptides (Figure 25). The calculated IC₅₀ values are shown in Table 10 below.

Clone	EC ₅₀ (ng/ml)		IC ₅₀ (ng/ml)
	L198-BSA peptide	L198-HIS fragment	L198-BSA peptide
6F4	13.5	18.5	8.2
8B3	8.4	1.3	3.1
12B1	15.2	8.7	4.8
15F8	24.7	15.0	6.5
15H11	3.2	3.6	4.5

Table 10. Summary of results from direct ELISA and inhibition ELISA of purified monoclonal anti-L198 antibodies

Selectivity evaluation and K_D determination of purified monoclonal antibodies by surface plasmon resonance: The five purified monoclonal antibodies 6F4, 8B3, 12B1, 15F8 and 15H11 were evaluated for selectivity, and K_D values for the target antigen were determined by surface plasmon resonance. All purified monoclonal antibodies demonstrated binding to the L198-HIS fragment (Figure 26), whereas no binding was shown to

recombinant full-length ApoE. The calculated k_{a1} , k_{d1} and K_{D1} values are shown in Table 11 below.

Clone	Biacore: L198-HIS fragment		
	k_a ($\times 10^4$ 1/Ms)	k_d ($\times 10^{-6}$ 1/s)	K_D (nM)
6F4	51	7.1	2.2
8B3	4.2	302	12
12B1	77	128	20
15F8	74	205	40
15H11	61	7.2	1.6

Table 11. Summary of results from surface plasmon resonance of purified
5 monoclonal anti-L198 antibodies

Selectivity evaluation and human target binding in Alzheimer's disease brain extracts by Western blot analysis: The purified monoclonal antibodies selective for the N-terminal neo-epitope of ApoE fragments starting at amino acid L198 (6F4, 8B3, 12B1, 15F8 and 15H11) were tested for their ability to bind selectively to ApoE fragments ≤ 12 kDa in human brain extracts from an Alzheimer's disease patient, and without any binding to full-length ApoE. Western blot analysis demonstrated binding of three of the monoclonal antibodies (6F4, 8B3 and 12B1) to two ApoE fragments approximately 12 kDa and 10 kDa in size, without any visual binding to full-length ApoE (Figure 27A). Re-staining of the Western blot membranes with a polyclonal anti-ApoE antibody showed staining of full-length ApoE, high molecular weight (HMW) ApoE fragments (~ 20 -25 kDa) and a distinct 12 and 10 kDa ApoE fragment (Figure 27B).

20 Selectivity evaluation and human target binding in Alzheimer's disease brain extracts by immunoprecipitation/Western blot analysis: The monoclonal antibodies selective for the N-terminal neo-epitope of ApoE fragments starting at amino acid L198 (6F4, 8B3, 12B1, 15F8 and 15H11) were tested for their ability to find and bind selectively to ApoE fragments ≤ 12 kDa in solution, in
25 human brain extracts from Alzheimer's disease patients.

Immunoprecipitation (IP) of brain extracts from Alzheimer's disease patients using the monoclonal anti-L198 antibodies, followed by Western blot

using an anti-ApoE C-terminal antibody, demonstrated immunoprecipitation of a 12 kDa ApoE fragment by two of the monoclonal anti-L198 antibodies; 12B1 and 15F8 (Figure 27C). No full-length ApoE was immunoprecipitated from the brain extracts. Results from the Western blot also show intense bands for the IP antibody heavy and light chain.

Example 16

Generation and screening of antibodies to the N-terminal ApoE fragment neo-epitope A199

10

This Example describes the immunization of BALB/c and C57Bl/6 mice and subsequent generation and screening of hybridoma cell lines.

Materials and methods

15 Peptide synthesis: The immunogen used in this experiment was designed to incorporate one of the N-terminal neo-epitopes of the neurotoxic ApoE fragment identified in Examples 1-7. As its N-terminal sequence, the immunogen comprised the amino acid residues corresponding to amino acid residues 199-205 in full-length ApoE. This N-terminal sequence was coupled
20 C-terminally to a 6-aminocaproic acid linker (Acp; also denoted aminohexanoic acid linker (Ahx)), followed by a cysteine residue for the purposes of conjugation to for example keyhole limpet hemocyanin (KLH) or bovine serum albumin (BSA) as indicated. The entire immunogen sequence used was AGQPLQE-Acp-C (SEQ ID NO:55). SEQ ID NO:55 was prepared
25 by Innovagen AB and delivered at 96.9 % purity. In addition, a shorter peptide AGQPLQ-Acp-C (SEQ ID NO:51), corresponding to amino acids 199-204, was used for screening for positive clones against the neo-epitope. SEQ ID NO:51 was prepared by Innovagen AB and delivered at 96.7 % purity.

30 Additionally, as in Examples 9 and 13, ApoE-derived peptides were prepared which incorporated the other two identified putative N-terminal neo-epitopes of the neurotoxic ApoE fragment, as well as a negative control peptide without any of the identified neo-epitopes. These peptides were LAGQPL-Acp-C (SEQ ID NO:52, prepared by Innovagen AB and delivered at

95.2 % purity), GQPLQE-Acp-C (SEQ ID NO:50, prepared by Innovagen AB and delivered at 95.5 % purity) and the negative control peptide AATVGSLAGQPLQER-Acp-C (SEQ ID NO:53, prepared by Innovagen AB and delivered at 97.8 % purity).

- 5 Immunization: 6-8 weeks old BALB/c or C57Bl/6 mice were immunized with SEQ ID NO:55 conjugated to KLH. In the first injection (s.c.), the immunogen was given together with Freund's complete adjuvant. In subsequent injections (s.c.) except the last one, the immunogen was given in Freund's incomplete adjuvant. Plasma samples were collected three weeks
10 and five weeks after the first immunization. Every mouse received between 2-3 injections. The final immunization (booster dose) was administered intraperitoneally (i.p.) without adjuvant.

- Plasma screening by direct ELISA: Plasma samples were analyzed by ELISA for reactivity against the target peptide SEQ ID NO:55 conjugated to
15 bovine serum albumin, BSA, and against the recombinant ApoE fragment A199-HIS (SEQ ID NO:58) to determine when to stop immunizations and initiate hybridoma generation. In addition, negative control peptide (SEQ ID NO:53) and ApoE 1-272 (SEQ ID NO:56) were tested for reactivity. Briefly, 96-well full area plates were coated with 1 µg/ml antigen, i.e. A199 N-terminal
20 neo-epitope peptide (ApoE sequence 199-206 incorporated in SEQ ID NO:55) coupled to BSA, negative control peptide (ApoE sequence 192-206 incorporated in SEQ ID NO:53) coupled to BSA, or purified recombinant C-terminal ApoE fragment A199-HIS (SEQ ID NO:58) or ApoE 1-272-HIS (SEQ ID NO:56). ELISA was run according to a standard protocol and a
25 peroxidase-conjugated goat anti-mouse IgG antibody was used as detection antibody. Only mice that responded well to immunizations and gave positive responses towards the target peptide SEQ ID NO:55 conjugated to BSA and the recombinant ApoE fragment A199-HIS (SEQ ID NO:58), and a negative response to ApoE 1-272 (SEQ ID NO:56) were considered interesting for
30 fusion to hybridomas.

Generation of hybridomas: Two selected mice received a final booster dose of immunogen i.p. (no adjuvant) 4 days before generation of hybridomas. Generation of hybridomas was performed by fusion with Sp2/0

cells and the fused cells were plated in 96-well plates. Primary screening was performed by ELISA using BSA-conjugated peptide and ApoE fragment, and positive clones were then screened against the negative control peptide and ApoE 1-272 fragment. Supernatant from the best responders (20 clones)
5 were characterized further for selection of clones to be processed for limiting dilution assay to ensure monoclonality. After a limiting dilution assay, unpurified supernatant was subjected to further characterization. The clones of interest were cryopreserved and expanded for production and purification of antibody (Innovagen AB) and subjected to sequencing (Absolute
10 Antibodies).

Hybridoma screening by direct ELISA: ELISA experiments to identify hybridoma clones that produced antibodies with reactivity against the target epitope were performed according to standard protocols as described above under "Plasma screening by direct ELISA" and in the corresponding sections
15 of Examples 9 and 13. During screening of hybridomas, and to reach monoclonality, six different antigens were used at a concentration of 1 µg/ml. These were the two A199 N-terminal neo-epitope peptides (ApoE sequence 199-205 incorporated in SEQ ID NO:55 and ApoE sequence 199-204 incorporated in SEQ ID NO:51) coupled to BSA, negative control peptide
20 (SEQ ID NO:53) coupled to BSA, recombinant C-terminal ApoE fragment A199-HIS (SEQ ID NO:58), recombinant ApoE4 1-272 fragment (SEQ ID NO:56), and recombinant full-length ApoE4 (SEQ ID NO:45, Abcam, cat. no. ab50243).

For the hybridoma screening ELISAs, "positive" wells were selected
25 based on positive response towards the target peptide SEQ ID NO:55 conjugated to BSA and the recombinant ApoE fragment A199-HIS (SEQ ID NO:58) and the presence of clone(s). The identified positive clones were then subjected to negative screenings using the same ELISA protocol, with negative control peptide (SEQ ID NO:53) coupled to BSA, and ApoE fragment
30 1-272 (SEQ ID NO:56) as coat for the plates. Supernatants from the top 20 clones that showed no binding in the negative screening were further characterized by determination of IgG concentration, by establishing concentration-response curves towards the A199 N-terminal neo-epitope

peptide coupled to BSA, the recombinant ApoE fragment A199-HIS and ApoE4 full-length protein (performed as in Examples 9 and 13), and by studying human target engagement by Western blot.

Determination of IgG concentration by direct ELISA: A standard ELISA
5 protocol was used to determine the IgG concentration in the hybridoma supernatants. To be able to measure IgG concentration, plates were coated with a 0.5 µg/ml solution of an α-mouse IgG, F(ab')₂ specific antibody, and a reference antibody with a known IgG concentration was used as standard. The optical density at 450 nm was plotted against the antibody concentration
10 to generate concentration-response curves. IgG concentrations for the hybridoma supernatants were calculated from the curve fit.

Selectivity evaluation and human target binding in Alzheimer's disease brain extracts by Western blot analysis: Western blot analysis was used to evaluate selective binding of monoclonal antibodies with affinity for the N-
15 terminal neo-epitope of ApoE fragments ≤12 kDa, starting at A199, without binding to ApoE full-length protein, in a human brain extract from an Alzheimer's disease patient. The same protocol as in Examples 10 and 13 was used, and a total protein content of 60 µg in the RIPA 2 % SDS brain extract was mixed with 2x Laemmli sample buffer.

20 Based on the result from IgG concentration, ELISA and Western blot, the top four candidate clones were subjected to at least two rounds of limiting dilution assay to ensure monoclonality, and screened for binding to the A199-HIS fragment (SEQ ID NO:58) and the target peptide SEQ ID NO:55 conjugated to BSA. Clones that still bound A199-HIS fragment and continued
25 to grow were considered especially interesting for further characterization. Antibodies were produced from these clones and purified to generate purified monoclonal antibodies (Innovagen AB), and the clones were sent for sequencing (Absolute Antibodies).

30 *Results*

Generation of monoclonal antibodies by hybridoma technology:
Antibodies that bind selectively to the N-terminal neo-epitope starting at amino acid A199 of the ApoE protein were generated by immunizations using

an ApoE specific sequence consisting of the seven first amino acids following the N-terminal in the 199-299 ApoE fragment. The shortness of the immunization peptide was considered necessary in order to enable generation of antibodies that bind selectively to the N-terminal neo-epitope starting at amino acid A199 of the ApoE protein, without any binding to the linear epitope found in full-length ApoE protein. The ApoE-specific sequence peptide 199-205, conjugated to keyhole limpet haemocyanin (KLH) via an Acp linker and a cysteine residue, was used in the immunizations. The plasma samples were analyzed by ELISA for reactivity against the corresponding peptide conjugated to BSA (to avoid detecting reactivity towards KLH), and against recombinant ApoE fragment. The best responders of the immunized mice (one C57BL/6 mouse and one BALB/c mouse) were chosen for hybridoma generation.

ELISA screening for antibodies that bind selectively to the N-terminal neo-epitope of ApoE fragment starting at A199: Generated hybridoma clones were screened for reactivity towards the recombinant ApoE fragment A199-HIS, as well as reactivity towards the target peptide SEQ ID NO:55 conjugated to BSA. In addition, lack of reactivity towards recombinant full-length ApoE4, recombinant ApoE fragment 1-272-HIS, and negative control peptide SEQ ID NO:53 conjugated to BSA was evaluated. Twenty clones were identified as antibodies selective for the N-terminal neo-epitope of ApoE fragments starting at amino acid A199 and were evaluated further.

Determination of IgG concentration: The range of IgG concentration was between 2.9-26.6 µg/ml. Antibody concentration was normalized in cell medium, so that the starting concentration in ELISA experiments would be the same.

ELISA screening of top twenty antibodies that bind selectively to the N-terminal neo-epitope of ApoE fragment starting at A199: All antibodies were reactive towards the target peptide SEQ ID NO:51 conjugated to BSA and the recombinant ApoE fragment A199-HIS, but not towards recombinant full-length ApoE4. The top eight responders based on ELISA and IgG concentration were further evaluated by Western blot.

Selectivity evaluation and human target binding in Alzheimer's disease brain extracts by Western blot analysis: All antibodies were able to bind to the correct fragments in a human brain extract. Of the twenty top responders, four were chosen based on IgG concentration, direct ELISA, and binding to

5 human target. These clones were subjected to limiting dilution assay to ensure monoclonality and continued reactivity towards the recombinant ApoE fragment A199-HIS and the target peptide SEQ ID NO:55 conjugated to BSA, but not towards recombinant full-length ApoE4. All four clones were identified as antibodies selective for the N-terminal neo-epitope of ApoE fragments

10 starting at amino acid A199, and denoted 36A12, 38G9, 63F6 and 67G3. All were produced and purified as monoclonal antibodies and clones were sent for sequencing.

Example 17

15 Hybridoma sequencing and isotype determination of antibodies to the N-terminal ApoE fragment neo-epitope A199

Materials and methods

Hybridoma sequencing: Hybridoma clones producing the monoclonal

20 antibodies generated and characterized in Example 16, having a demonstrated selectivity for the N-terminal neo-epitope of ApoE fragment starting at amino acid A199, and a proven human target binding in brain extracts from Alzheimer's disease, were sent to Absolute Antibody for sequencing. Briefly, hybridoma sequencing was performed by whole

25 transcriptome shotgun sequencing. The DNA and protein sequences of the mature VH and VL regions were identified.

Results

Hybridoma sequencing: Hybridoma clones with a demonstrated

30 selectivity for the N-terminal neo-epitope of ApoE fragment starting at amino acid A199, in addition to a demonstrated binding to human target in brain extracts from Alzheimer's disease, were sequenced.

The following hybridoma clones were sequenced: 36A12, 38G9, 63F6 and 67G3. The amino acid sequences of the entire antibodies were obtained. Amino acid sequences obtained for the respective variable heavy (VH) and variable light (VL) chains are given in Table 12 below.

5

Region	Antibody	SEQ ID NO:
36A12		
VH	EVKLEESGGGLVQPGGSMKLSKVASGFTFSNYWMNWVRQSPEKGLE WIGEIKLKSNNYGTHYAESVKGRFTISRDDSKSSVYLQMNNLRAEDTGI YYCTRDTEVVAGAHWGQGTTLTVSA	109
VL	NIMMTQSPSSLAIVSAGEKVTMSCKSSQSVLYNSNQKNYLAWYQQKP GQSPKLLIYWASTRDSGVPDRFTGSGSGTDFTLTISVRAEDLAVYFC QQYLSSLTFGAGTKLELK	110
38G9		
VH	EVKLEESGGGLVQPGGSMKLSKVASGFTFSNYWMNWVRQSPEKGLE WVGEIKLKSNNYGTHYAESVKGRFTISRDDSKSSVYLQMNNLRAEDT GIYYCTRDTEVVAGAHWGQGTTLTVSA	111
VL	NIMMTQSPSSLAIVSAGEKVTMSCKSSQSVLYSSNQKNYLAWYQQKP GQSPKLLIYWASTRESGVPDRFTGSGSGTDFTLTISVQAEDLAVYFC QQYLSSLTFGAGTKLELK	112
63F6		
VH	QVQLQQPGTELVKPGASVKLSCKASGYTFTNYWMHWVKQRPGQGLE WIGNINPRNGGTNYNEKFKSKATLTVDKSSSTDYMQLSSTSEDSAVY YCARGLYDYDFDYWGQGTTLTVSS	113
VL	DVLTMTQTPSLPVS LGDQASISCRSSQSIVHGNGNTYLQWFLQKPGQ SPKLLIYKVSSRFSGVPDRFSGSGSGTDFTLKISRVEAEDLGVYYCFQ GSHVPFTFGSGTKLEIK	114
67G3		
VH	QVQLQQPGTELVKPGASVKLSCKASGYTFTRYWLHWVKQRPGQGLE WIGNINPTNGGANYNEKFKNKATLTVDKSSSTAYMQLSSTSEDSAVY YCSRGAYDYDCDYWGQGTTLTVSS	115
VL	DVLTMTQTPSLPVS LGDQASISCRSSQRIVHSNGNIYLQWYLQKPGQS PKLLIYKVS NRFSGVPDRFSGSGSGTDFTLKISRVEAEDLGVYYCFQ SHVPYTFGGGTKLEIK	116

Table 12. Variable region sequences

The complementarity determining regions (CDRs) were identified using the Kabat definition for the primary VH and VL sequences and are given in

10 Table 13 below.

Antibody	CDR H1	CDR H2	CDR H3
36A12	NYWMN (SEQ ID NO:62)	EIKLKSNNYGTHYAESVKG (SEQ ID NO:92)	DTEVVAGAH (SEQ ID NO:93)
38G9	NYWMN (SEQ ID NO:62)	EIKLKSNNYGTHYAESVKG (SEQ ID NO:92)	DTEVVAGAH (SEQ ID NO:93)
63F6	NYWMH (SEQ ID NO:94)	NINPRNGGTNYNEKFKS (SEQ ID NO:95)	GLYDYDFDY (SEQ ID NO:96)
67G3	RYWLH (SEQ ID NO:97)	NINPTNGGANYNEKFKN (SEQ ID NO:98)	GAYDYDCDY (SEQ ID NO:99)
	CDR L1	CDR L2	CDR L3
36A12	KSSQSVLYNSNQKNYLA (SEQ ID NO:100)	WASTRDS (SEQ ID NO:101)	QQYLSSLT (SEQ ID NO:102)
38G9	KSSQSVLYSSNQKNYLA (SEQ ID NO:103)	WASTRES (SEQ ID NO:104)	QQYLSSLT (SEQ ID NO:102)
63F6	RSSQSIVHGNNGNTYLQ (SEQ ID NO:105)	KVSSRFS (SEQ ID NO:106)	FQGSHVPFT (SEQ ID NO:107)
67G3	RSSQRIVHSNGNIYLQ (SEQ ID NO:108)	KVSNRFS (SEQ ID NO:25)	FQGSHVPYT (SEQ ID NO:28)

Table 13. CDR region sequences

The antibodies were isotyped based on their sequences, and their
5 respective sub-class and light chain are summarized in Table 14 below.

Clone	Sub-class	Light chain
36A12	IgG1	K
38G9	IgG1	K
63F6	IgG2b	K
67G3	IgG2b	K

Table 14. Isotyping results

Example 18

10 Characterization of purified monoclonal antibodies to the N-terminal ApoE fragment neo-epitope A199

This example describes the characterization of the purified monoclonal
antibodies described in Examples 16 and 17 by various methods, including
15 direct ELISA, inhibition ELISA, surface plasmon resonance and Western blot
on human brain extract.

Materials and methods

Selectivity evaluation of purified monoclonal antibodies by direct

ELISA: The direct ELISA described below was used to evaluate the binding selectivity of the purified monoclonal antibodies produced in Example 17. The ability to selectively bind to the A199 N-terminal neo-epitope ApoE peptide (BSA-conjugated SEQ ID NO:51) and to the recombinant A199-HIS C-terminal ApoE fragment resulting from cleavage in the hinge region (SEQ ID NO:58) was compared with binding to the L198, G200 and negative control peptides (BSA-conjugated SEQ ID NO:52, 50 and 53, respectively), and to recombinant full-length ApoE4 (ApoE sequence aa 1-299; SEQ ID NO:45).

The screening was performed according to standard ELISA protocols. Briefly, 1 µg/ml solutions of BSA-conjugated neo-epitope peptides and negative control peptide, and 0.1 µM recombinant C-terminal ApoE fragment and full-length ApoE (Abcam, cat. no. ab50243) were prepared by dilution in PBS. 50 µl/well were added to an ELISA half-area 96 well microtiter plate, and the plate was sealed with adhesive sealer and incubated over night at 4 °C. After discarding the solution, the plates were blocked with 150 µl/well of protein-free blocking solution (Pierce) for 1 h at room temperature with shake (600-900 rpm). The plates were washed four times with washing buffer containing 0.28 mM NaH₂PO₄, 2.5 mM Na₂HPO₄, 150 mM NaCl, 0.1 % TWEEN®-20 and 0.0075 % Kathon CG. Purified monoclonal antibodies of interest were 3-fold serially diluted with a starting dilution at 3 µg/ml (mixed 1:2 with dilution buffer (PBS with 0.1 % BSA and 0.05 % TWEEN®-20)). An anti-ApoE C-terminal antibody was used as a reference antibody (ApoE E-8 mouse monoclonal, Santa Cruz Biotechnology, cat. no. sc-393302). 50 µl/well were added into the ELISA plates and incubated for 2 h at room temperature with shake (600-900 rpm). The plates were washed four times as described previously. Detection antibody (HRP-conjugated anti-mouse IgG, Southern Biotech, cat. no. 1030-05, diluted 1:10000 in dilution buffer) was added 50 µl/well, and the plates were incubated for 1 h at room temperature with shake (600-900 rpm). After another wash (as described previously), 50 µl/well of K-Blue® aqueous substrate (Neogen) were added, and the reaction was stopped after 5-15 min with 50 µl/well of 0.5 M H₂SO₄. The optical density at

450 nm was read using an ELISA reader (Tecan). The optical density was plotted against the antibody concentration to generate concentration-response curves (Figure 28) and the EC₅₀ values were determined from the log agonist concentration response curve.

- 5 Selectivity evaluation and IC₅₀ determination of purified monoclonal antibodies by inhibition ELISA: The inhibition ELISA described below was used to evaluate the binding strength and selectivity for the purified monoclonal antibodies described in Examples 16 and 17. The ability of the purified monoclonal antibodies to bind to the N-terminal neo-epitope of
10 synthetic ApoE peptides starting at amino acid A199 was evaluated in comparison with their ability to bind to synthetic ApoE peptides starting at amino acids L198 and G200, as well as to the negative control peptide (aa 192-206) in solution.

- In brief, the purified monoclonal antibody to be tested was allowed to
15 interact with the N-terminal neo-epitope of synthetic ApoE peptides conjugated to BSA and starting at amino acid A199, L198 or G200, or to BSA-conjugated negative control peptide (SEQ ID NO:51, 52, 50 and 53, respectively). Thereafter, the mix was added to a microtiter plate coated with the BSA-coupled A199 synthetic ApoE peptide. If the purified monoclonal
20 antibody binds to any of the antigens in the pre-incubation step (the synthetic ApoE peptides), the antibody is prevented from binding to the synthetic A199 ApoE peptide immobilized on the microtiter plate. This leads to inhibition of the ELISA detection signal.

- 0.5 µg/ml solution of N-terminal neo-epitope peptide A199 (SEQ ID
25 NO:51) conjugated to BSA was prepared by dilution in PBS. 50 µl/well were added to an ELISA half-area 96 well microtiter plate, the plate was sealed with adhesive sealer and incubated over night at 4 °C. After discarding the solution, the plates were blocked with protein-free blocking solution (Pierce) (150 µl/well) at room temperature for 90 min with shaking (900 rpm). The
30 blocked plate was washed four times with washing buffer containing 0.28 mM NaH₂PO₄, 2.5 mM Na₂HPO₄, 150 mM NaCl, 0.1 % TWEEN®-20 and 0.0075 % Kathon CG.

Serial dilutions of each synthetic ApoE peptide were prepared by 4-fold dilution starting from 10000 ng/ml in 96 well storage plates (30 μ l final volume). Each purified monoclonal antibody to be tested was added (30 μ l) to the serial dilutions of each of the synthetic ApoE peptides at a final
5 concentration of 0.05 μ g/ml per well. Samples were pre-incubated for 45 min at room temperature with shaking (900 rpm).

The pre-incubated samples were transferred (50 μ l/well) to the blocked ELISA plates, and plates were incubated for 10 min at room temperature without shaking. Plates were washed as described above. Alkaline
10 phosphatase conjugated anti-mouse IgG detection antibody (Mabtech, cat. no. 3310-4) was diluted 1:1000 and added to each plate (50 μ l/well). The plate was sealed and incubated with shaking (900 rpm) for 45 min at room temperature and subsequently washed as described above. Alkaline
15 phosphatase substrate (50 μ l/well) was added to the plate and the optical density was read every 10 min at a wavelength of 405 nm for up to 120 min. The IC₅₀ values were determined from a log inhibitor concentration response curve (Figure 29).

Selectivity evaluation and K_D determination of antibodies by surface plasmon resonance: Binding interactions between the antigens and
20 antibodies were evaluated by surface plasmon resonance (SPR) using a Biacore 8K instrument (GE Healthcare) according to standard procedures.

To determine the binding affinity of the monoclonal antibodies for N-terminal neo-epitopes compared to full-length ApoE, single-cycle kinetics experiments were conducted. The recombinant fragments were immobilized
25 onto a CM5 Sensor Chip (GE Healthcare, cat. no. 29104988) using an amine coupling kit (GE Healthcare, cat. no. BR100050) according to the manufacturer's instructions. Reference (no immobilized antigen) and active surfaces were treated with the same conditions using the amine coupling reagents on flow cell 1 (Fc1) and flow cell 2 (Fc2) respectively. The
30 immobilization level for the active surfaces was kept at approximately 150-200 response units (RU). The same protocol setup was used to immobilize full-length ApoE on the CM5 chip surface.

Purified antibodies were prepared in 2-fold serial dilution from 14 to 0.3 nM (in 5-7 steps). Next, the prepared serial dilution of purified antibodies was injected (30 μ l/min, contact time 360 s, dissociation time 2500 s) over both flow cells of the sensor chip. The interaction series was done in triplets.

- 5 Values were blank subtracted, and a bivalent analyte binding kinetics fit model was used for the evaluation.

In all SPR experiments, 1xPBS-P+ (GE Healthcare, cat. no. 28995084) was used to dilute antibodies and target antigens. Experiments were performed at 25 °C.

- 10 Selectivity evaluation and human target binding in Alzheimer's disease brain extracts by Western blot analysis: The Western blot analysis described below was used to evaluate selective binding of monoclonal antibodies with affinity for the N-terminal neo-epitope of ApoE fragments $\leq$ 12 kDa, starting at A199, without binding to ApoE full-length protein, in human brain extracts
- 15 from an Alzheimer's disease patient. Fresh frozen brain tissue from the Alzheimer's disease patient was homogenized in RIPA 2 % SDS extraction buffer, followed by centrifugation at 16000 x g for 1 h. The subsequent supernatant was subjected to standard protein concentration determination.

- RIPA 2 % SDS brain extract containing approximately 60 μ g total
- 20 protein was mixed with 2x Laemmli sample buffer, boiled for 5 min at 95 °C and loaded onto SDS-PAGE gels (Bolt™ 12 % Bis-Tris Plus 10 well, Thermo Fisher, cat. no. NW00120BOX). Gels were run for 30-40 min at 180 V, after which proteins were transferred from the gels onto nitrocellulose membranes using the Trans-Blot® Turbo™ system (Bio-Rad). Membranes were blocked in
- 25 Intercept® PBS Blocking Buffer (LI-COR) for 1 h and incubated over night at room temperature with monoclonal antibodies with affinity for the N-terminal neo-epitope of ApoE fragments $\leq$ 12 kDa starting at A199 (2 μ g/ml in Intercept® PBS Blocking Buffer (LI-COR) with 0.1 % TWEEN®-20).
- Membranes were washed and incubated for 1 h at room temperature with the
- 30 detection antibody anti-mouse-800CW (LI-COR, cat. no. 925-32210) diluted 1:25000 in Intercept® PBS Blocking Buffer (LI-COR) with 0.1 % TWEEN®-20. Membranes were washed and images acquired using Odyssey® FC (LI-COR).

To confirm that the obtained bands on the Western blot membranes were of ApoE origin, the membranes were re-stained over night with a polyclonal anti-ApoE antibody (Calbiochem, cat. no. 178479; immunogen ApoE aa 1-299), diluted 1:2000 in Intercept® PBS Blocking Buffer (LI-COR) with 0.1 % TWEEN®-20. Membranes were washed and incubated for 1 h at room temperature with detection antibody anti-goat-680RD (LI-COR, cat. no. 925-68074) diluted 1:25000 in Intercept® PBS Blocking Buffer (LI-COR) with 0.1 % TWEEN®-20. Membranes were washed and images acquired using Odyssey® FC (LI-COR).

10

Results

Selectivity evaluation of purified monoclonal antibodies by direct ELISA: The four purified monoclonal antibodies 36A12, 38G9, 63F6 and 67G3 were screened for reactivity towards the BSA-conjugated peptides 198-203, 199-204 or 200-205 and the ApoE recombinant fragment aa 199-299. In addition, absence of reactivity towards recombinant full-length ApoE (ApoE4 1-299) and the negative control peptide (SEQ ID NO:53) conjugated to BSA was studied.

All purified monoclonal antibodies demonstrated strong binding to the N-terminal neo-epitope peptide A199 (SEQ ID NO:51) and to the recombinant C-terminal ApoE fragment A199-HIS (aa 199-299; SEQ ID NO:58). At the highest concentration of antibody, antibodies 63F6 and 67G3 showed some binding to the negative control peptide (SEQ ID NO:53), and the 200-205 BSA-conjugated peptide (SEQ ID NO:50) (Figure 28). The calculated EC50 values are shown in Table 15 below.

As expected, the reference antibody directed to the ApoE C-terminal could detect and bind both the recombinant A199 fragment (aa 199-299) and the recombinant ApoE4 full-length protein (aa 1-299). Also as expected, because the epitope for the reference antibody is ApoE aa 274-299, no binding to the A199-BSA peptide was detected (Figure 28).

Selectivity evaluation and IC50 determination of purified monoclonal antibodies by inhibition ELISA: The four purified monoclonal antibodies 36A12, 38G9, 63F6 and 67G3 were screened for binding strength and

binding selectivity towards the N-terminal neo-epitope peptide A199-BSA (aa 199-204). In addition, antibodies were screened for lack of reactivity towards BSA-conjugated peptides 198-203, 200-205 and the negative control peptide 192-206.

- 5 All purified monoclonal antibodies demonstrated strong binding and selectivity for the A199-BSA peptide (aa 199-204), whereas there was no binding to 198-203, 200-205 or negative control BSA-conjugated peptides (Figure 29). The calculated IC₅₀ values are shown in Table 15 below.

Clone	EC ₅₀ (ng/ml)		IC ₅₀ (ng/ml)
	A199-BSA peptide	A199-HIS fragment	A199-BSA peptide
36A12	5.1	20.2	27.7
38G9	4.6	39.7	52.6
63F6	3.8	1.8	2.5
67G3	4.4	3.5	2.9

- 10 Table 15. Summary of results from direct ELISA and inhibition ELISA of purified monoclonal anti-A199 antibodies

- Selectivity evaluation and K_D determination of purified monoclonal antibodies by surface plasmon resonance: The four purified monoclonal antibodies 36A12, 38G9, 63F6 and 67G3 were evaluated for selectivity, and K_D values for the target antigen were determined by surface plasmon resonance.

- All purified monoclonal antibodies demonstrated binding to the A199-HIS fragment (Figure 30), whereas no binding was shown to recombinant full-length ApoE. The calculated k_{a1}, k_{d1} and K_{D1} values are shown in Table 16 below.

Clone	Biacore: A199-HIS fragment		
	k _a (x 10 ⁵ 1/Ms)	k _d (x 10 ⁻⁴ 1/s)	K _D (nM)
36A12	34.0	3.4	10
38G9	944	25	1450
63F6	1.5	207	137
67G3	1.8	172	83

Table 16. Summary of results from surface plasmon resonance of purified monoclonal anti-A199 antibodies

Selectivity evaluation and human target binding in Alzheimer's disease brain extracts by Western blot analysis: The monoclonal antibodies selective for the N-terminal neo-epitope of ApoE fragments starting at amino acid A199 (36A12, 38G9, 63F6 and 67G3) were tested for their ability to bind selectively to ApoE fragments ≤ 12 kDa in human brain extracts from an Alzheimer's disease patient, and without any binding to full-length ApoE. Western blot analysis demonstrated binding of all of the monoclonal antibodies to two ApoE fragments approximately 12 kDa and 10 kDa in size, without any visual binding to full-length ApoE (Figure 31A). Re-staining of the Western blot membranes with a polyclonal anti-ApoE antibody showed staining of full-length ApoE, high molecular weight (HMW) ApoE fragments (~ 20 -25 kDa) and a distinct 12 and 10 kDa ApoE fragment (Figure 31B).

Sequence Listing

Table 17 below lists the sequences referred to in this application.

SEQ ID NO:	Description/occurrence	Sequence
1	ApoE 200-299	GQPLQERAQAWGERLRARMEEMGSRTDRDLDEVK EQVAEVRAKLEEQAQQIRLQAEAFQARLKSWFEPLV EDMQRQWAGLVEKVQAAVGTSAAPVPSDNH
2	ApoE 199-299	AGQPLQERAQAWGERLRARMEEMGSRTDRDLDEV KEQVAEVRAKLEEQAQQIRLQAEAFQARLKSWFEPL VEDMQRQWAGLVEKVQAAVGTSAAPVPSDNH
3	ApoE 198-299	LAGQPLQERAQAWGERLRARMEEMGSRTDRDLDE VKEQVAEVRAKLEEQAQQIRLQAEAFQARLKSWFE PLVEDMQRQWAGLVEKVQAAVGTSAAPVPSDNH
4	Hexamer ag 198	LAGQPL
5	Hexamer ag 199	AGQPLQ
6	Hexamer ag 200	GQPLQE
7	Heptamer ag 198	LAGQPLQ
8	Heptamer ag 199	AGQPLQE
9	Octamer ag 198	LAGQPLQE
10-23	G200 HC CDRs	Table 4
24-33	G200/A199 LC CDRs	Tables 4 and 13
34-44	G200 VH and VL	Table 3
45	Human ApoE4	KVEQAVETEPEPELRQQTEWQSGQRWELALGRFW DYLRWVQTLSEQVQEELLSSQVTQELRALMDET MKELKAYKSELEEQLTPVAEETRARLSKELQAAQARLG ADMEDVRGRLVQYRGEVQAMLGQSTEELRVRLAS HLRKLRLRLRDADDLQKRLAVYQAGAREGAERGL SAIRERLGPLVEQGRVRAATVGSLAGQPLQERAQA WGERLRARMEEMGSRTDRDLDEVKEQVAEVRACL

		EEQAQQIRLQAEAFQARLKSWFEPLVEDMQRQWAGLVEKVQAAVGTSAAPVPSDNH
46	158-233 of FL ApoE	RLAVYQAGAREGAERGLSAIRERLGPLVEQGRVRAATVGSLAGQPLQERAQAWGERLRARMEEMGSRTRDRLDEVK
47	200-233 of FL ApoE	GQPLQERAQAWGERLRARMEEMGSRTRDRLDEVK
48	200-299-10xHIS	GQPLQERAQAWGERLRARMEEMGSRTRDRLDEVKEQVAEVRAKLEEQAQQIRLQAEAFQARLKSWFEPLVEDMQRQWAGLVEKVQAAVGTSAAPVPSDNHHHHHHHHHHHH
49	10xHIS-ApoE4-FL	HHHHHHHHHHKVEQAVETEPEPELRRQQTEWQSGQRWELALGRFWDYLRWVQTLSEQVQEELLSSQVTQELRALMDETMKELKAYKSELEEQLTPVAEETRARLSKELQAAQARLGADMEDVRGRLVQYRGEVQAMLGQSTEELRVRLASHLRKLRKLLRDADDLQKRLAVYQAGAREGAERGLSAIRERLGPLVEQGRVRAATVGSLAGQPLQERAQAWGERLRARMEEMGSRTRDRLDEVKEQVAEVRAKLEEQAQQIRLQAEAFQARLKSWFEPLVEDMQRQWAGLVEKVQAAVGTSAAPVPSDNH
50	G200 immunogen	GQPLQE-Acp-C
51	A199 immunogen	AGQPLQ-Acp-C
52	L198 immunogen	LAGQPL-Acp-C
53	Neg ctrl peptide	AATVGSLAGQPLQER-Acp-C
54	L198 new imm	LAGQPLQE-Acp-C
55	A199 new imm	AGQPLQE-Acp-C
56	10xHIS-ApoE4-1-272	HHHHHHHHHHKVEQAVETEPEPELRRQQTEWQSGQRWELALGRFWDYLRWVQTLSEQVQEELLSSQVTQELRALMDETMKELKAYKSELEEQLTPVAEETRARLSKELQAAQARLGADMEDVRGRLVQYRGEVQAMLGQSTEELRVRLASHLRKLRKLLRDADDLQKRLAVYQAGAREGAERGLSAIRERLGPLVEQGRVRAATVGSLAGQPLQERAQAWGERLRARMEEMGSRTRDRLDEVKEQVAEVRAKLEEQAQQIRLQAEAFQARLKSWFEPLVEDM
57	198-299-10xHIS	LAGQPLQERAQAWGERLRARMEEMGSRTRDRLDEVKEQVAEVRAKLEEQAQQIRLQAEAFQARLKSWFEPLVEDMQRQWAGLVEKVQAAVGTSAAPVPSDNHHHHHHHHHH
58	199-299-10xHIS	AGQPLQERAQAWGERLRARMEEMGSRTRDRLDEVKEQVAEVRAKLEEQAQQIRLQAEAFQARLKSWFEPLVEDMQRQWAGLVEKVQAAVGTSAAPVPSDNHHHHHHHHHH
59-70	L198/A199 HC CDRs	Tables 8 and 13
71-81	L198 LC CDRs	Table 8
82-91	L198 VH and VL	Table 7
92-99	A199 HC CDRs	Table 13
100-108	A199 LC CDRs	Table 13
109-116	A199 VH and VL	Table 12

CLAIMS

1. An antibody or antigen binding portion thereof that binds to a fragment of apolipoprotein E (ApoE), wherein the fragment has
- 5 - an apparent molecular weight of 12 kDa as measured by SDS-PAGE, and
- an N-terminus corresponding to an amino acid in full-length apolipoprotein E which is selected from the group consisting of amino acids L198, A199 and G200; and wherein the antibody or antigen binding portion
- 10 thereof binds to an epitope comprising the N-terminus of the fragment.
2. The antibody or antigen binding portion thereof according to claim 1, wherein the antibody or antigen binding portion thereof binds selectively to the ApoE fragment and does not bind to full-length apolipoprotein E.
- 15 3. The antibody or antigen binding portion thereof according to claim 1 or claim 2, wherein said fragment of apolipoprotein E is selected from
- i) a fragment consisting of the amino acid sequence of any one of SEQ ID NOs:1-3; and
- 20 ii) a fragment having at least 80% identity to any one of SEQ ID NOs: 1-3.
4. The antibody or antigen binding portion thereof according to any one of claims 1-3, wherein the antibody or antigen binding portion thereof
- 25 binds to an epitope comprising amino acid residues selected from the following:
- (i) amino acid residues 200-205 in full-length ApoE (GQPLQE);
- (ii) amino acid residues 199-204 in full-length ApoE (AGQPLQ);
- (iii) amino acid residues 199-205 in full-length ApoE (AGQPLQE);
- 30 (iv) amino acid residues 198-203 in full-length ApoE (LAGQPL);
- (v) amino acid residues 198-204 in full-length ApoE (LAGQPLQ); and
- (vi) amino acid residues 198-205 in full-length ApoE (LAGQPLQE).

5. The antibody or antigen binding portion thereof according to any one of claims 1-4, wherein said fragment of apolipoprotein E consists of the amino acid sequence of SEQ ID NO:1, optionally wherein the epitope comprises amino acid residues 200-205 in full-length apolipoprotein E.

5

6. A method of producing an antibody or an antigen binding portion thereof, comprising a step of immunizing a host mammal with a peptide immunogen comprising an N-terminal amino acid sequence selected from the group consisting of LAGQPL (SEQ ID NO:4), AGQPLQ (SEQ ID NO:5),
10 GQPLQE (SEQ ID NO:6), LAGQPLQ (SEQ ID NO:7), AGQPLQE (SEQ ID NO:8) and LAGQPLQE (SEQ ID NO:9).

7. The method according to claim 6, wherein said N-terminal amino acid sequence is GQPLQE (SEQ ID NO:6).

15

8. An antibody or antigen binding portion thereof, obtainable by a method according to claim 6 or claim 7.

9. The antibody or antigen binding portion thereof according to any
20 one of claims 1-5 or 8, wherein the antibody or antigen binding portion thereof comprises a variable heavy chain domain (VH) comprising three CDR sequences (CDR-H1, CDR-H2 and CDR-H3), wherein the three VH CDR sequences are independently selected from:

- CDR-H1 selected from the group consisting of SEQ ID NO: 10, 15,
25 18 and 21;

- CDR-H2 selected from the group consisting of SEQ ID NO: 11, 13, 16, 19 and 22; and

- CDR-H3 selected from the group consisting of SEQ ID NO: 12, 14, 17, 20 and 23.

30

10. The antibody or antigen binding portion thereof according to any one of claims 1-5 and 8-9, wherein the antibody or antigen binding portion thereof comprises a variable light chain domain (VL) comprising three CDR

sequences (CDR-L1, CDR-L2 and CDR-L3), wherein the three VL CDR sequences are independently selected from:

- CDR-L1 selected from the group consisting of SEQ ID NO: 24, 27, 29, 31 and 32;
- 5 - CDR-L2 being SEQ ID NO: 25; and
- CDR-L3 selected from the group consisting of SEQ ID NO: 26, 28, 30 and 33.

11. The antibody or antigen binding portion thereof according to any
10 one of claims 1-5 and 8-10, wherein the antibody or antigen binding portion thereof comprises a heavy chain variable domain (VH) comprising or consisting of an amino acid sequence selected from:

- i) the group consisting of SEQ ID NOs: 34, 36, 38, 40, 42 and 43; and
- ii) a sequence having at least 70 % identity to any one of SEQ ID
15 NOs: 34, 36, 38, 40, 42 and 43.

12. The antibody or antigen binding portion thereof according to any
one of claims 1-5 and 8-11, wherein the antibody or antigen binding portion thereof comprises a light chain variable domain (VL) comprising or consisting
20 of an amino acid sequence selected from:

- i) the group consisting of SEQ ID NOs: 35, 37, 39, 41 and 44; and
- ii) a sequence having at least 70 % identity to any one of SEQ ID
NO:35, 37, 39, 41 and 44.

25 13. The antibody or antigen binding portion thereof according to any one of claims 1-5 or 8, wherein the antibody or antigen binding portion thereof comprises a variable heavy chain domain (VH) comprising three CDR sequences (CDR-H1, CDR-H2 and CDR-H3), wherein the three VH CDR sequences are independently selected from:

- 30 - CDR-H1 selected from the group consisting of SEQ ID NO: 59, 62 and 65;
- CDR-H2 selected from the group consisting of SEQ ID NO: 60, 63, 66, 68 and 70; and

- CDR-H3 selected from the group consisting of SEQ ID NO: 61, 64, 67 and 69.

14. The antibody or antigen binding portion thereof according to any one of claims 1-5, 8 and 13, wherein the antibody or antigen binding portion thereof comprises a variable light chain domain (VL) comprising three CDR sequences (CDR-L1, CDR-L2 and CDR-L3), wherein the three VL CDR sequences are independently selected from:

- CDR-L1 selected from the group consisting of SEQ ID NO: 71, 74, 76, 79 and 80;

- CDR-L2 selected from the group consisting of SEQ ID NO: 72 and 77; and

- CDR-L3 selected from the group consisting of SEQ ID NO: 73, 75, 78 and 81.

15

15. The antibody or antigen binding portion thereof according to any one of claims 1-5, 8 and 13-14, wherein the antibody or antigen binding portion thereof comprises a heavy chain variable domain (VH) comprising or consisting of an amino acid sequence selected from:

i) the group consisting of SEQ ID NOs: 82, 84, 86, 88 and 90; and
ii) a sequence having at least 70%, at least 80%, at least 90%, or at least 95% identity to any one of SEQ ID NOs: 82, 84, 86, 88 and 90.

16. The antibody or antigen binding portion thereof according to any one of claims 1-5, 8 and 13-15, wherein the antibody or antigen binding portion thereof comprises a light chain variable domain (VL) comprising or consisting of an amino acid sequence selected from:

i) the group consisting of SEQ ID NOs: 83, 85, 87, 89 and 91; and
ii) a sequence having at least 70%, at least 80%, at least 90%, or at least 95% identity to any one of SEQ ID NO: 83, 85, 87, 89 and 91.

17. The antibody or antigen binding portion thereof according to any one of claims 1-5 or 8, wherein the antibody or antigen binding portion thereof

comprises a variable heavy chain domain (VH) comprising three CDR sequences (CDR-H1, CDR-H2 and CDR-H3), wherein the three VH CDR sequences are independently selected from:

- CDR-H1 selected from the group consisting of SEQ ID NO: 62, 94 and 97;
- CDR-H2 selected from the group consisting of SEQ ID NO: 92, 95 and 98; and
- CDR-H3 selected from the group consisting of SEQ ID NO: 93, 96 and 99.

10

18. The antibody or antigen binding portion thereof according to any one of claims 1-5, 8 and 17, wherein the antibody or antigen binding portion thereof comprises a variable light chain domain (VL) comprising three CDR sequences (CDR-L1, CDR-L2 and CDR-L3), wherein the three VL CDR

15 sequences are independently selected from:

- CDR-L1 selected from the group consisting of SEQ ID NO: 100, 103, 105 and 108;
- CDR-L2 selected from the group consisting of SEQ ID NO: 25, 101, 104 and 106; and
- CDR-L3 selected from the group consisting of SEQ ID NO: 28, 102 and 107.

20

19. The antibody or antigen binding portion thereof according to any one of claims 1-5, 8 and 17-18, wherein the antibody or antigen binding portion thereof comprises a heavy chain variable domain (VH) comprising or consisting of an amino acid sequence selected from:

25

- i) the group consisting of SEQ ID NOs: 109, 111, 113 and 115; and
- ii) a sequence having at least 70%, at least 80%, at least 90%, or at least 95% identity to any one of SEQ ID NOs: 109, 111, 113 and 115.

30

20. The antibody or antigen binding portion thereof according to any one of claims 1-5, 8 and 17-19, wherein the antibody or antigen binding

portion thereof comprises a light chain variable domain (VL) comprising or consisting of an amino acid sequence selected from:

- i) the group consisting of SEQ ID NOs: 110, 112, 114 and 116; and
- ii) a sequence having at least 70%, at least 80%, at least 90%, or at least 95% identity to any one of SEQ ID NO: 110, 112, 114 and 116.

21. A pharmaceutical composition comprising an antibody or antigen binding portion thereof according to any one of claims 1-5 and 8-20 and at least one pharmaceutically acceptable excipient or carrier.

10

22. An antibody or antigen binding portion thereof according to any one of claims 1-5 and 8-20, or pharmaceutical composition according to claim 21, for use in therapy.

23. A method of detecting or diagnosing a neurological disorder or a disorder characterized by a loss of cognitive memory capacity in a subject, the method comprising contacting a sample obtained from the subject with an antibody or antigen binding portion thereof according to any one of claims 1-5 and 8-20.

20

Fig. 1

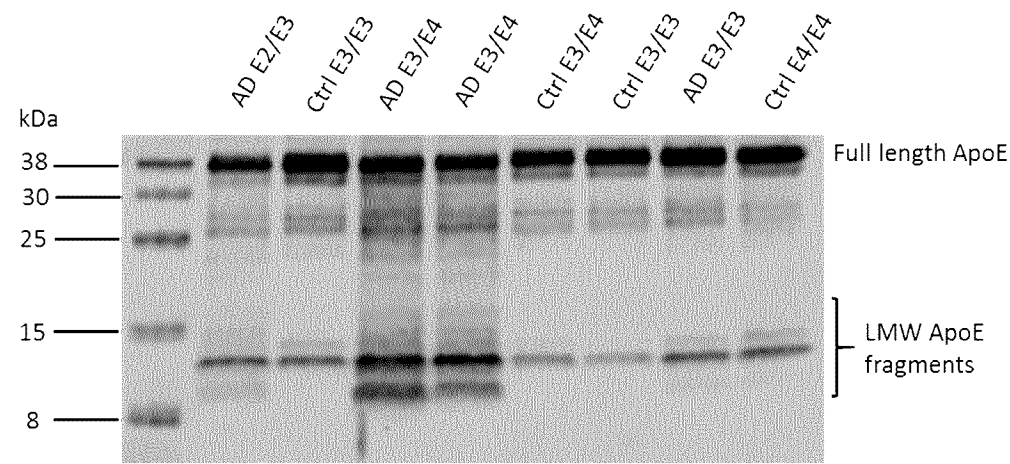


Fig. 2

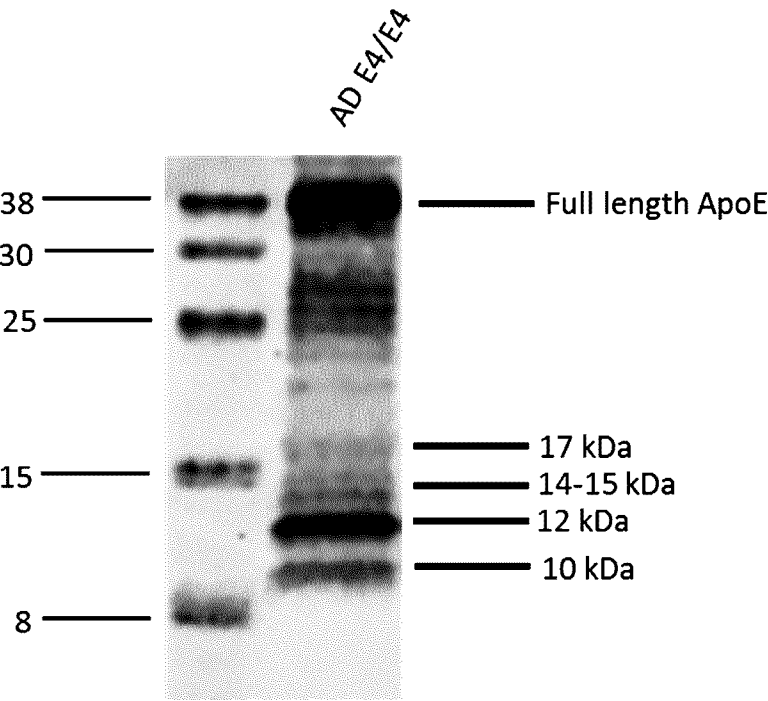


Fig. 3

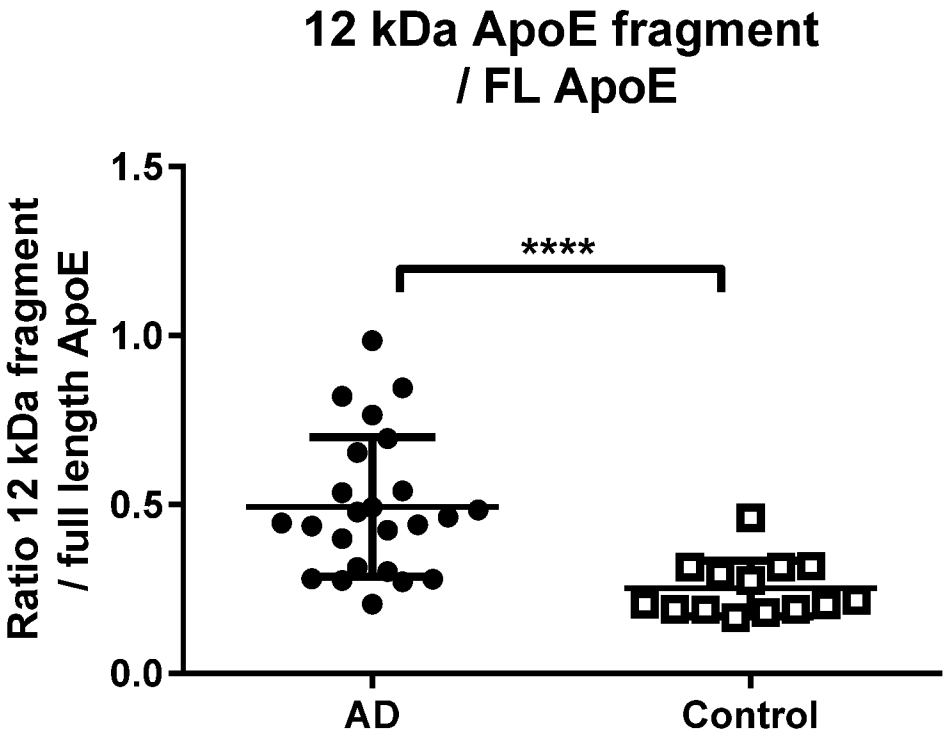


Fig. 4

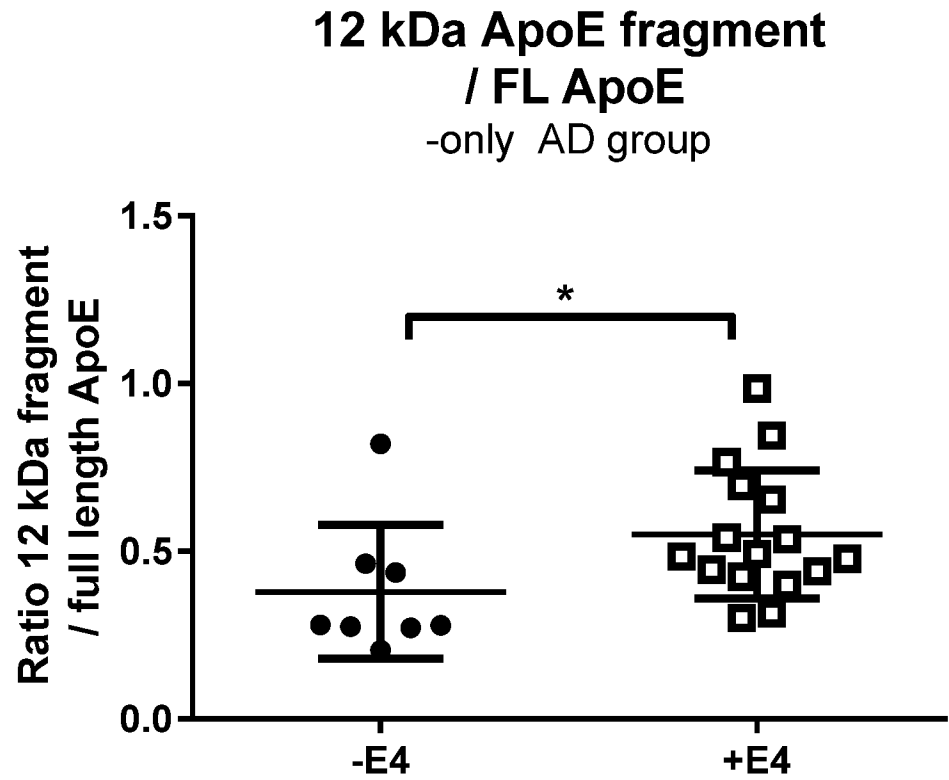


Fig. 5

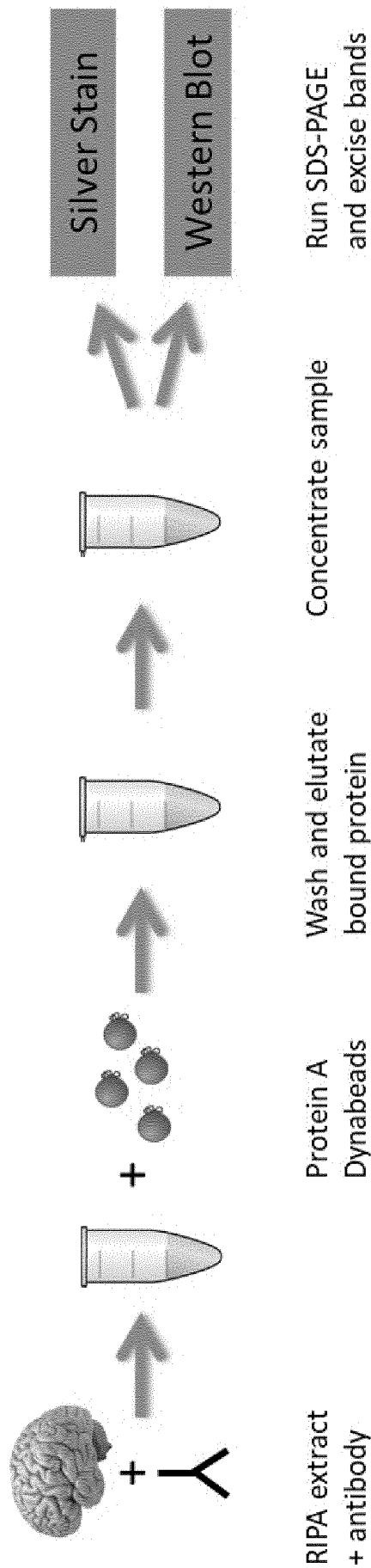


Fig. 6

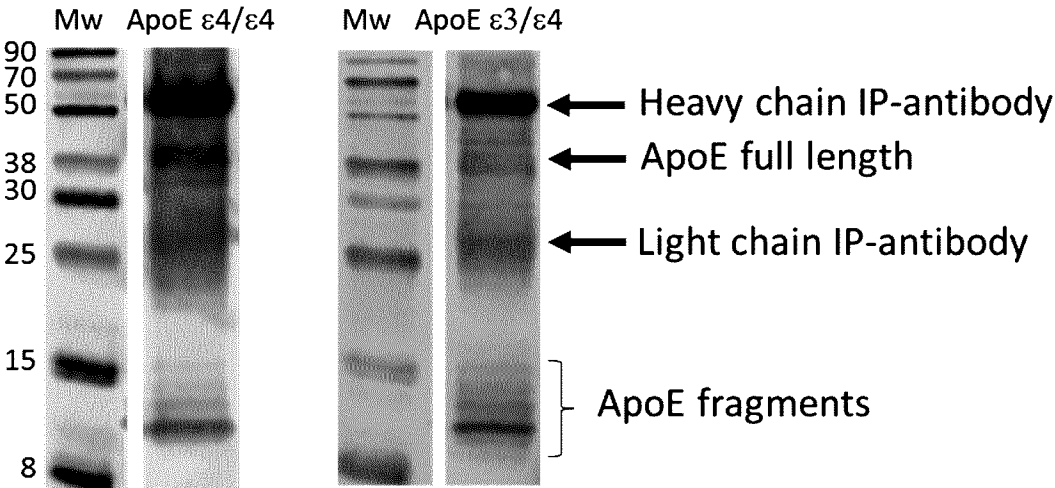


Fig. 7

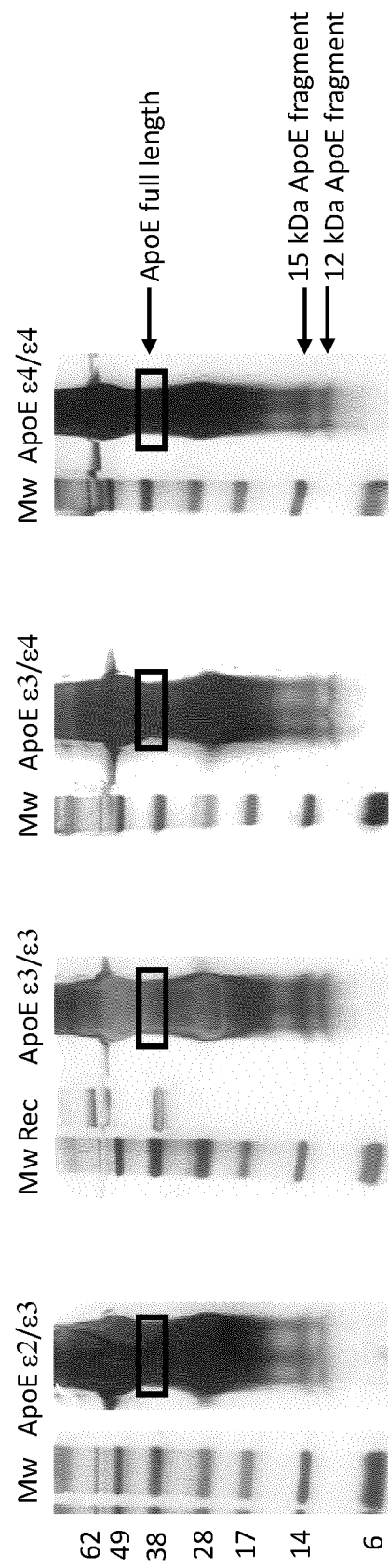


Fig. 8

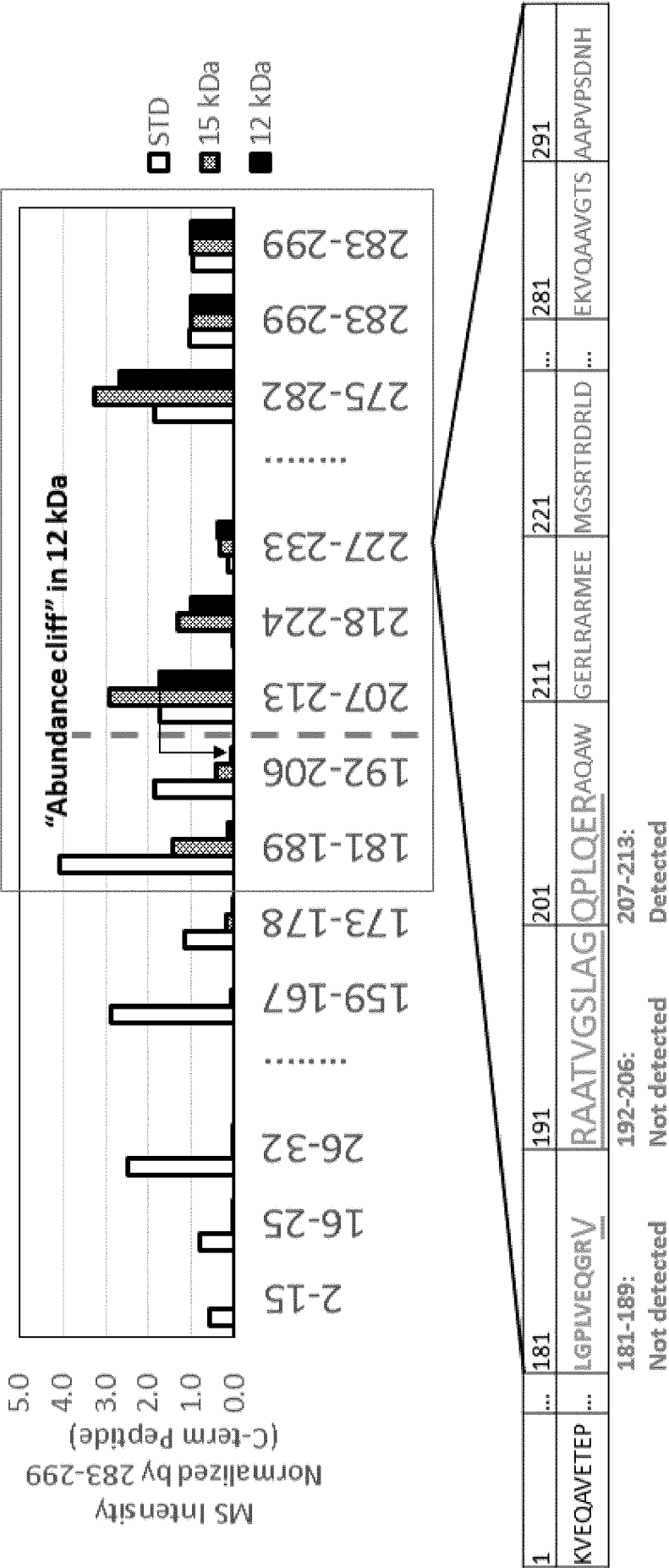
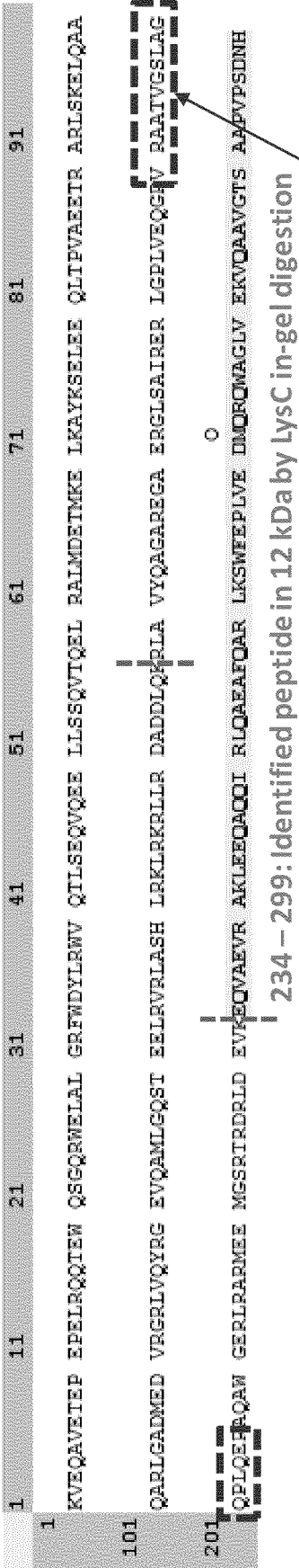
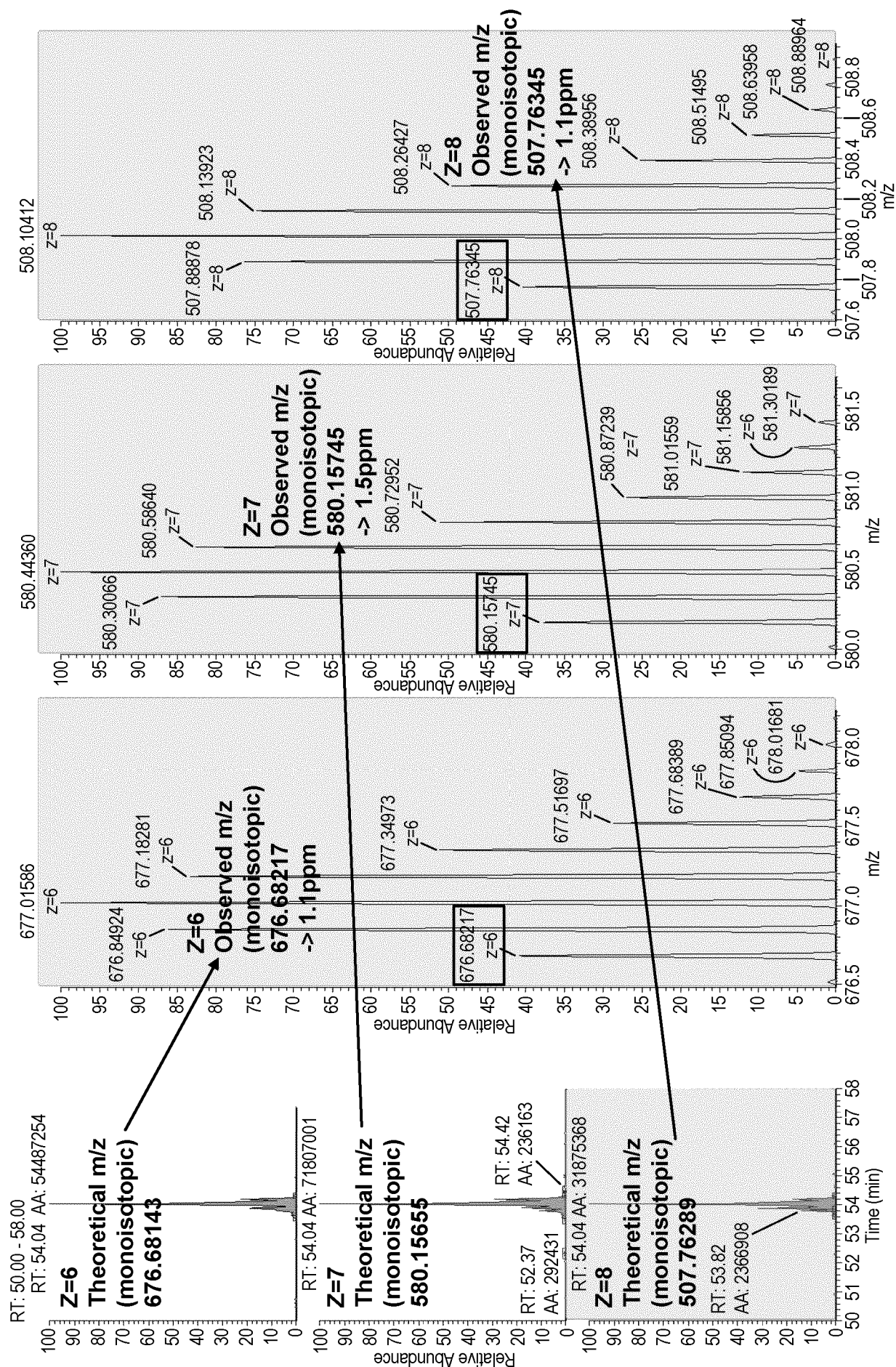


Fig. 9



190 - 206:
Possible cleavage sites (Based on Tryptic experiment)

Fig. 10



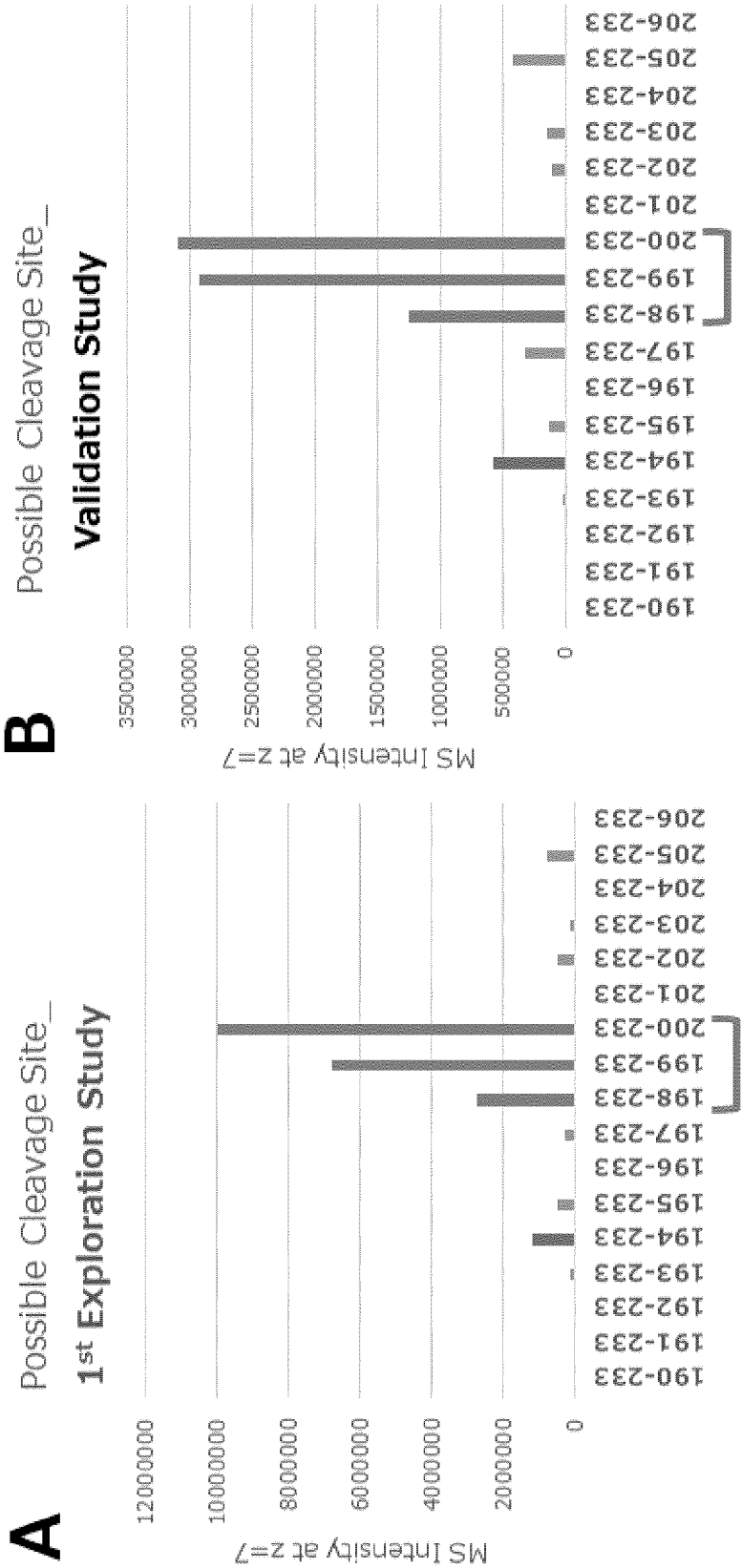


Fig. 11

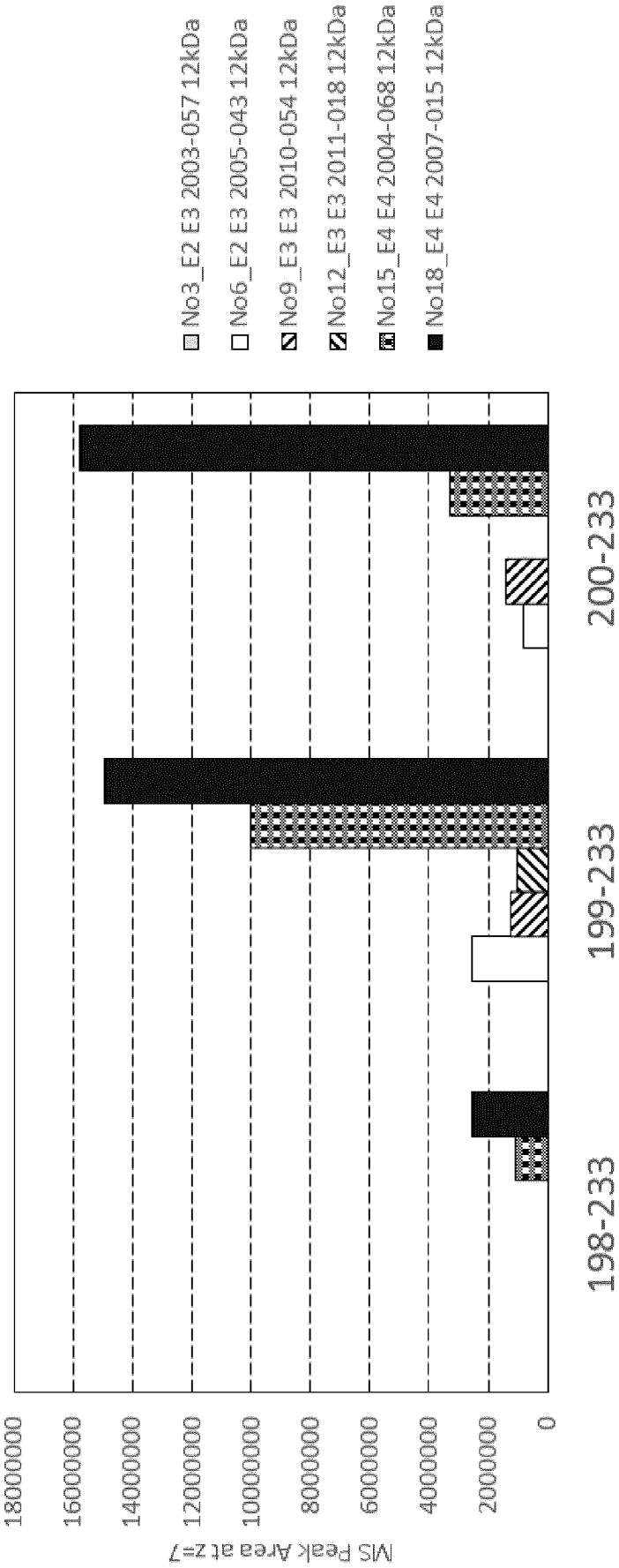


Fig. 12

Fig. 13

A) Neuro2A

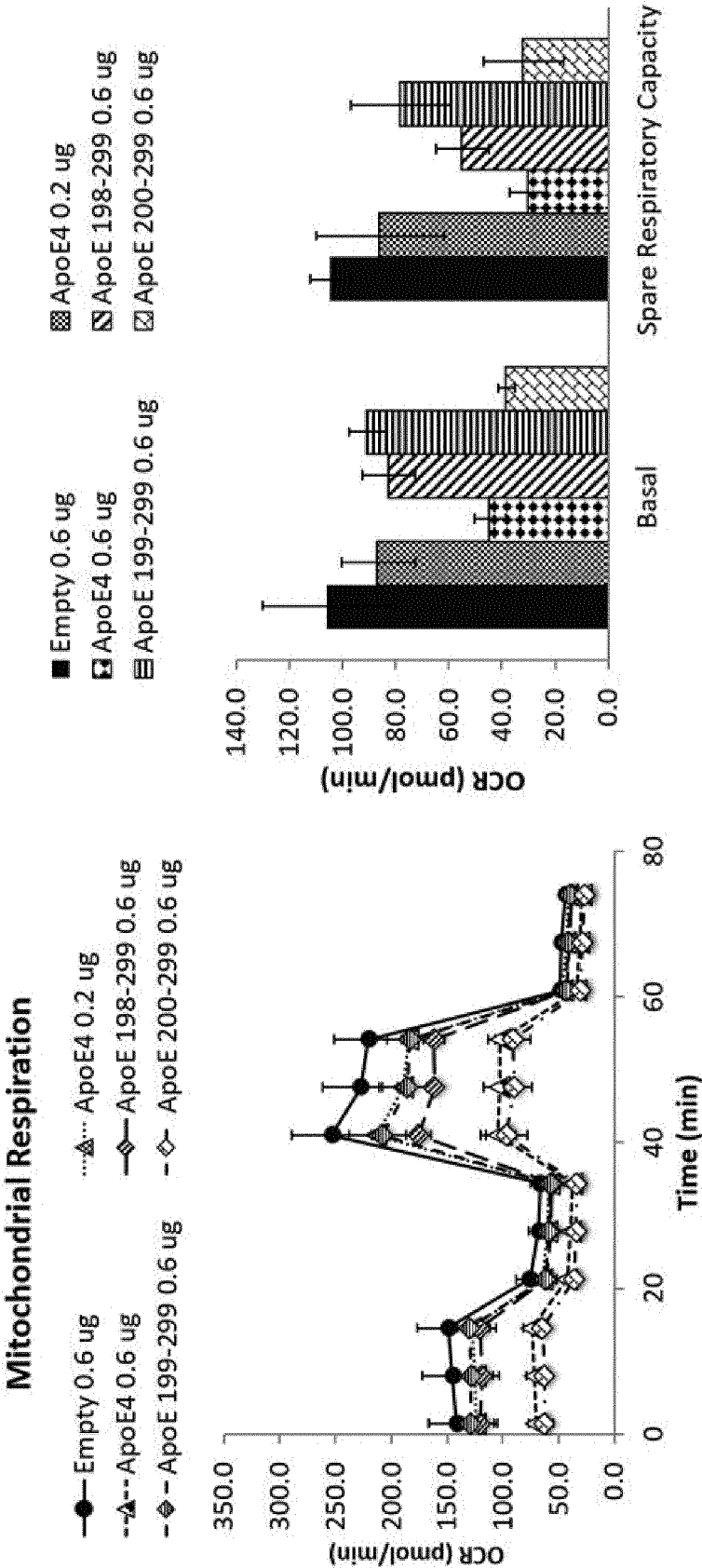


Fig. 13 continued

B) Rat primary hippocampal neuron

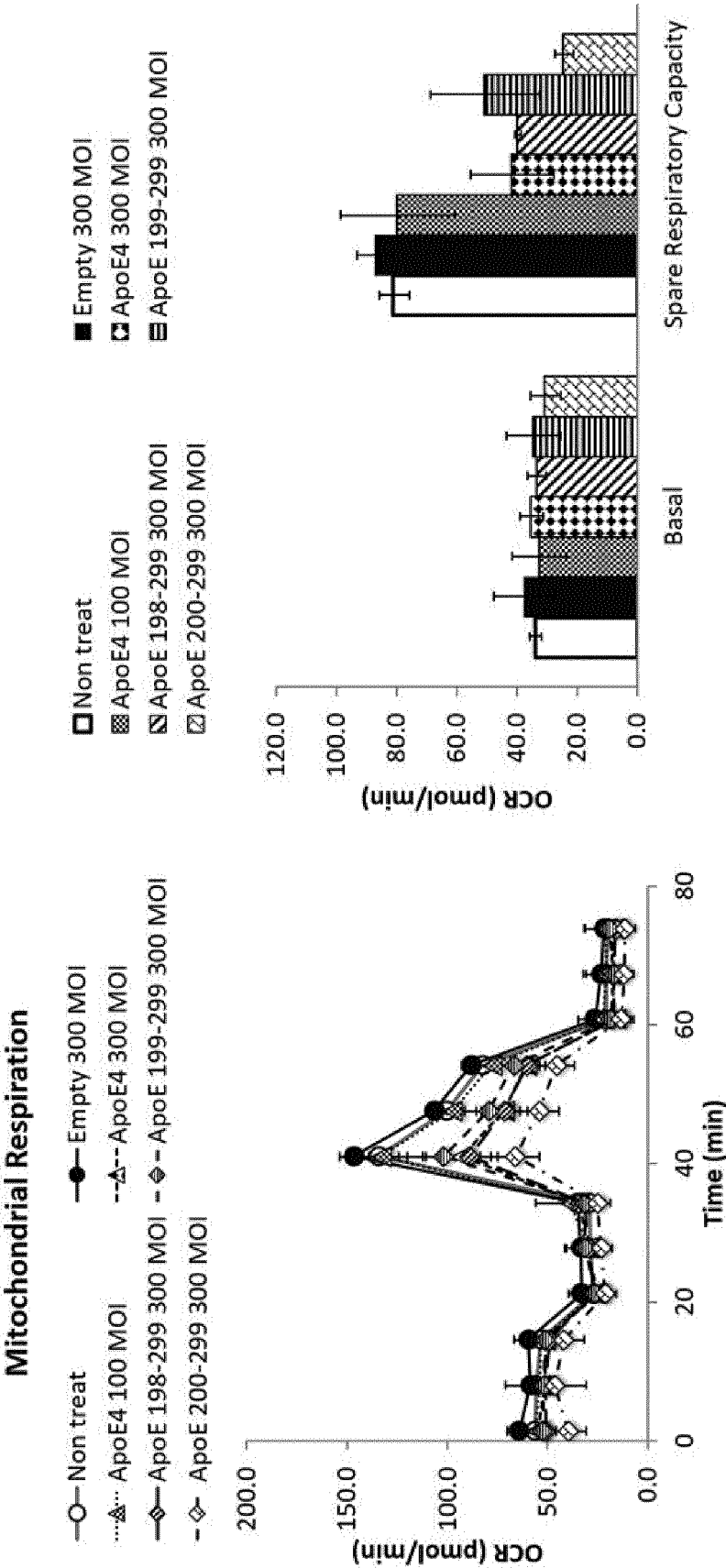
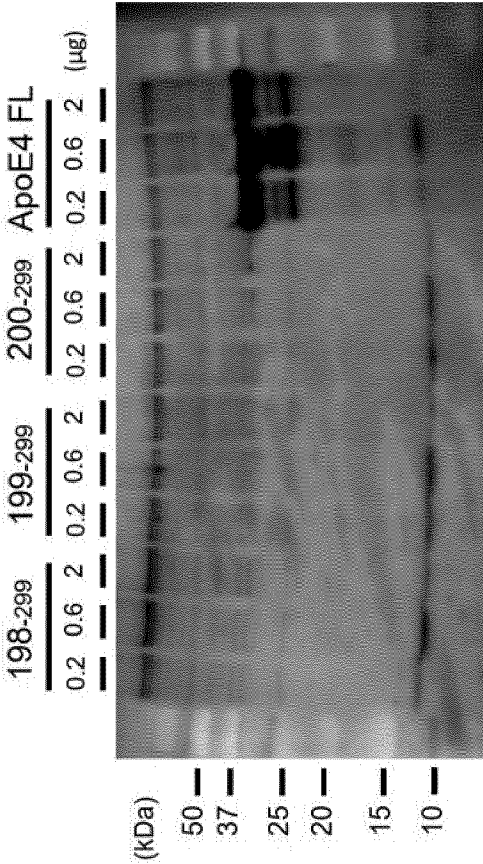


Fig. 13 continued

C) Expression check

<Neuro2A>



<Rat primary hippocampal neuron>

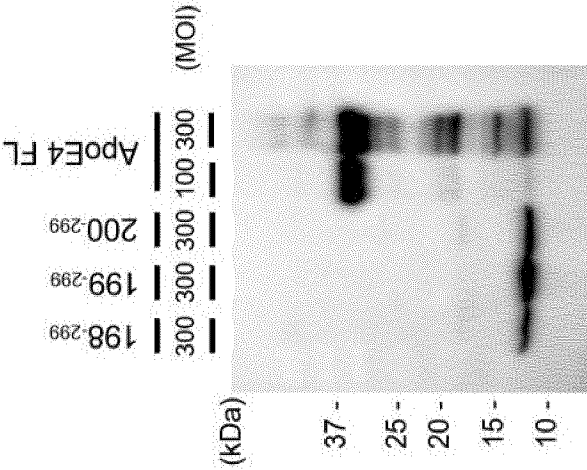


Fig. 14

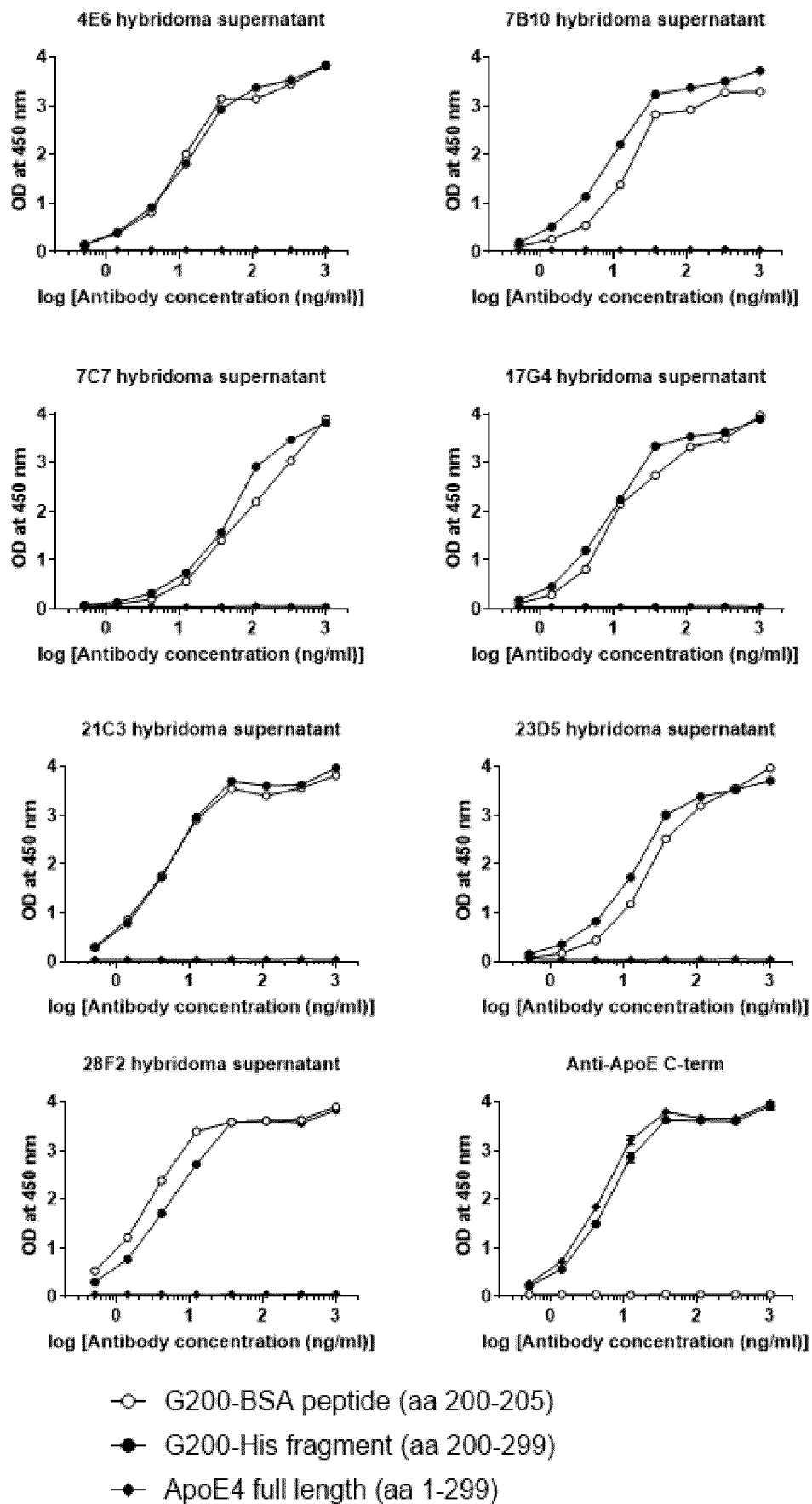
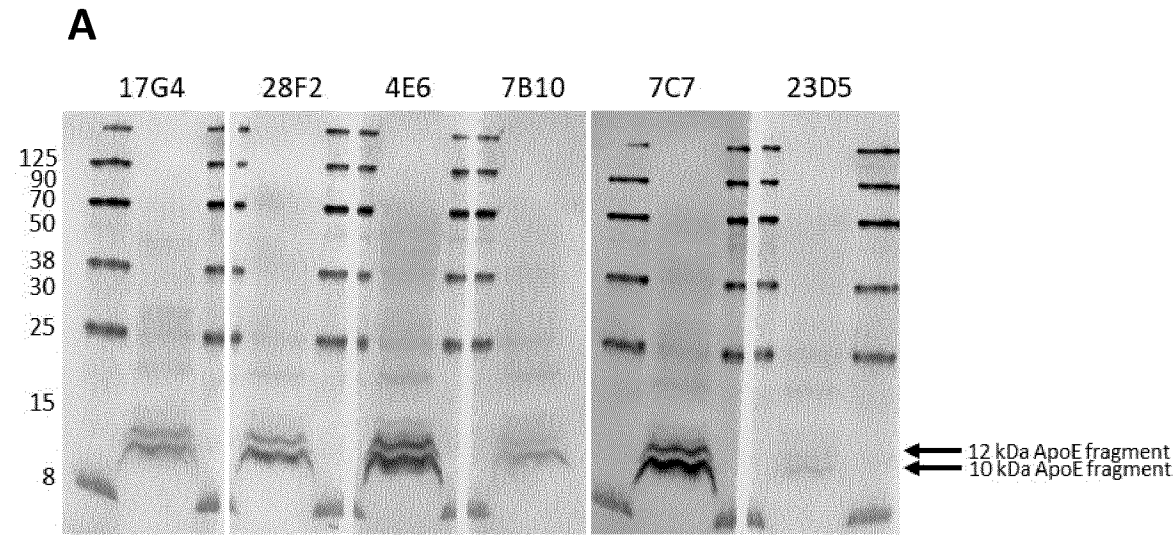
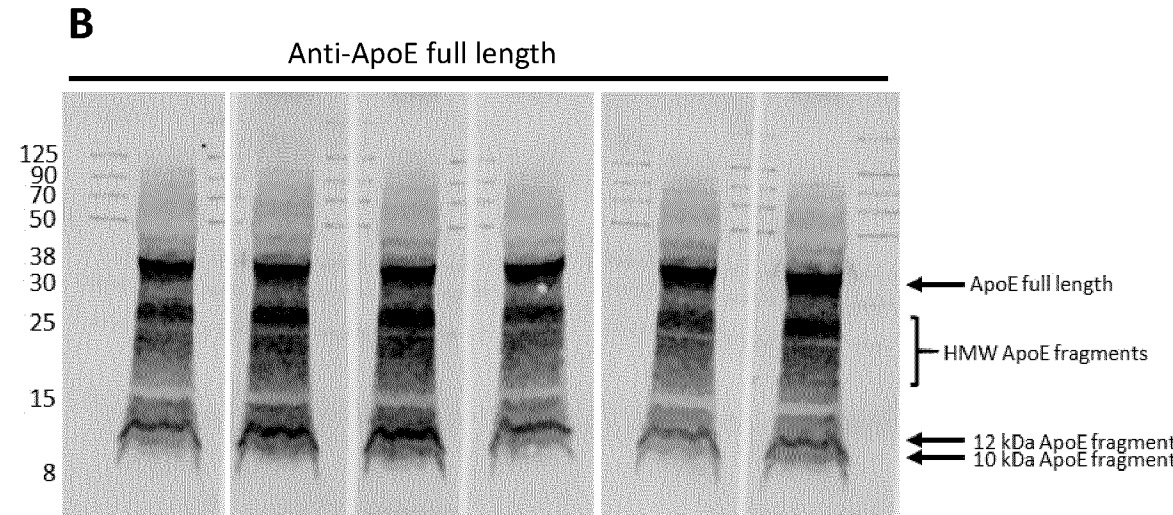


Fig. 15



WB primary antibody: Supernatants from hybridoma clones



WB primary antibody: Anti-ApoE antibody (Calbiochem, cat. no. 178479)

Fig. 16

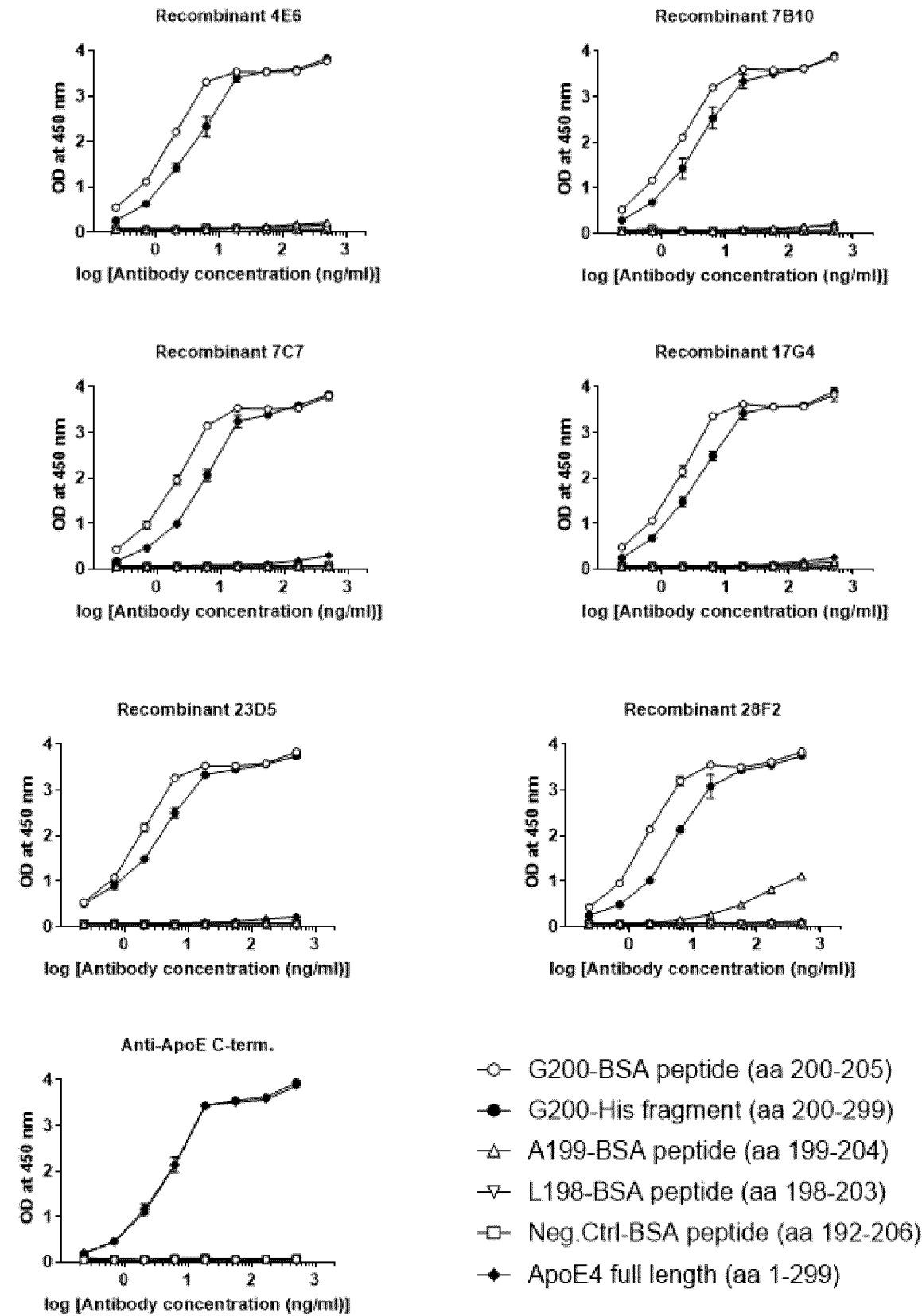


Fig. 17

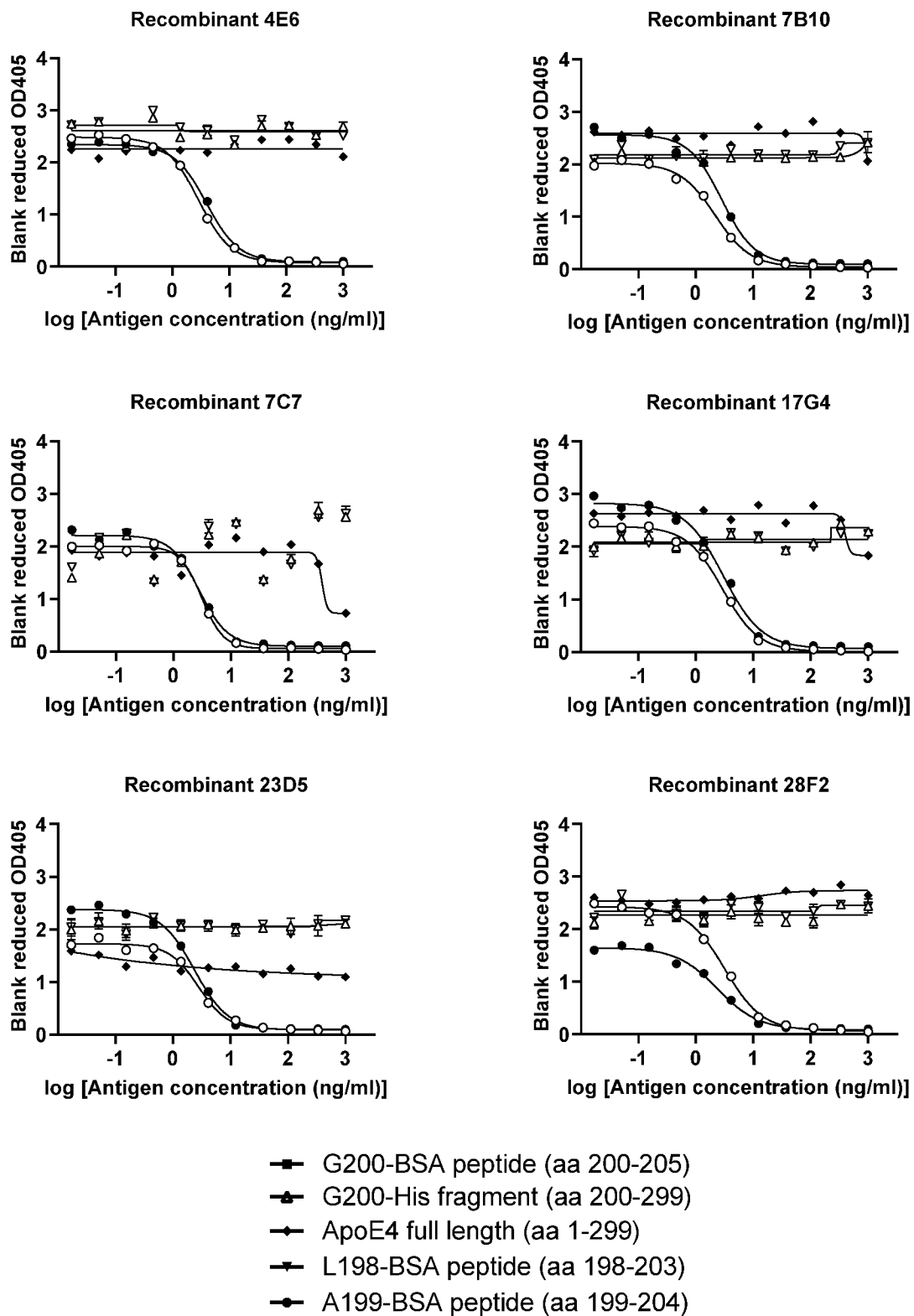


Fig. 18

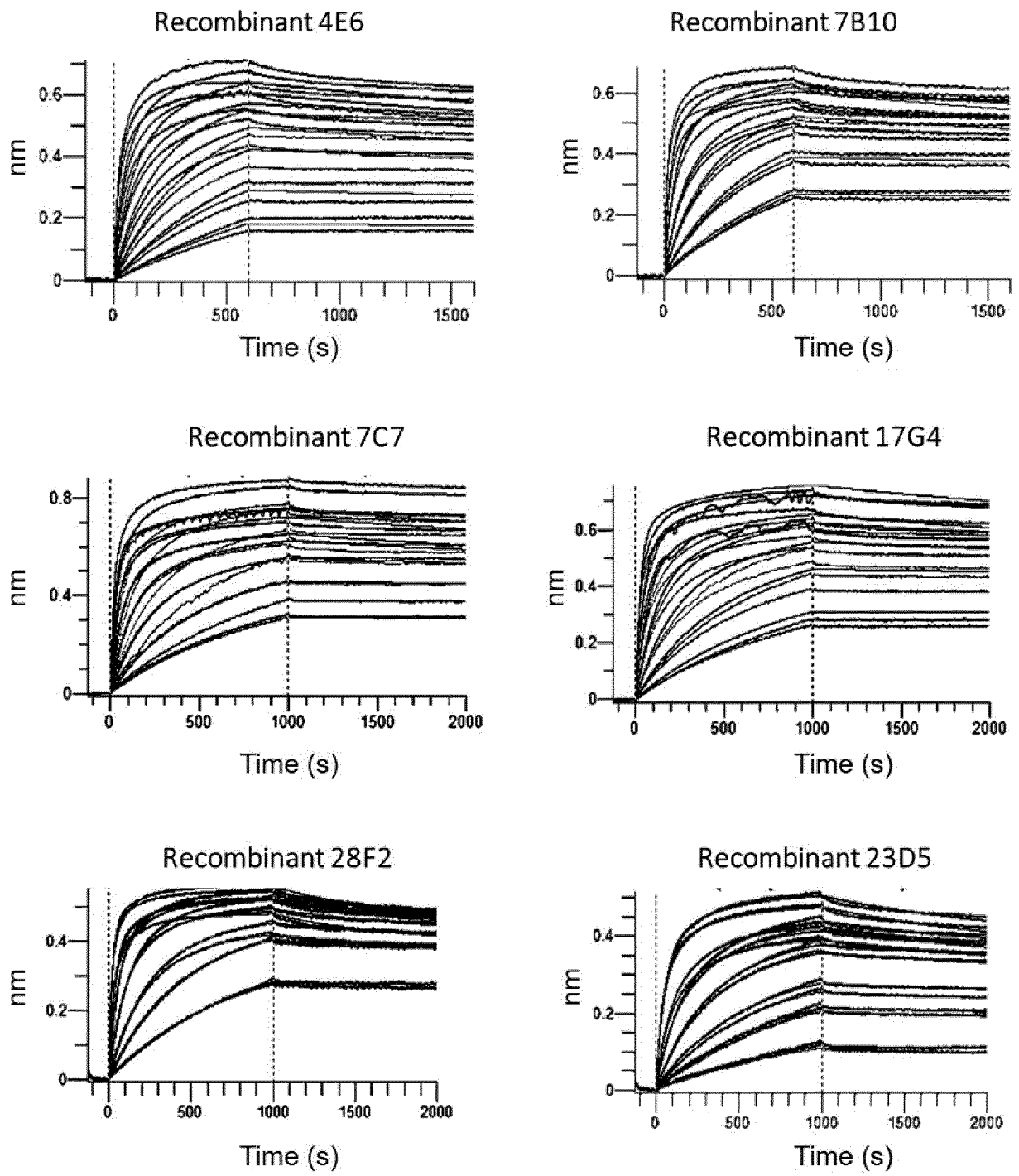


Fig. 19

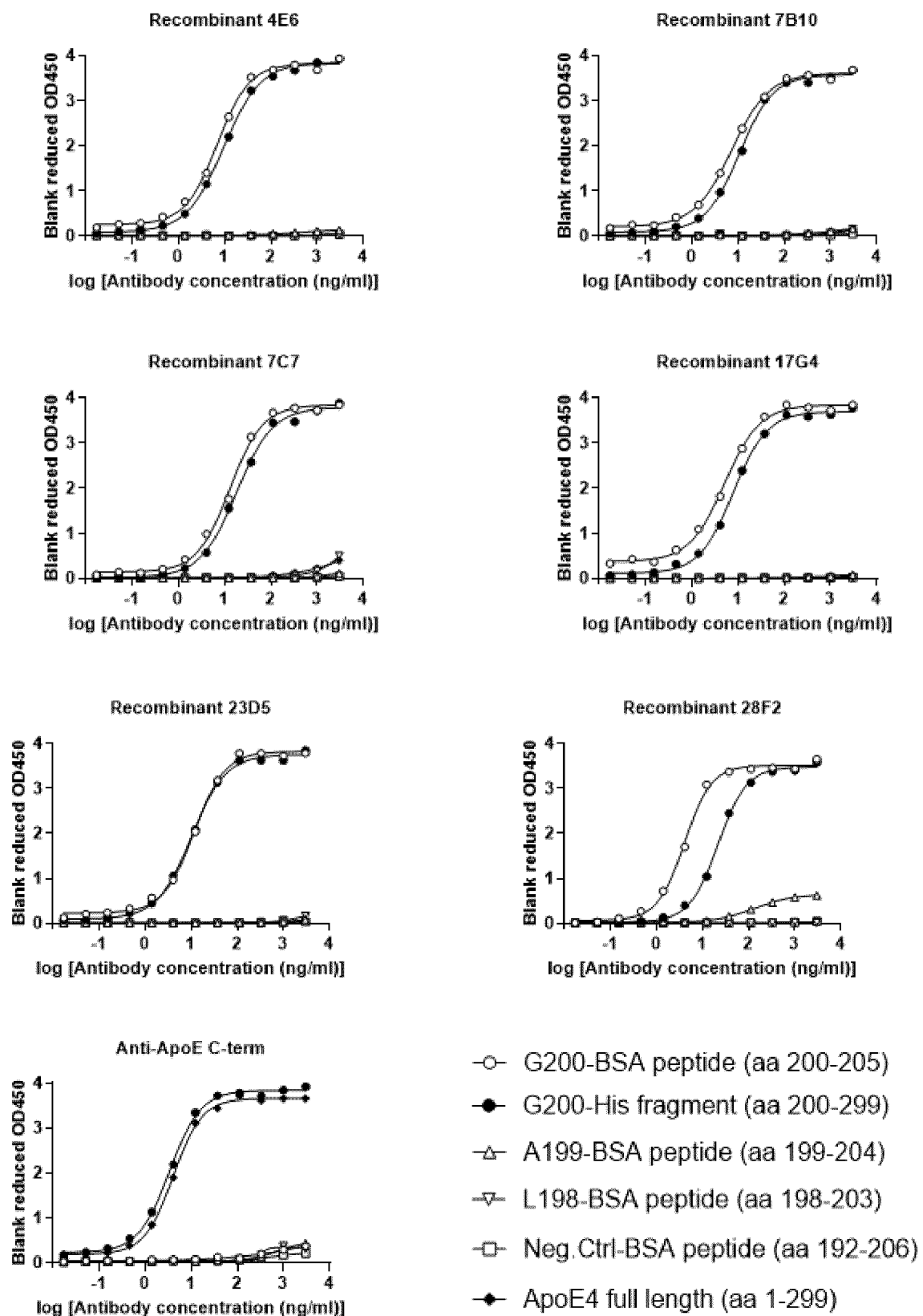
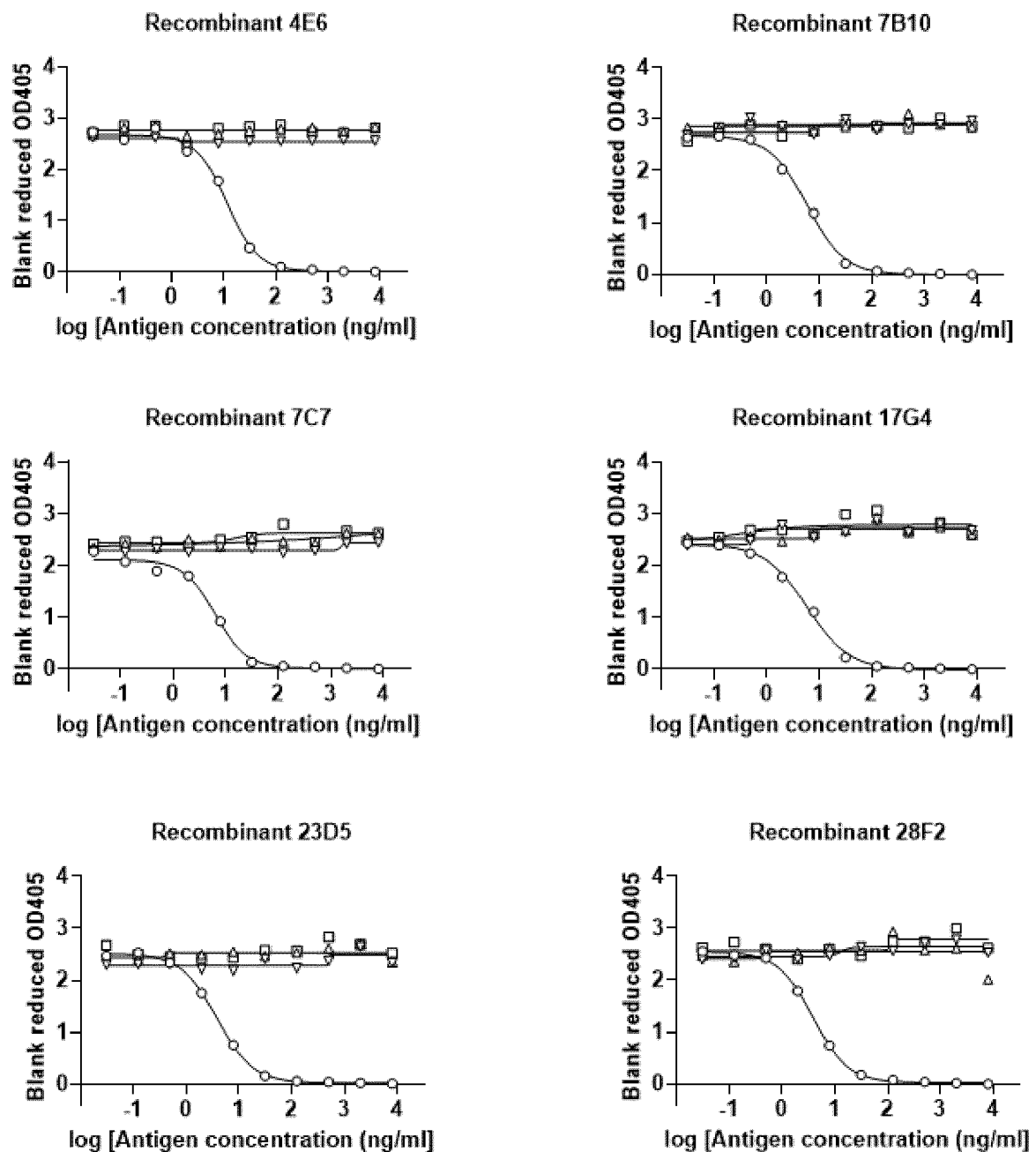


Fig. 20



- G200-BSA peptide (aa 200-205)
- △ A199-BSA peptide (aa 199-204)
- ▽ L198-BSA peptide (aa 198-203)
- Neg. Ctrl-BSA peptide (aa 192-206)

Fig. 21

Biacore: recombinant G200

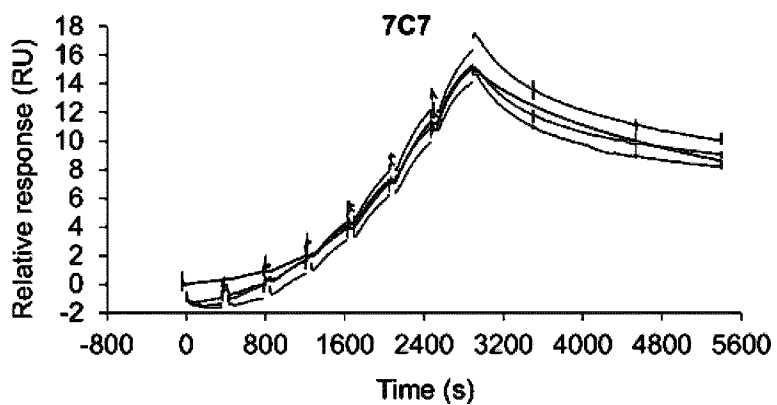
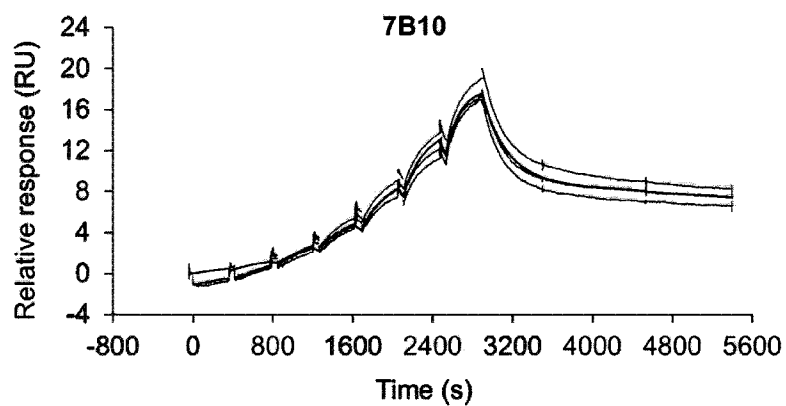
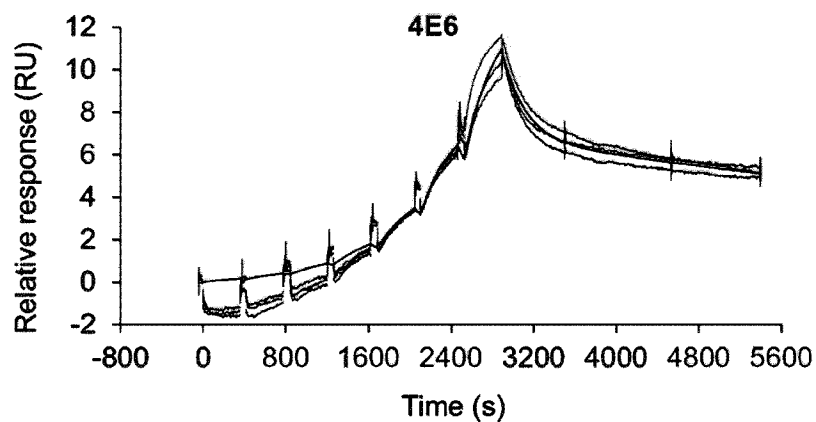


Fig. 21 continued

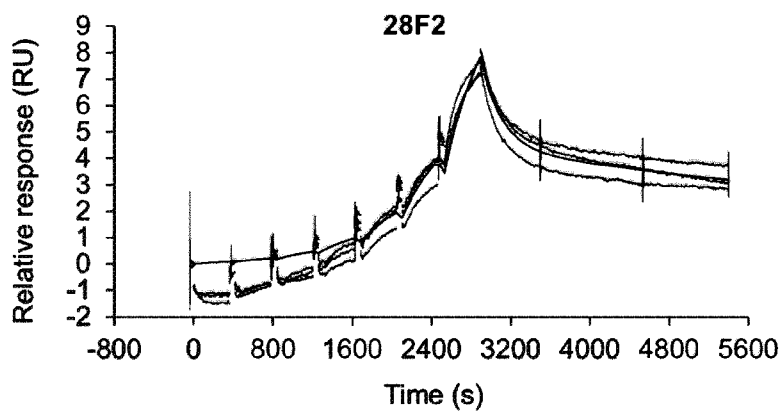
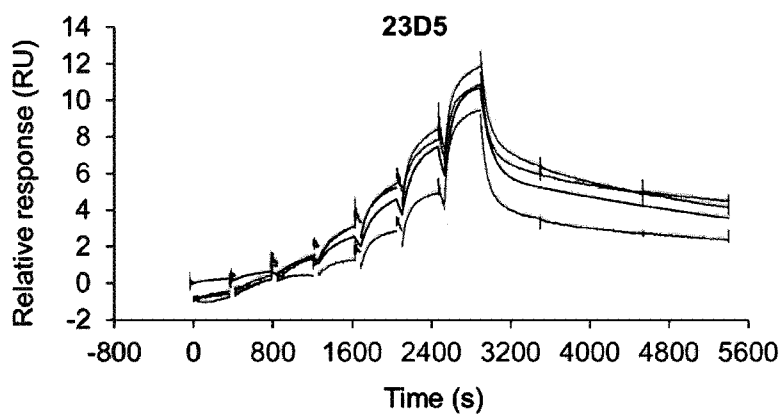
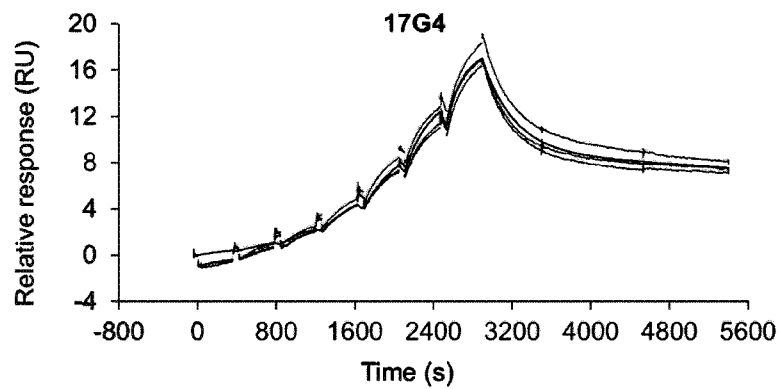
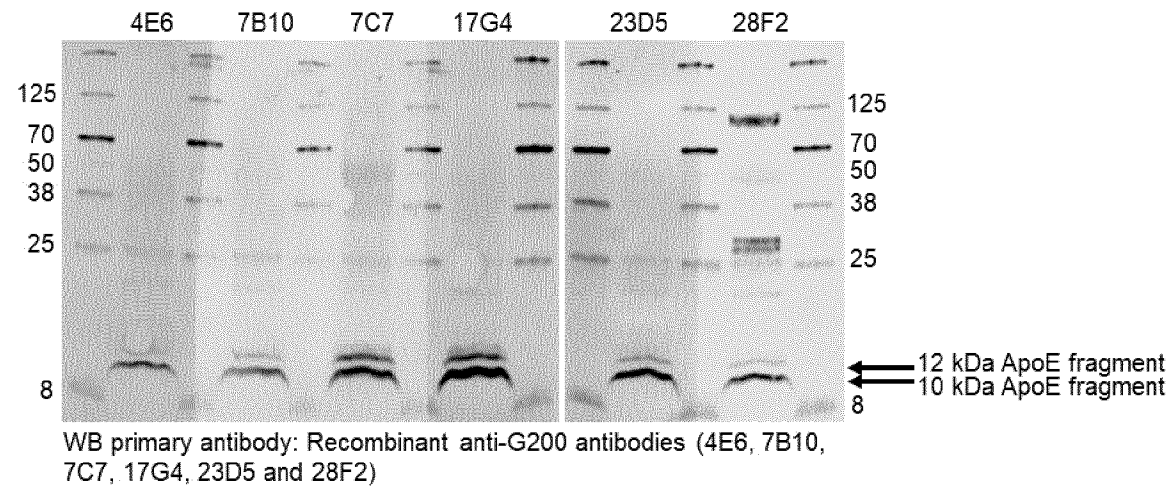
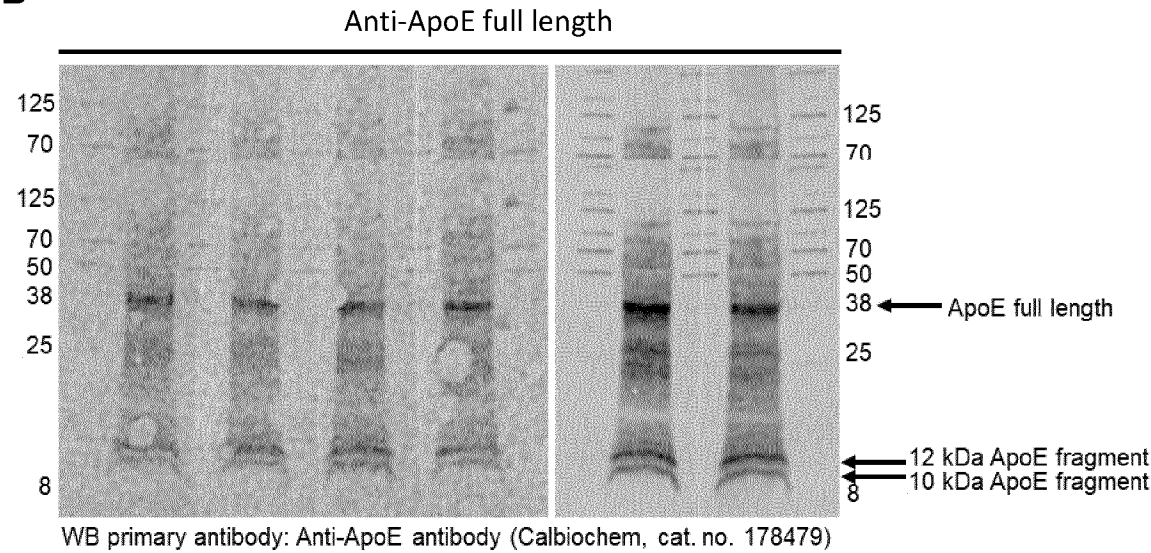


Fig. 22

A



B



C

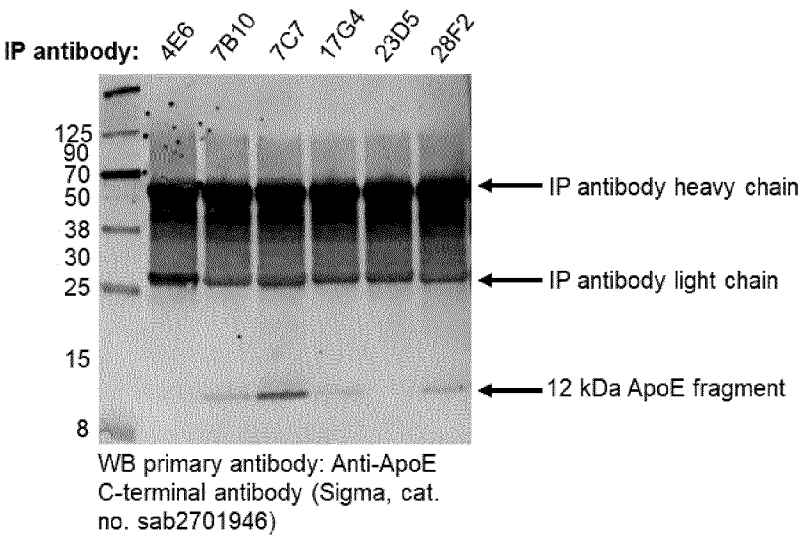


Fig. 23

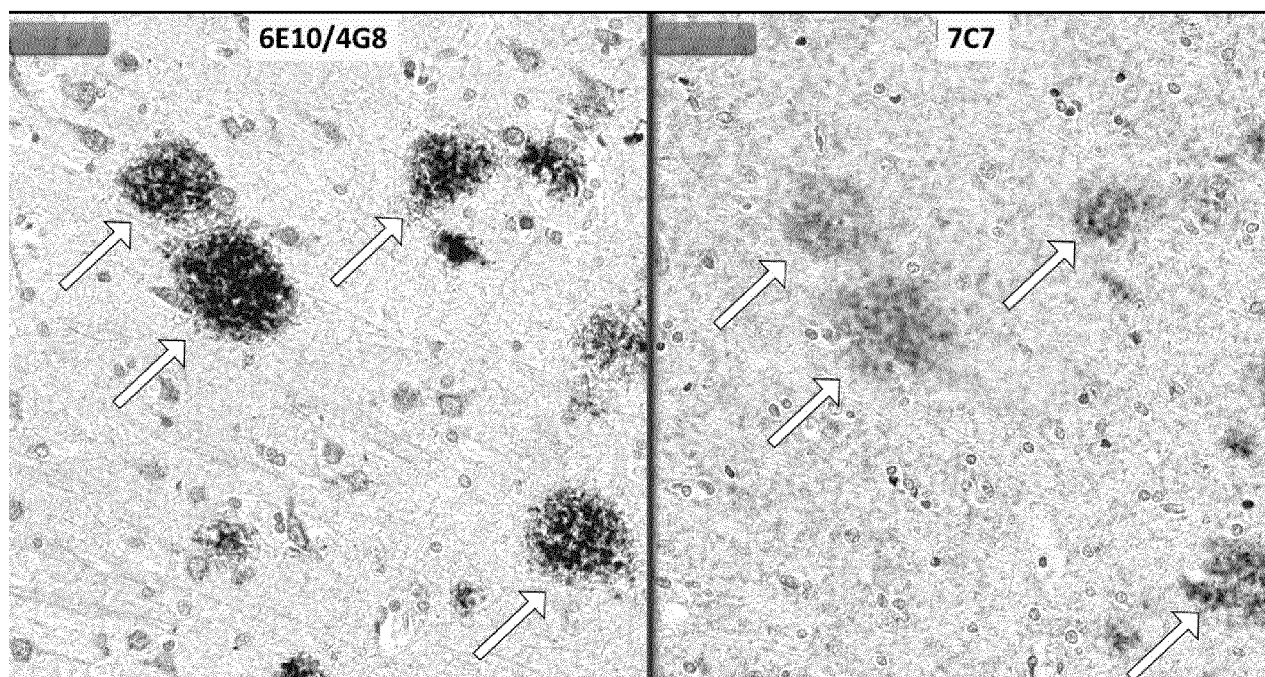
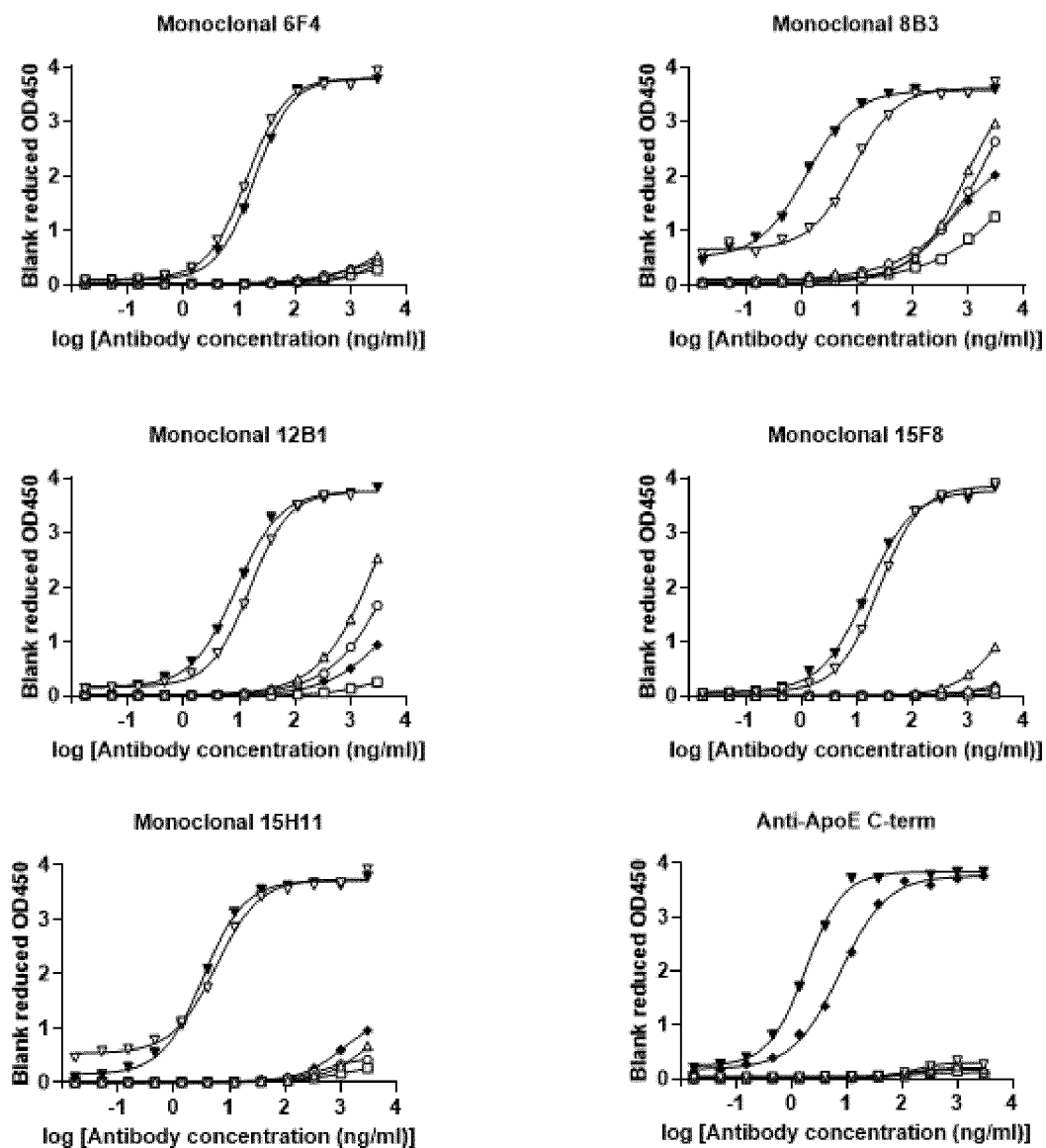


Fig. 24



- ▽ L198-BSA peptide (aa 198-203)
- ▼ L198-His fragment (aa 198-299)
- G200-BSA peptide (aa 200-205)
- △ A199-BSA peptide (aa 199-204)
- Neg.Ctrl-BSA peptide (aa 192-206)
- ◆ ApoE4 full length (aa 1-299)

Fig. 25

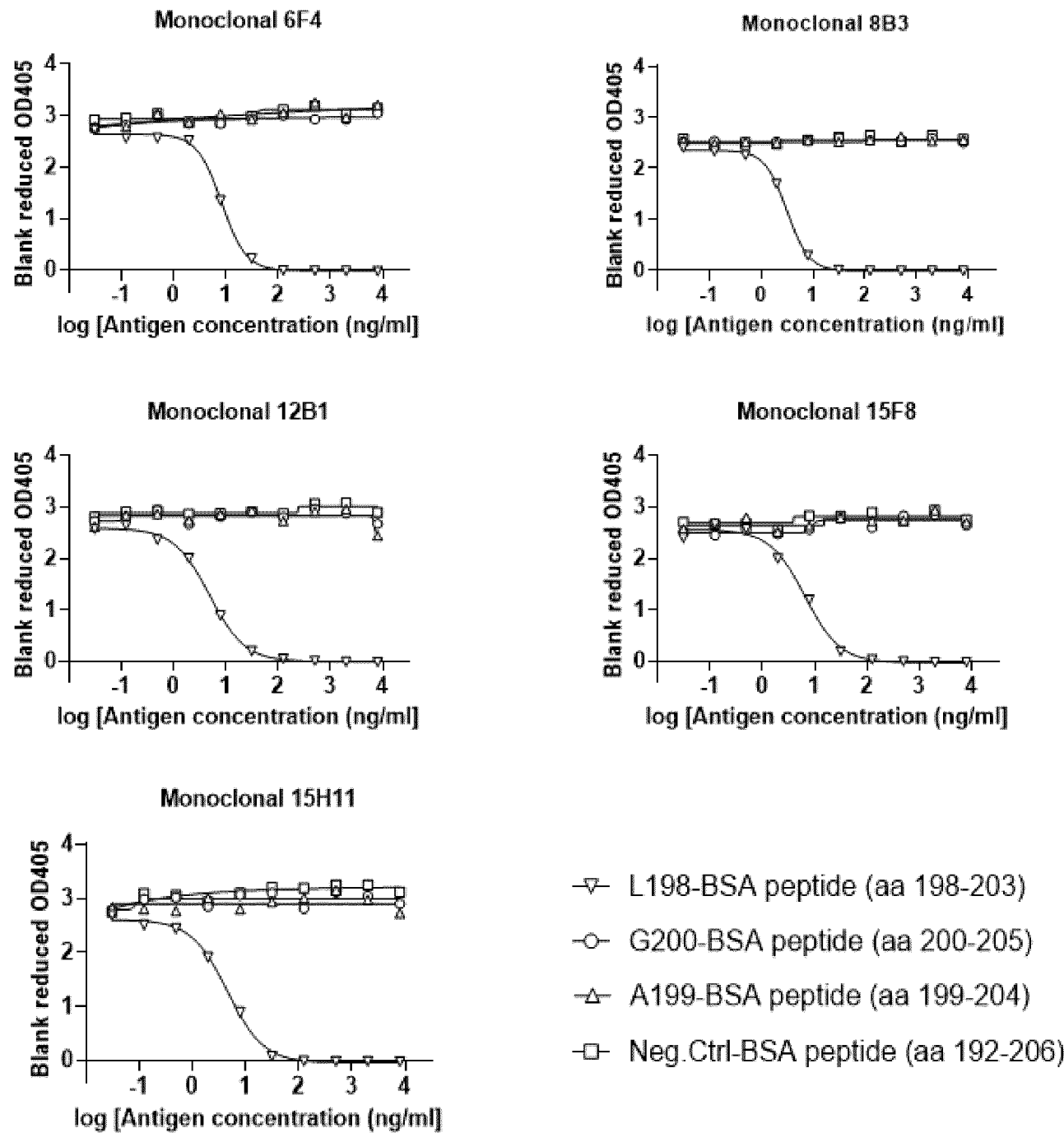


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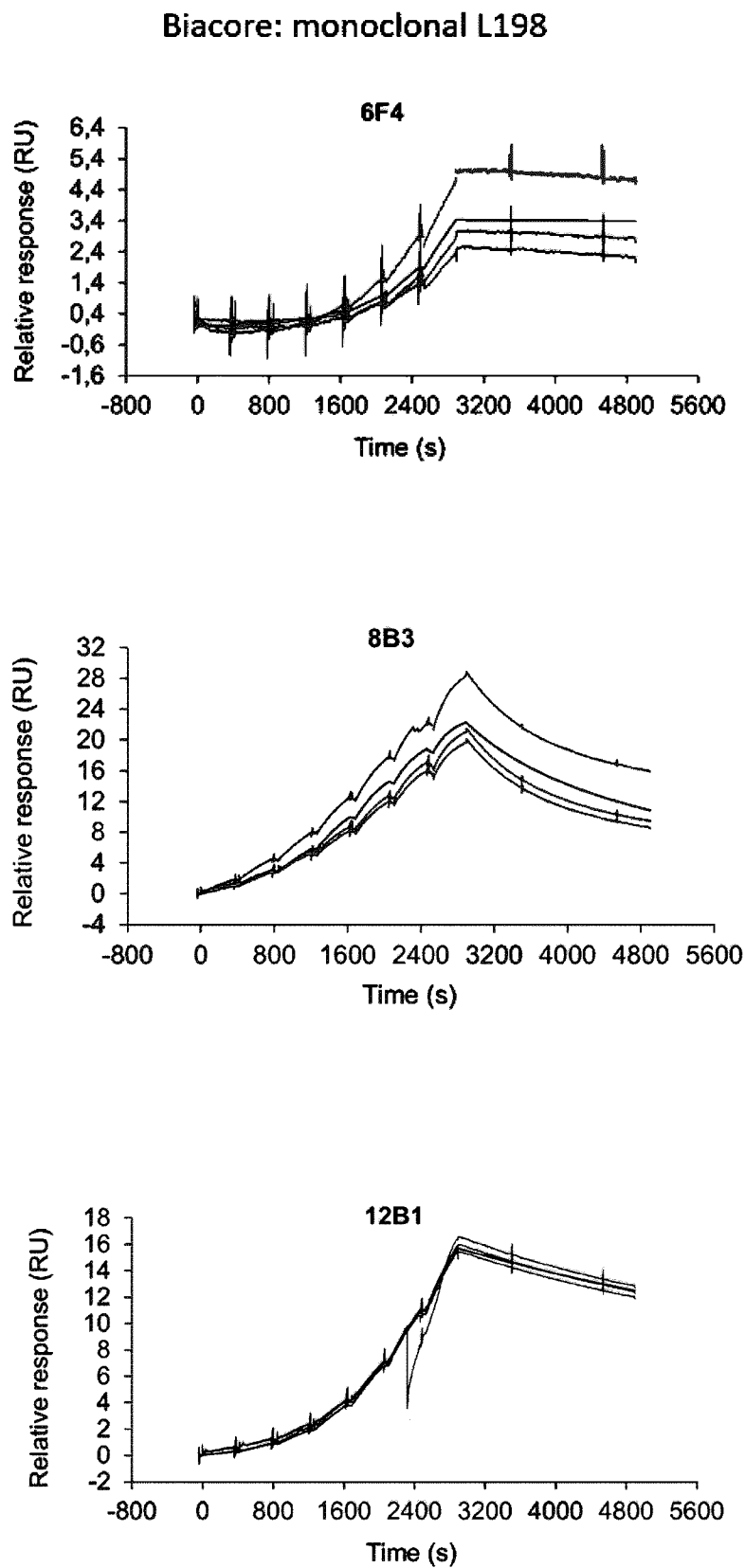


Fig. 26 continued

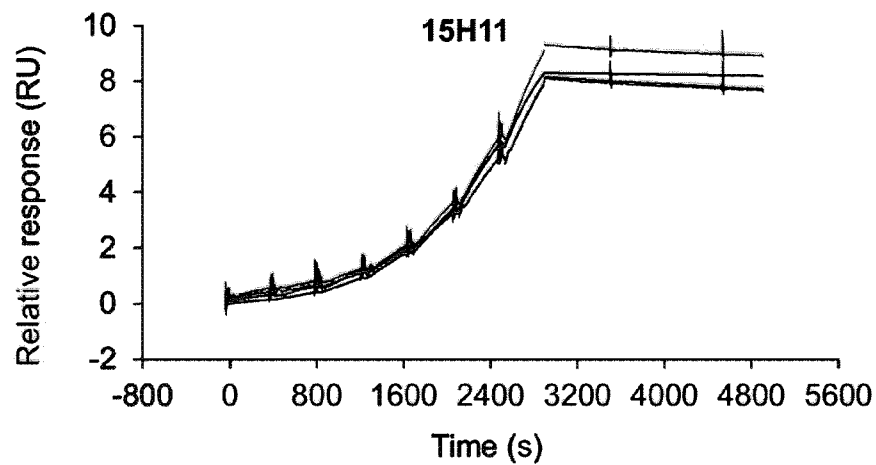
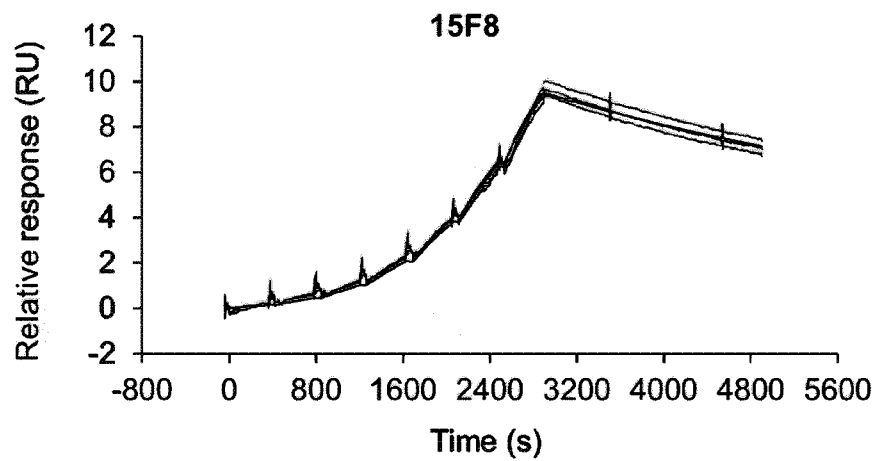
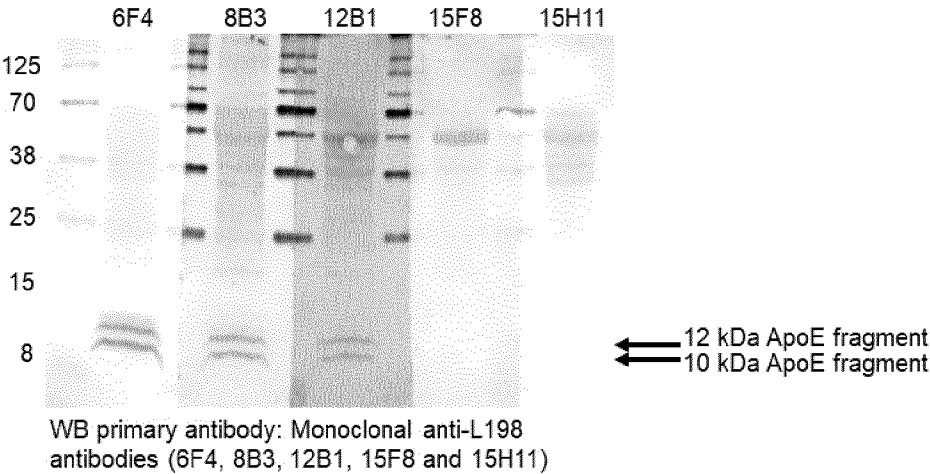
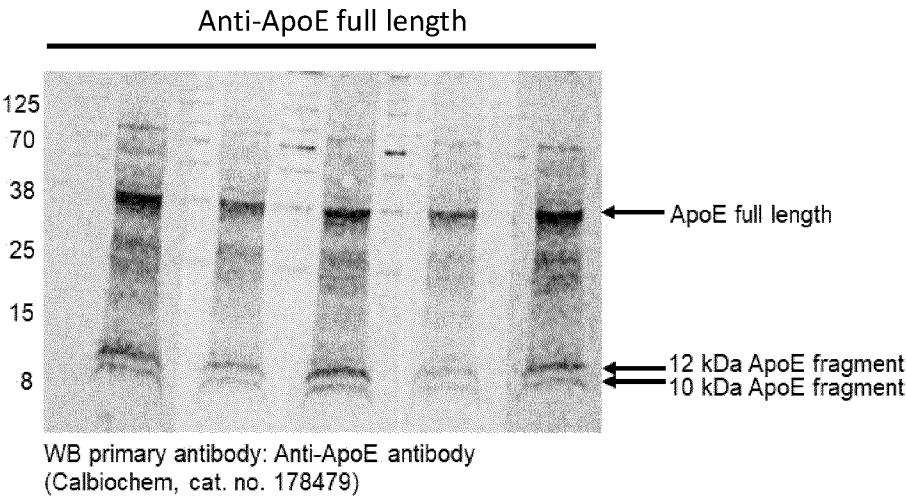


Fig. 27

A



B



C

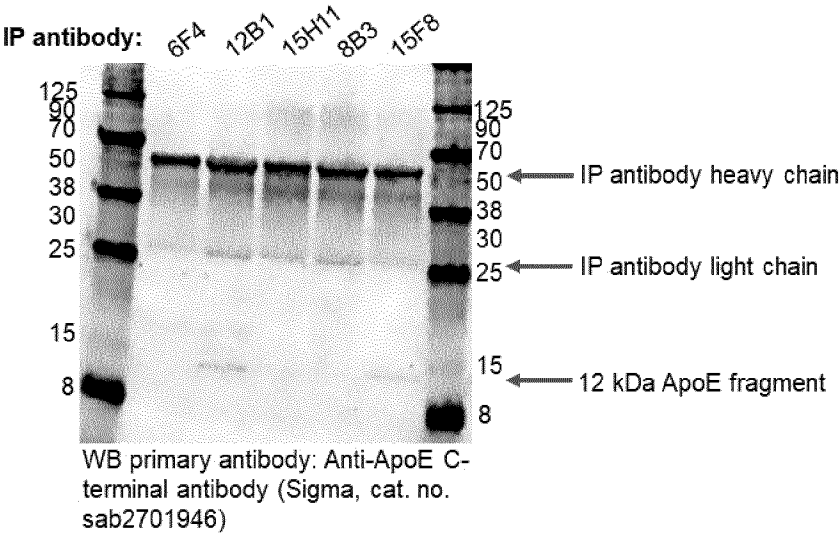


Fig. 28

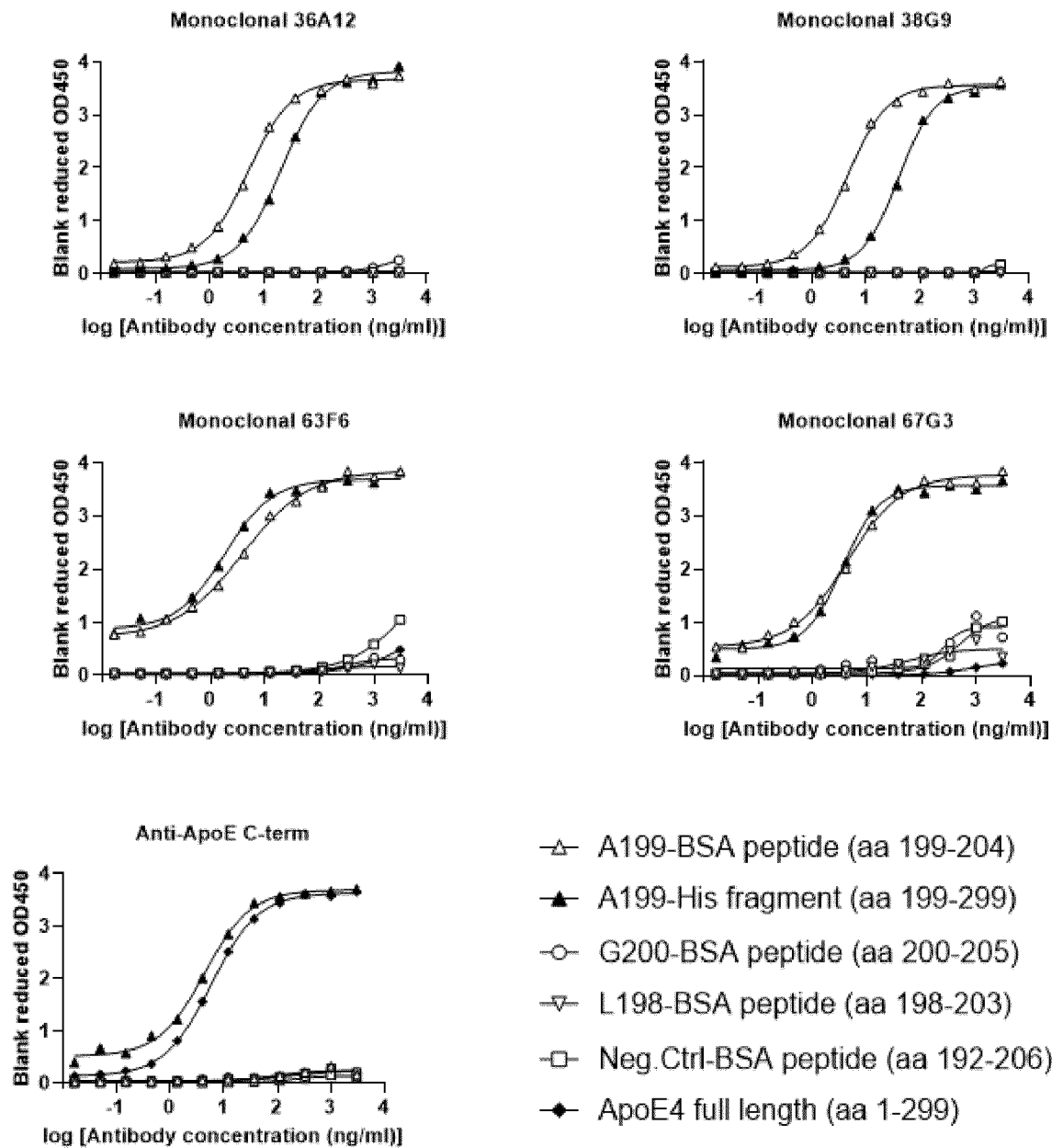
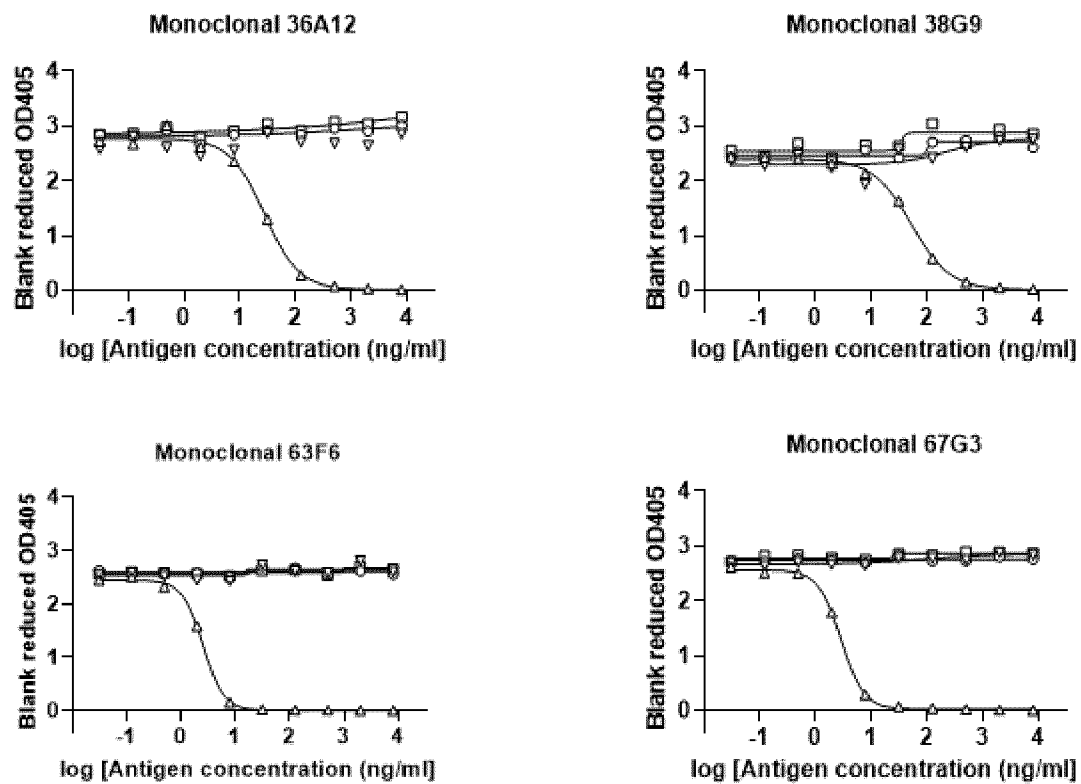


Fig. 29



- △— A199-BSA peptide (aa 199-204)
- G200-BSA peptide (aa 200-205)
- ▽— L198-BSA peptide (aa 198-203)
- Neg.Ctrl-BSA peptide (aa 192-206)

Fig. 30

Biacore: monoclonal A199

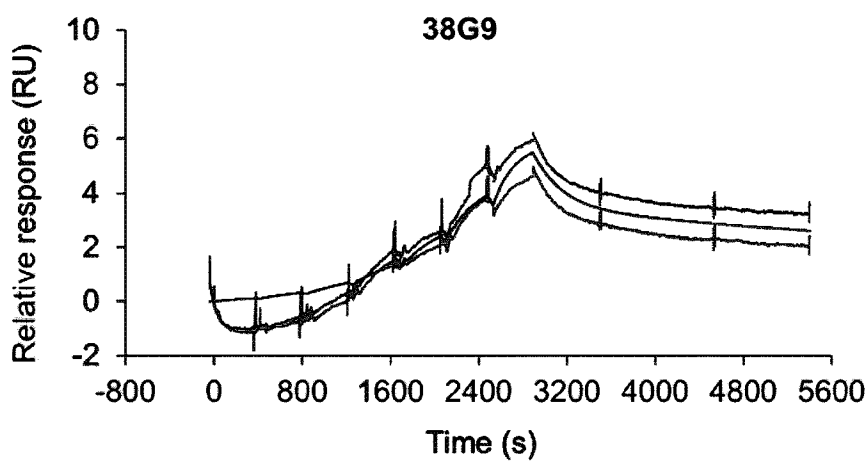
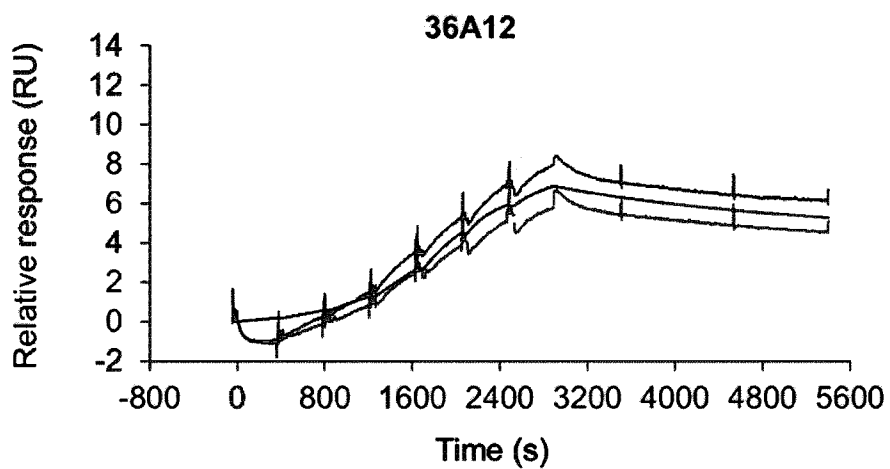


Fig. 30 continued

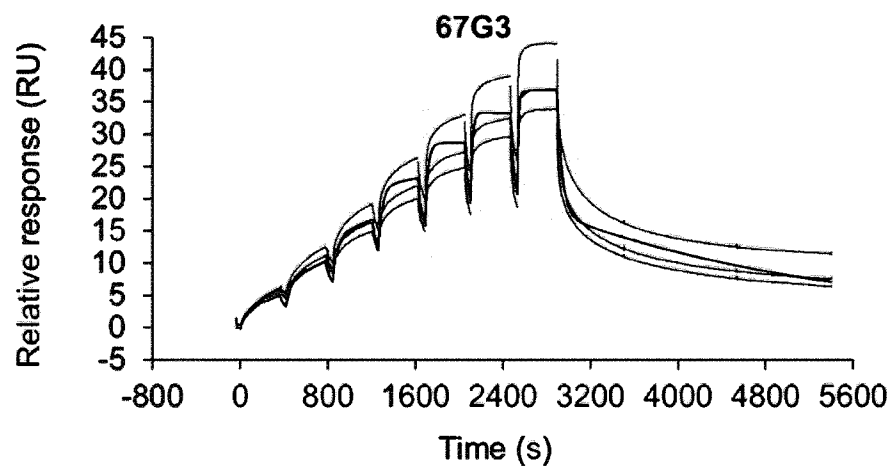
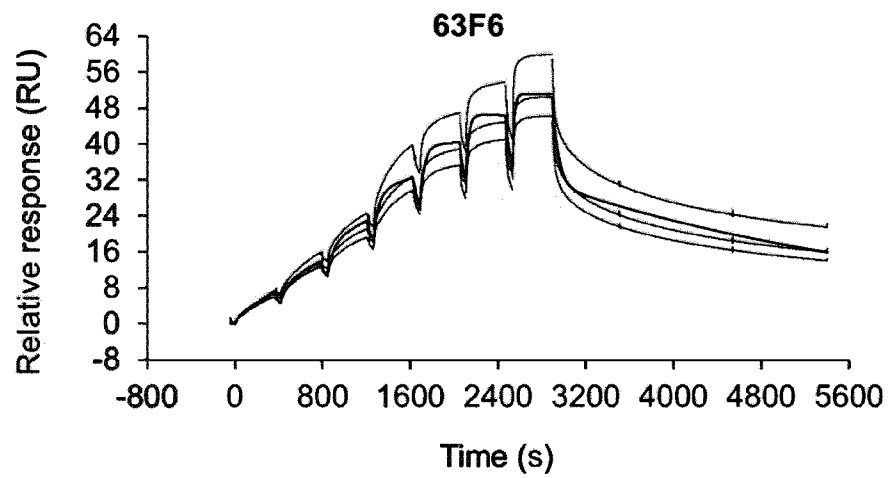
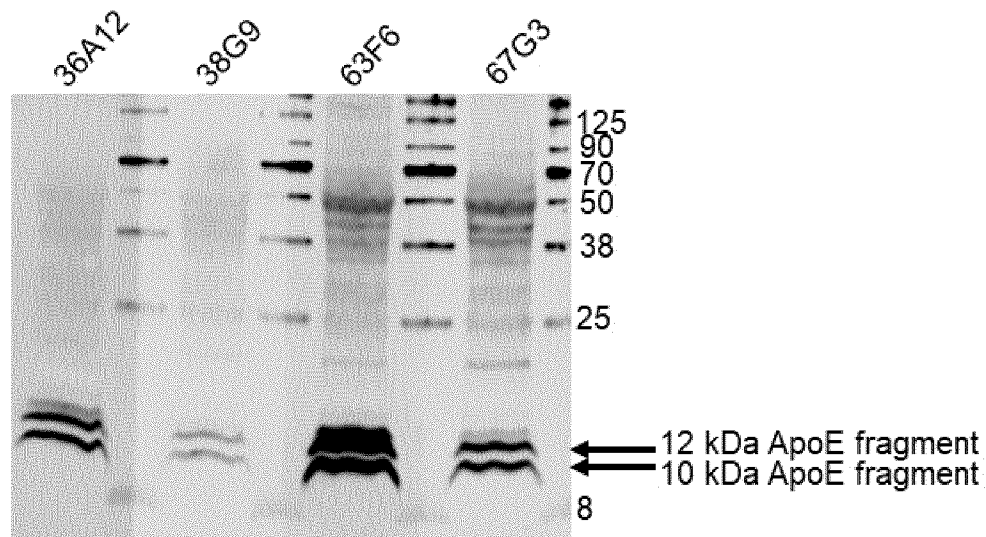


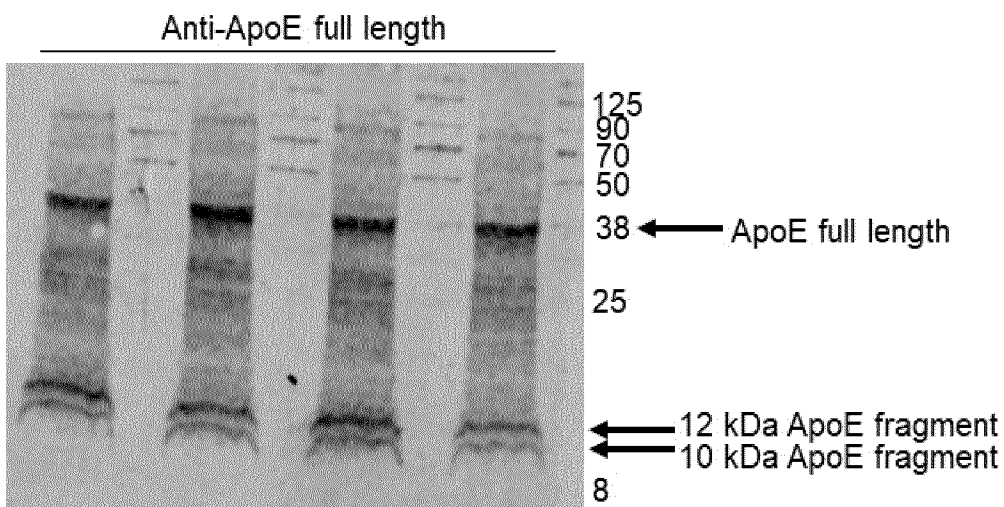
Fig. 31

A



WB primary antibody: Recombinant anti-A199 antibodies (36A12, 38G9, 63F6 and 68G3)

B



WB primary antibody: Anti-ApoE antibody (Calbiochem, cat. no.178479)

SEQUENCE LISTING

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<120> NEW ANTIBODY

<130> NLW/P207695W000

<150> EP19183405

<151> 2019-06-28

<160> 116

<170> PatentIn version 3.5

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Val Lys Glu Gln Val Ala Glu Val Arg Ala Lys Leu Glu Glu Gln Ala
35 40 45

Gln Gln Ile Arg Leu Gln Ala Glu Ala Phe Gln Ala Arg Leu Lys Ser
50 55 60

Trp Phe Glu Pro Leu Val Glu Asp Met Gln Arg Gln Trp Ala Gly Leu
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Val Glu Lys Val Gln Ala Ala Val Gly Thr Ser Ala Ala Pro Val Pro
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Ser Asp Asn His
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20 25 30

Glu Val Lys Glu Gln Val Ala Glu Val Arg Ala Lys Leu Glu Glu Gln
35 40 45

Ala Gln Gln Ile Arg Leu Gln Ala Glu Ala Phe Gln Ala Arg Leu Lys
50 55 60

Ser Trp Phe Glu Pro Leu Val Glu Asp Met Gln Arg Gln Trp Ala Gly
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Leu Val Glu Lys Val Gln Ala Ala Val Gly Thr Ser Ala Ala Pro Val
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Pro Ser Asp Asn His
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20 25 30

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35 40 45

Gln Ala Gln Gln Ile Arg Leu Gln Ala Glu Ala Phe Gln Ala Arg Leu
50 55 60

Lys Ser Trp Phe Glu Pro Leu Val Glu Asp Met Gln Arg Gln Trp Ala
65 70 75 80

Gly Leu Val Glu Lys Val Gln Ala Ala Val Gly Thr Ser Ala Ala Pro
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Val Pro Ser Asp Asn His
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Leu Ala Gly Gln Pro Leu Gln
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Ala Gly Gln Pro Leu Gln Glu
1 5

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Gly

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Gly

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20

25

30

Ala Met Ser Trp Val Arg Gln Ser Pro Glu Lys Arg Leu Glu Trp Val
 35 40 45

Ala Glu Ile Ser Gly Ser Gly Ser Arg Asp His Tyr Thr Asp Ser Val
 50 55 60

Thr Gly Arg Phe Thr Val Ser Arg Asp Asn Ala Lys Asn Thr Leu Tyr
 65 70 75 80

Leu Glu Met Ser Ser Leu Arg Ser Glu Asp Thr Ala Ile Tyr Tyr Cys
 85 90 95

Ala Arg Gln Leu Thr Gly Thr Asp Tyr Tyr Gly Thr Asp Tyr Trp Gly
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Gln Gly Thr Ser Val Thr Val Ser Ser Val Leu
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 20 25 30

Asn Gly Asn Thr Tyr Leu Glu Trp Tyr Leu Gln Arg Pro Gly Gln Ser
 35 40 45

Pro Lys Leu Leu Ile Tyr Lys Val Ser Asn Arg Phe Ser Gly Val Pro
 50 55 60

Asp Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Lys Ile
 65 70 75 80

Ser Arg Val Glu Ala Glu Asp Leu Gly Val Tyr Tyr Cys Phe Gln Gly
 85 90 95

Ser His Leu Pro Tyr Thr Phe Gly Gly Gly Thr Lys Leu Glu Ile Lys
 100 105 110

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20 25 30

Ala Met Ser Trp Val Arg Gln Phe Pro Asp Lys Arg Leu Asp Trp Val
35 40 45

Ala Glu Ile Ser Ser Gly Gly Gly Ser Thr Asn Tyr Leu Asp Thr Val
50 55 60

Thr Gly Arg Phe Thr Ile Ser Arg Asp Asn Ala Lys Asn Thr Leu Tyr
65 70 75 80

Leu Glu Met Asn Ser Leu Arg Ser Glu Asp Thr Ala Met Tyr Tyr Cys
85 90 95

Ala Arg Gln Leu Val Gly Thr Asp Tyr Tyr Gly Thr Asp Tyr Trp Gly
100 105 110

Gln Gly Thr Ser Val Thr Val Ser Ser
115 120

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20 25 30

Asn Gly Asn Thr Tyr Leu Glu Trp Tyr Leu Gln Lys Pro Gly Gln Ser
35 40 45

Pro Lys Leu Leu Ile Tyr Lys Val Ser Asn Arg Phe Ser Gly Val Pro
50 55 60

Asp Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Lys Ile
65 70 75 80

Ser Arg Val Glu Ala Glu Asp Leu Gly Val Tyr Tyr Cys Phe Gln Gly
85 90 95

Ser His Val Pro Tyr Thr Phe Gly Gly Gly Thr Lys Leu Glu Ile Lys
100 105 110

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Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe Ser Ser Phe
20 25 30

Ala Met Ser Trp Val Arg Gln Ser Pro Glu Arg Arg Leu Glu Trp Val
35 40 45

Ala Glu Ile Ser Arg Gly Gly Gly Tyr Ala Phe Tyr Ser Asp Thr Val
50 55 60

Thr Gly Arg Phe Thr Ile Ser Arg Asp Asn Ala Arg Asn Thr Leu Phe
65 70 75 80

Leu Glu Met Gly Ser Leu Arg Ser Glu Asp Thr Ala Met Tyr Tyr Cys
85 90 95

Ser Arg Gln Leu Thr Gly Thr Asp Tyr Tyr Ala Met Asp Tyr Trp Gly
100 105 110

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

Asn Gly Asn Thr Tyr Leu Glu Trp Tyr Leu Gln Lys Pro Gly Gln Ser
35 40 45

Pro Lys Leu Leu Ile Tyr Lys Val Ser Asn Arg Phe Ser Gly Val Pro
50 55 60

Asp Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Lys Ile
65 70 75 80

Ser Arg Val Glu Ala Glu Asp Leu Gly Val Tyr Tyr Cys Phe Gln Gly
85 90 95

Ser Gln Val Pro Tyr Thr Phe Gly Gly Gly Thr Lys Leu Glu Leu Lys

100

105

110

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Ser Leu Lys Leu Ser Cys Ser Ala Ser Gly Phe Thr Phe Ser Arg Tyr
 20 25 30

Ala Met Ser Trp Val Arg Gln Ser Pro Glu Lys Arg Leu Glu Trp Val
 35 40 45

Ala Glu Ile Asn Ser Gly Gly Ser Tyr Ser Phe Tyr Ser Asp Thr Val
 50 55 60

Thr Gly Arg Phe Thr Ile Ser Arg Asp Asn Ala Lys Asn Thr Leu Phe
 65 70 75 80

Leu Glu Met Ser Ser Leu Arg Ser Glu Asp Thr Ala Ile Tyr Tyr Cys
 85 90 95

Ala Arg Gln Leu Thr Gly Thr Asp Tyr Tyr Gly Thr Asp Tyr Trp Gly
 100 105 110

Gln Gly Thr Ser Val Thr Val Ser Ser
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 1 5 10 15

Asp Gln Ala Ser Ile Ser Cys Arg Ser Ser Gln Ser Leu Leu Tyr Ser
 20 25 30

Asn Gly Asn Thr Tyr Leu Glu Trp Tyr Leu Gln Lys Pro Gly Gln Ser
 35 40 45

Pro Lys Leu Leu Ile Tyr Lys Val Ser Asn Arg Phe Ser Gly Val Pro
 50 55 60

Asp Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Lys Ile
 65 70 75 80

Ser Arg Val Glu Ala Glu Asp Leu Gly Val Tyr Tyr Cys Phe Gln Gly
85 90 95

Ser His Val Pro Tyr Thr Phe Gly Gly Gly Thr Lys Leu Glu Ile Lys
100 105 110

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Ser Leu Lys Leu Ser Cys Ser Ala Ser Gly Phe Thr Phe Ser Arg Tyr
20 25 30

Ala Met Ser Trp Val Arg Gln Ser Pro Glu Lys Arg Leu Glu Trp Val
35 40 45

Ala Glu Ile Asn Ser Gly Gly Ser Tyr Ser Phe Tyr Ser Asp Thr Val
50 55 60

Thr Gly Arg Phe Thr Ile Ser Arg Asp Asn Ala Lys Asn Thr Leu Phe
65 70 75 80

Leu Glu Met Ser Ser Leu Arg Ser Glu Asp Thr Ala Ile Tyr Tyr Cys
85 90 95

Ala Arg Gln Leu Ser Gly Thr Asp Tyr Tyr Gly Thr Asp Tyr Trp Gly
100 105 110

Gln Gly Thr Ser Val Thr Val Ser Ser
115 120

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Thr Val Lys Ile Ser Cys Lys Ala Ser Gly Tyr Thr Phe Thr Asn Tyr
20 25 30

Ala Met His Trp Met Lys Gln Ala Pro Gly Lys Ala Leu Lys Trp Met
35 40 45

Gly Trp Ile Asn Thr Tyr Thr Gly Glu Pro Thr Phe Ala Asp Asp Phe
50 55 60

Lys Gly Arg Phe Ala Phe Ser Leu Glu Thr Ser Ala Ser Thr Ala Tyr
65 70 75 80

Leu Gln Ile Asn Ser Leu Lys Asn Glu Asp Met Ala Thr Tyr Phe Cys
85 90 95

Ala Arg Glu Gly Tyr Tyr Asp Arg Ser His Tyr Phe Asp Tyr Trp Gly
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Gln Gly Thr Thr Leu Thr Val Ser Ser
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20 25 30

Asp Gly Asn Thr Tyr Leu Glu Trp Tyr Leu Gln Lys Pro Gly Gln Ser
35 40 45

Pro Lys Leu Leu Ile Tyr Lys Val Ser Asn Arg Phe Ser Gly Val Pro
50 55 60

Asp Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Lys Ile
65 70 75 80

Ser Arg Val Glu Ala Glu Asp Leu Gly Val Tyr Tyr Cys Leu Gln Gly
85 90 95

Ser His Ile Pro Phe Thr Phe Gly Ser Gly Thr Lys Leu Glu Ile Lys
100 105 110

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20 25 30

Phe Trp Asp Tyr Leu Arg Trp Val Gln Thr Leu Ser Glu Gln Val Gln
35 40 45

Glu Glu Leu Leu Ser Ser Gln Val Thr Gln Glu Leu Arg Ala Leu Met
 50 55 60
 Asp Glu Thr Met Lys Glu Leu Lys Ala Tyr Lys Ser Glu Leu Glu Glu
 65 70 75 80
 Gln Leu Thr Pro Val Ala Glu Glu Thr Arg Ala Arg Leu Ser Lys Glu
 85 90 95
 Leu Gln Ala Ala Gln Ala Arg Leu Gly Ala Asp Met Glu Asp Val Arg
 100 105 110
 Gly Arg Leu Val Gln Tyr Arg Gly Glu Val Gln Ala Met Leu Gly Gln
 115 120 125
 Ser Thr Glu Glu Leu Arg Val Arg Leu Ala Ser His Leu Arg Lys Leu
 130 135 140
 Arg Lys Arg Leu Leu Arg Asp Ala Asp Asp Leu Gln Lys Arg Leu Ala
 145 150 155 160
 Val Tyr Gln Ala Gly Ala Arg Glu Gly Ala Glu Arg Gly Leu Ser Ala
 165 170 175
 Ile Arg Glu Arg Leu Gly Pro Leu Val Glu Gln Gly Arg Val Arg Ala
 180 185 190
 Ala Thr Val Gly Ser Leu Ala Gly Gln Pro Leu Gln Glu Arg Ala Gln
 195 200 205
 Ala Trp Gly Glu Arg Leu Arg Ala Arg Met Glu Glu Met Gly Ser Arg
 210 215 220
 Thr Arg Asp Arg Leu Asp Glu Val Lys Glu Gln Val Ala Glu Val Arg
 225 230 235 240
 Ala Lys Leu Glu Glu Gln Ala Gln Gln Ile Arg Leu Gln Ala Glu Ala
 245 250 255
 Phe Gln Ala Arg Leu Lys Ser Trp Phe Glu Pro Leu Val Glu Asp Met
 260 265 270
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 290 295

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

Val Arg Ala Ala Thr Val Gly Ser Leu Ala Gly Gln Pro Leu Gln Glu
35 40 45

Arg Ala Gln Ala Trp Gly Glu Arg Leu Arg Ala Arg Met Glu Glu Met
50 55 60

Gly Ser Arg Thr Arg Asp Arg Leu Asp Glu Val Lys
65 70 75

<210> 47

<211> 34

<212> PRT

<213> Homo sapiens

<400> 47

Gly Gln Pro Leu Gln Glu Arg Ala Gln Ala Trp Gly Glu Arg Leu Arg
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Ala Arg Met Glu Glu Met Gly Ser Arg Thr Arg Asp Arg Leu Asp Glu
20 25 30

Val Lys

<210> 48

<211> 110

<212> PRT

<213> Homo sapiens

<400> 48

Gly Gln Pro Leu Gln Glu Arg Ala Gln Ala Trp Gly Glu Arg Leu Arg
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Ala Arg Met Glu Glu Met Gly Ser Arg Thr Arg Asp Arg Leu Asp Glu
20 25 30

Val Lys Glu Gln Val Ala Glu Val Arg Ala Lys Leu Glu Glu Gln Ala
35 40 45

Gln Gln Ile Arg Leu Gln Ala Glu Ala Phe Gln Ala Arg Leu Lys Ser
50 55 60

Trp Phe Glu Pro Leu Val Glu Asp Met Gln Arg Gln Trp Ala Gly Leu
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Val Glu Lys Val Gln Ala Ala Val Gly Thr Ser Ala Ala Pro Val Pro
85 90 95

Ser Asp Asn His His His His His His His His His His His His
100 105 110

<210> 49
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<212> PRT
<213> Homo sapiens

<400> 49

His His His His His His His His His His Lys Val Glu Gln Ala Val
1 5 10 15

Glu Thr Glu Pro Glu Pro Glu Leu Arg Gln Gln Thr Glu Trp Gln Ser
20 25 30

Gly Gln Arg Trp Glu Leu Ala Leu Gly Arg Phe Trp Asp Tyr Leu Arg
35 40 45

Trp Val Gln Thr Leu Ser Glu Gln Val Gln Glu Glu Leu Leu Ser Ser
50 55 60

Gln Val Thr Gln Glu Leu Arg Ala Leu Met Asp Glu Thr Met Lys Glu
65 70 75 80

Leu Lys Ala Tyr Lys Ser Glu Leu Glu Glu Gln Leu Thr Pro Val Ala
85 90 95

Glu Glu Thr Arg Ala Arg Leu Ser Lys Glu Leu Gln Ala Ala Gln Ala
100 105 110

Arg Leu Gly Ala Asp Met Glu Asp Val Arg Gly Arg Leu Val Gln Tyr
115 120 125

Arg Gly Glu Val Gln Ala Met Leu Gly Gln Ser Thr Glu Glu Leu Arg
130 135 140

Val Arg Leu Ala Ser His Leu Arg Lys Leu Arg Lys Arg Leu Leu Arg
145 150 155 160

Asp Ala Asp Asp Leu Gln Lys Arg Leu Ala Val Tyr Gln Ala Gly Ala
165 170 175

Arg Glu Gly Ala Glu Arg Gly Leu Ser Ala Ile Arg Glu Arg Leu Gly
180 185 190

Pro Leu Val Glu Gln Gly Arg Val Arg Ala Ala Thr Val Gly Ser Leu
195 200 205

Ala Gly Gln Pro Leu Gln Glu Arg Ala Gln Ala Trp Gly Glu Arg Leu

210	215	220
Arg Ala Arg Met Glu Glu Met Gly Ser Arg Thr Arg Asp Arg Leu Asp 225 230 235 240		
Glu Val Lys Glu Gln Val Ala Glu Val Arg Ala Lys Leu Glu Glu Gln 245 250 255		
Ala Gln Gln Ile Arg Leu Gln Ala Glu Ala Phe Gln Ala Arg Leu Lys 260 265 270		
Ser Trp Phe Glu Pro Leu Val Glu Asp Met Gln Arg Gln Trp Ala Gly 275 280 285		
Leu Val Glu Lys Val Gln Ala Ala Val Gly Thr Ser Ala Ala Pro Val 290 295 300		
Pro Ser Asp Asn His 305		

<210> 50
 <211> 8
 <212> PRT
 <213> Artificial sequence

<220>
 <223> Synthetic peptide

<220>
 <221> MOD_RES
 <222> (7)..(7)
 <223> Acp

<400> 50

Gly Gln Pro Leu Gln Glu Xaa Cys
1 5

<210> 51
 <211> 8
 <212> PRT
 <213> Artificial sequence

<220>
 <223> Synthetic peptide

<220>
 <221> MOD_RES
 <222> (7)..(7)
 <223> Acp

<400> 51

Ala Gly Gln Pro Leu Gln Xaa Cys
1 5

<210> 52
 <211> 8

<212> PRT
<213> Artificial sequence

<220>
<223> Synthetic peptide

<220>
<221> MOD_RES
<222> (7)..(7)
<223> Acp

<400> 52

Leu Ala Gly Gln Pro Leu Xaa Cys
1 5

<210> 53
<211> 17
<212> PRT
<213> Artificial sequence

<220>
<223> Synthetic peptide

<220>
<221> MOD_RES
<222> (16)..(16)
<223> Acp

<400> 53

Ala Ala Thr Val Gly Ser Leu Ala Gly Gln Pro Leu Gln Glu Arg Xaa
1 5 10 15

Cys

<210> 54
<211> 10
<212> PRT
<213> Artificial sequence

<220>
<223> Synthetic peptide

<220>
<221> MOD_RES
<222> (9)..(9)
<223> Acp

<400> 54

Leu Ala Gly Gln Pro Leu Gln Glu Xaa Cys
1 5 10

<210> 55
<211> 9
<212> PRT
<213> Artificial sequence

<220>
<223> Synthetic peptide

<220>
<221> MOD_RES
<222> (8)..(8)
<223> Acp

<400> 55

Ala Gly Gln Pro Leu Gln Glu Xaa Cys
1 5

<210> 56
<211> 282
<212> PRT
<213> Homo sapiens

<400> 56

His His His His His His His His His His Lys Val Glu Gln Ala Val
1 5 10 15

Glu Thr Glu Pro Glu Pro Glu Leu Arg Gln Gln Thr Glu Trp Gln Ser
20 25 30

Gly Gln Arg Trp Glu Leu Ala Leu Gly Arg Phe Trp Asp Tyr Leu Arg
35 40 45

Trp Val Gln Thr Leu Ser Glu Gln Val Gln Glu Glu Leu Leu Ser Ser
50 55 60

Gln Val Thr Gln Glu Leu Arg Ala Leu Met Asp Glu Thr Met Lys Glu
65 70 75 80

Leu Lys Ala Tyr Lys Ser Glu Leu Glu Glu Gln Leu Thr Pro Val Ala
85 90 95

Glu Glu Thr Arg Ala Arg Leu Ser Lys Glu Leu Gln Ala Ala Gln Ala
100 105 110

Arg Leu Gly Ala Asp Met Glu Asp Val Arg Gly Arg Leu Val Gln Tyr
115 120 125

Arg Gly Glu Val Gln Ala Met Leu Gly Gln Ser Thr Glu Glu Leu Arg
130 135 140

Val Arg Leu Ala Ser His Leu Arg Lys Leu Arg Lys Arg Leu Leu Arg
145 150 155 160

Asp Ala Asp Asp Leu Gln Lys Arg Leu Ala Val Tyr Gln Ala Gly Ala
165 170 175

Arg Glu Gly Ala Glu Arg Gly Leu Ser Ala Ile Arg Glu Arg Leu Gly
180 185 190

Pro Leu Val Glu Gln Gly Arg Val Arg Ala Ala Thr Val Gly Ser Leu

195

200

205

Ala Gly Gln Pro Leu Gln Glu Arg Ala Gln Ala Trp Gly Glu Arg Leu
 210 215 220

Arg Ala Arg Met Glu Glu Met Gly Ser Arg Thr Arg Asp Arg Leu Asp
 225 230 235 240

Glu Val Lys Glu Gln Val Ala Glu Val Arg Ala Lys Leu Glu Glu Gln
 245 250 255

Ala Gln Gln Ile Arg Leu Gln Ala Glu Ala Phe Gln Ala Arg Leu Lys
 260 265 270

Ser Trp Phe Glu Pro Leu Val Glu Asp Met
 275 280

<210> 57
 <211> 112
 <212> PRT
 <213> Homo sapiens

<400> 57

Leu Ala Gly Gln Pro Leu Gln Glu Arg Ala Gln Ala Trp Gly Glu Arg
 1 5 10 15

Leu Arg Ala Arg Met Glu Glu Met Gly Ser Arg Thr Arg Asp Arg Leu
 20 25 30

Asp Glu Val Lys Glu Gln Val Ala Glu Val Arg Ala Lys Leu Glu Glu
 35 40 45

Gln Ala Gln Gln Ile Arg Leu Gln Ala Glu Ala Phe Gln Ala Arg Leu
 50 55 60

Lys Ser Trp Phe Glu Pro Leu Val Glu Asp Met Gln Arg Gln Trp Ala
 65 70 75 80

Gly Leu Val Glu Lys Val Gln Ala Ala Val Gly Thr Ser Ala Ala Pro
 85 90 95

Val Pro Ser Asp Asn His His His His His His His His His His His
 100 105 110

<210> 58
 <211> 111
 <212> PRT
 <213> Homo sapiens

<400> 58

Ala Gly Gln Pro Leu Gln Glu Arg Ala Gln Ala Trp Gly Glu Arg Leu
 1 5 10 15

Arg Ala Arg Met Glu Glu Met Gly Ser Arg Thr Arg Asp Arg Leu Asp
20 25 30

Glu Val Lys Glu Gln Val Ala Glu Val Arg Ala Lys Leu Glu Glu Gln
35 40 45

Ala Gln Gln Ile Arg Leu Gln Ala Glu Ala Phe Gln Ala Arg Leu Lys
50 55 60

Ser Trp Phe Glu Pro Leu Val Glu Asp Met Gln Arg Gln Trp Ala Gly
65 70 75 80

Leu Val Glu Lys Val Gln Ala Ala Val Gly Thr Ser Ala Ala Pro Val
85 90 95

Pro Ser Asp Asn His His His His His His His His His His His
100 105 110

<210> 59
<211> 5
<212> PRT
<213> Mus musculus

<400> 59

Asp Tyr Trp Met Asn
1 5

<210> 60
<211> 19
<212> PRT
<213> Mus musculus

<400> 60

Gln Ile Arg Leu Lys Ser Asp Asn Tyr Val Thr His Tyr Val Glu Ser
1 5 10 15

Leu Lys Gly

<210> 61
<211> 11
<212> PRT
<213> Mus musculus

<400> 61

His Ile Leu Leu Arg Arg Arg Tyr Phe Asp Val
1 5 10

<210> 62
<211> 5
<212> PRT
<213> Mus musculus

<400> 62

Asn Tyr Trp Met Asn

1 5

<210> 63
<211> 19
<212> PRT
<213> Mus musculus

<400> 63

Gln Ile Arg Leu Lys Ser Asp Asn Tyr Ala Thr Arg Tyr Ala Glu Ser
1 5 10 15

Val Lys Gly

<210> 64
<211> 11
<212> PRT
<213> Mus musculus

<400> 64

His Ile Leu Leu Arg Tyr Tyr Ala Leu Asp Phe
1 5 10

<210> 65
<211> 5
<212> PRT
<213> Mus musculus

<400> 65

Lys Tyr Trp Met Asn
1 5

<210> 66
<211> 19
<212> PRT
<213> Mus musculus

<400> 66

Gln Ile Arg Leu Arg Ser Asp Asn Tyr Ala Thr His Tyr Ala Glu Ser
1 5 10 15

Val Lys Gly

<210> 67
<211> 11
<212> PRT
<213> Mus musculus

<400> 67

His Tyr Tyr Gly Ser Ser Gly Tyr Val Asp Val
1 5 10

<210> 68
<211> 19
<212> PRT

<213> Mus musculus

<400> 68

Gln Ile Lys Leu Arg Ser Asp Asn Tyr Ala Thr His Tyr Ala Glu Ser
1 5 10 15

Val Lys Gly

<210> 69

<211> 11

<212> PRT

<213> Mus musculus

<400> 69

His Tyr Leu Asp Ser Gly Gly Tyr Phe Asp Val
1 5 10

<210> 70

<211> 19

<212> PRT

<213> Mus musculus

<400> 70

Gln Ile Arg Leu Lys Ser Asp Asn Tyr Ala Thr His Tyr Ala Glu Ser
1 5 10 15

Val Lys Gly

<210> 71

<211> 11

<212> PRT

<213> Mus musculus

<400> 71

Glu Ala Ser Gln Asn Val Gly Thr Asn Val Ala
1 5 10

<210> 72

<211> 7

<212> PRT

<213> Mus musculus

<400> 72

Ser Ala Ser Tyr Arg Tyr Ser
1 5

<210> 73

<211> 9

<212> PRT

<213> Mus musculus

<400> 73

His Gln Phe Asn Thr Tyr Pro Tyr Thr
1 5

<210> 74
<211> 11
<212> PRT
<213> Mus musculus

<400> 74

Lys Ala Ser Gln Asn Val Tyr Thr Asp Val Ala
1 5 10

<210> 75
<211> 9
<212> PRT
<213> Mus musculus

<400> 75

Gln Gln Tyr Asn Ser Phe Pro Tyr Thr
1 5

<210> 76
<211> 11
<212> PRT
<213> Mus musculus

<400> 76

Lys Ala Ser Gln Asn Val Gly Thr Asn Val Ala
1 5 10

<210> 77
<211> 7
<212> PRT
<213> Mus musculus

<400> 77

Ser Ala Ser Gln Arg Tyr Ser
1 5

<210> 78
<211> 9
<212> PRT
<213> Mus musculus

<400> 78

Gln Gln Tyr Asn Ser Tyr Pro Tyr Thr
1 5

<210> 79
<211> 11
<212> PRT
<213> Mus musculus

<400> 79

Lys Ala Ser Gln Asn Val Gly Ile Asn Val Ala
1 5 10

<210> 80

<211> 11
<212> PRT
<213> Mus musculus

<400> 80

Lys Ala Ser Gln Asn Val Gly Asn Asn Val Ala
1 5 10

<210> 81
<211> 9
<212> PRT
<213> Mus musculus

<400> 81

His Gln Tyr Asn Ser Tyr Pro Tyr Thr
1 5

<210> 82
<211> 122
<212> PRT
<213> Mus musculus

<400> 82

Glu Val Asn Leu Glu Glu Ser Gly Gly Asp Leu Val Gln Pro Gly Arg
1 5 10 15

Ser Leu Lys Leu Ser Cys Val Ala Ser Gly Phe Thr Phe Ser Asp Tyr
20 25 30

Trp Met Asn Trp Val Arg Gln Ser Pro Glu Lys Gly Leu Glu Trp Val
35 40 45

Ala Gln Ile Arg Leu Lys Ser Asp Asn Tyr Val Thr His Tyr Val Glu
50 55 60

Ser Leu Lys Gly Arg Phe Thr Ile Ser Arg Asp Asp Ser Arg Ser Cys
65 70 75 80

Val Tyr Leu Gln Met Asn Asn Leu Arg Ala Glu Asp Thr Gly Ile Tyr
85 90 95

Tyr Cys Thr Gly His Ile Leu Leu Arg Arg Arg Tyr Phe Asp Val Trp
100 105 110

Gly Thr Gly Thr Thr Val Thr Val Ser Ser
115 120

<210> 83
<211> 107
<212> PRT
<213> Mus musculus

<400> 83

Asp Ile Val Met Thr Gln Ser Gln Lys Phe Met Ser Thr Ser Val Gly
1 5 10 15

Asp Arg Val Ser Val Thr Cys Glu Ala Ser Gln Asn Val Gly Thr Asn
20 25 30

Val Ala Trp Tyr Gln Gln Lys Pro Gly Gln Ser Pro Lys Gly Leu Ile
35 40 45

Tyr Ser Ala Ser Tyr Arg Tyr Ser Gly Val Pro Asp Arg Phe Thr Gly
50 55 60

Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Thr Val Gln Ser
65 70 75 80

Glu Asp Leu Ala Glu Tyr Phe Cys His Gln Phe Asn Thr Tyr Pro Tyr
85 90 95

Thr Phe Gly Gly Gly Thr Thr Leu Glu Ile Ile
100 105

<210> 84
<211> 122
<212> PRT
<213> Mus musculus

<400> 84

Glu Val Lys Val Glu Glu Ser Gly Gly Gly Val Val Gln Pro Gly Gly
1 5 10 15

Ser Met Lys Leu Ser Cys Leu Ala Ser Gly Phe Thr Phe Arg Asn Tyr
20 25 30

Trp Met Asn Trp Val Arg Gln Ser Pro Glu Lys Gly Leu Glu Trp Val
35 40 45

Ala Gln Ile Arg Leu Lys Ser Asp Asn Tyr Ala Thr Arg Tyr Ala Glu
50 55 60

Ser Val Lys Gly Arg Phe Thr Ile Ser Arg Asp Asp Ser Lys Ser Ser
65 70 75 80

Val Tyr Leu Gln Met Asn Asn Leu Arg Ala Glu Asp Thr Gly Thr Tyr
85 90 95

Tyr Cys Ser Gly His Ile Leu Leu Arg Tyr Tyr Ala Leu Asp Phe Trp
100 105 110

Gly Gln Gly Thr Ser Val Thr Val Ser Ser
115 120

<210> 85
<211> 107
<212> PRT
<213> Mus musculus

<400> 85

Asp Ile Val Leu Thr Gln Ser Gln Lys Phe Met Ser Thr Ser Val Gly
1 5 10 15

Asp Arg Val Ser Val Thr Cys Lys Ala Ser Gln Asn Val Tyr Thr Asp
20 25 30

Val Ala Trp Tyr Gln Gln Lys Pro Gly Gln Ser Pro Lys Gly Met Ile
35 40 45

Tyr Ser Ala Ser Tyr Arg Tyr Ser Gly Val Pro Asp Arg Phe Thr Gly
50 55 60

Ser Gly Ser Gly Thr Asp Phe Thr Leu Lys Ile Ser Asn Val Gln Ser
65 70 75 80

Glu Asp Leu Ala Glu Tyr Phe Cys Gln Gln Tyr Asn Ser Phe Pro Tyr
85 90 95

Thr Phe Gly Gly Gly Thr Lys Leu Glu Ile Lys
100 105

<210> 86

<211> 122

<212> PRT

<213> Mus musculus

<400> 86

Glu Val Lys Leu Glu Glu Ser Gly Gly Gly Leu Val Gln Pro Gly Gly
1 5 10 15

Ser Met Lys Leu Ser Cys Val Ala Ser Gly Ile Thr Phe Ser Lys Tyr
20 25 30

Trp Met Asn Trp Val Arg Gln Cys Pro Glu Lys Gly Leu Glu Trp Val
35 40 45

Ala Gln Ile Arg Leu Arg Ser Asp Asn Tyr Ala Thr His Tyr Ala Glu
50 55 60

Ser Val Lys Gly Arg Phe Thr Ile Ser Arg Asp Asp Ser Lys Ser Ser
65 70 75 80

Val Tyr Leu Gln Met Asn Thr Leu Arg Ala Glu Asp Thr Gly Ile Tyr
85 90 95

Tyr Cys Thr His His Tyr Tyr Gly Ser Ser Gly Tyr Val Asp Val Trp
100 105 110

Gly Thr Gly Thr Thr Val Thr Val Ser Ser
115 120

<210> 87
<211> 107
<212> PRT
<213> Mus musculus

<400> 87

Asp Ile Val Met Thr Gln Ser Gln Lys Phe Met Ser Thr Ser Val Gly
1 5 10 15

Asp Arg Val Ser Val Thr Cys Lys Ala Ser Gln Asn Val Gly Thr Asn
20 25 30

Val Ala Trp Tyr Gln Gln Lys Pro Gly Gln Cys Pro Lys Gly Met Ile
35 40 45

Leu Ser Ala Ser Gln Arg Tyr Ser Gly Val Pro Asp Arg Phe Thr Gly
50 55 60

Ser Gly Phe Gly Thr Glu Phe Thr Leu Thr Ile Ser Asn Val Gln Ser
65 70 75 80

Glu Asp Leu Ala Glu Tyr Phe Cys Gln Gln Tyr Asn Ser Tyr Pro Tyr
85 90 95

Thr Phe Gly Gly Gly Thr Lys Leu Glu Arg Lys
100 105

<210> 88
<211> 122
<212> PRT
<213> Mus musculus

<400> 88

Glu Val Lys Leu Glu Glu Ser Gly Gly Gly Leu Val His Pro Gly Gly
1 5 10 15

Ser Met Lys Leu Ser Cys Val Gly Ser Gly Phe Thr Phe Ser Asn Tyr
20 25 30

Trp Met Asn Trp Val Arg Gln Ser Pro Glu Lys Gly Leu Glu Trp Val
35 40 45

Ala Gln Ile Lys Leu Arg Ser Asp Asn Tyr Ala Thr His Tyr Ala Glu
50 55 60

Ser Val Lys Gly Lys Phe Thr Ile Ser Arg Asp Asp Ala Lys Ser Ser
65 70 75 80

Val Tyr Leu Gln Met Asp Asn Leu Arg Ala Glu Asp Thr Gly Ile Tyr
85 90 95

Tyr Cys Ser His His Tyr Leu Asp Ser Gly Gly Tyr Phe Asp Val Trp
100 105 110

Gly Thr Gly Thr Ser Val Thr Val Ser Ser
115 120

<210> 89
<211> 107
<212> PRT
<213> Mus musculus

<400> 89

Asp Ile Val Met Thr Gln Ser Gln Lys Phe Met Ser Thr Ser Val Gly
1 5 10 15

Asp Arg Val Ser Val Thr Cys Lys Ala Ser Gln Asn Val Gly Ile Asn
20 25 30

Val Ala Trp Tyr Gln Asp Lys Pro Gly Gln Ser Pro Lys Gly Leu Ile
35 40 45

Leu Ser Ala Ser Tyr Arg Tyr Ser Gly Val Pro Asp Arg Phe Thr Gly
50 55 60

Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Asn Val Gln Ser
65 70 75 80

Glu Asp Leu Ala Glu Tyr Phe Cys Gln Gln Tyr Asn Ser Tyr Pro Tyr
85 90 95

Thr Phe Gly Gly Gly Thr Lys Leu Glu Ile Lys
100 105

<210> 90
<211> 122
<212> PRT
<213> Mus musculus

<400> 90

Glu Val Lys Leu Glu Glu Ser Gly Gly Gly Leu Val Gln Pro Gly Gly
1 5 10 15

Ala Met Lys Leu Ser Cys Val Ala Ser Gly Ile Thr Phe Ser Asn Tyr
20 25 30

Trp Met Asn Trp Val Arg Gln Cys Pro Glu Lys Gly Leu Glu Trp Val
35 40 45

Ala Gln Ile Arg Leu Lys Ser Asp Asn Tyr Ala Thr His Tyr Ala Glu
50 55 60

Ser Val Lys Gly Arg Phe Thr Ile Ser Arg Asp Asp Ser Lys Ser Ser
65 70 75 80

Val Tyr Leu Gln Met Asn Thr Leu Arg Ala Glu Asp Thr Gly Ile Tyr

85

90

95

Tyr Cys Thr His His Tyr Tyr Gly Ser Ser Gly Tyr Val Asp Val Trp
 100 105 110

Gly Thr Gly Thr Thr Val Thr Val Ser Ser
 115 120

<210> 91
 <211> 107
 <212> PRT
 <213> Mus musculus

<400> 91

Asp Ile Val Met Thr Gln Ser Gln Lys Phe Met Ser Thr Ser Val Gly
 1 5 10 15

Asp Arg Val Ser Val Thr Cys Lys Ala Ser Gln Asn Val Gly Asn Asn
 20 25 30

Val Ala Trp Tyr Gln Lys Lys Pro Gly Gln Cys Pro Lys Gly Met Ile
 35 40 45

Leu Ser Ala Ser Gln Arg Tyr Ser Gly Val Pro Asp Arg Phe Thr Gly
 50 55 60

Ser Gly Phe Gly Thr Glu Phe Thr Leu Ile Ile Ser Asn Val Gln Ser
 65 70 75 80

Glu Asp Leu Ala Glu Tyr Phe Cys His Gln Tyr Asn Ser Tyr Pro Tyr
 85 90 95

Thr Phe Gly Gly Gly Thr Lys Leu Glu Arg Lys
 100 105

<210> 92
 <211> 19
 <212> PRT
 <213> Mus musculus

<400> 92

Glu Ile Lys Leu Lys Ser Asn Asn Tyr Gly Thr His Tyr Ala Glu Ser
 1 5 10 15

Val Lys Gly

<210> 93
 <211> 9
 <212> PRT
 <213> Mus musculus

<400> 93

Asp Thr Glu Val Val Ala Gly Ala His

1 5

<210> 94
<211> 5
<212> PRT
<213> Mus musculus

<400> 94

Asn Tyr Trp Met His
1 5

<210> 95
<211> 17
<212> PRT
<213> Mus musculus

<400> 95

Asn Ile Asn Pro Arg Asn Gly Gly Thr Asn Tyr Asn Glu Lys Phe Lys
1 5 10 15

Ser

<210> 96
<211> 9
<212> PRT
<213> Mus musculus

<400> 96

Gly Leu Tyr Asp Tyr Asp Phe Asp Tyr
1 5

<210> 97
<211> 5
<212> PRT
<213> Mus musculus

<400> 97

Arg Tyr Trp Leu His
1 5

<210> 98
<211> 17
<212> PRT
<213> Mus musculus

<400> 98

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

Asn

<210> 99
<211> 9
<212> PRT

<213> Mus musculus

<400> 99

Gly Ala Tyr Asp Tyr Asp Cys Asp Tyr
1 5

<210> 100

<211> 17

<212> PRT

<213> Mus musculus

<400> 100

Lys Ser Ser Gln Ser Val Leu Tyr Asn Ser Asn Gln Lys Asn Tyr Leu
1 5 10 15

Ala

<210> 101

<211> 7

<212> PRT

<213> Mus musculus

<400> 101

Trp Ala Ser Thr Arg Asp Ser
1 5

<210> 102

<211> 8

<212> PRT

<213> Mus musculus

<400> 102

Gln Gln Tyr Leu Ser Ser Leu Thr
1 5

<210> 103

<211> 17

<212> PRT

<213> Mus musculus

<400> 103

Lys Ser Ser Gln Ser Val Leu Tyr Ser Ser Asn Gln Lys Asn Tyr Leu
1 5 10 15

Ala

<210> 104

<211> 7

<212> PRT

<213> Mus musculus

<400> 104

Trp Ala Ser Thr Arg Glu Ser
1 5

<210> 105
<211> 16
<212> PRT
<213> Mus musculus

<400> 105

Arg Ser Ser Gln Ser Ile Val His Gly Asn Gly Asn Thr Tyr Leu Gln
1 5 10 15

<210> 106
<211> 7
<212> PRT
<213> Mus musculus

<400> 106

Lys Val Ser Ser Arg Phe Ser
1 5

<210> 107
<211> 9
<212> PRT
<213> Mus musculus

<400> 107

Phe Gln Gly Ser His Val Pro Phe Thr
1 5

<210> 108
<211> 16
<212> PRT
<213> Mus musculus

<400> 108

Arg Ser Ser Gln Arg Ile Val His Ser Asn Gly Asn Ile Tyr Leu Gln
1 5 10 15

<210> 109
<211> 120
<212> PRT
<213> Mus musculus

<400> 109

Glu Val Lys Leu Glu Glu Ser Gly Gly Gly Leu Val Gln Pro Gly Gly
1 5 10 15

Ser Met Lys Leu Ser Cys Val Ala Ser Gly Phe Thr Phe Ser Asn Tyr
20 25 30

Trp Met Asn Trp Val Arg Gln Ser Pro Glu Lys Gly Leu Glu Trp Ile
35 40 45

Gly Glu Ile Lys Leu Lys Ser Asn Asn Tyr Gly Thr His Tyr Ala Glu
50 55 60

Ser Val Lys Gly Arg Phe Thr Ile Ser Arg Asp Asp Ser Lys Ser Ser
65 70 75 80

Val Tyr Leu Gln Met Asn Asn Leu Arg Ala Glu Asp Thr Gly Ile Tyr
85 90 95

Tyr Cys Thr Arg Asp Thr Glu Val Val Ala Gly Ala His Trp Gly Gln
100 105 110

Gly Thr Leu Val Thr Val Ser Ala
115 120

<210> 110
<211> 112
<212> PRT
<213> Mus musculus

<400> 110

Asn Ile Met Met Thr Gln Ser Pro Ser Ser Leu Ala Val Ser Ala Gly
1 5 10 15

Glu Lys Val Thr Met Ser Cys Lys Ser Ser Gln Ser Val Leu Tyr Asn
20 25 30

Ser Asn Gln Lys Asn Tyr Leu Ala Trp Tyr Gln Gln Lys Pro Gly Gln
35 40 45

Ser Pro Lys Leu Leu Ile Tyr Trp Ala Ser Thr Arg Asp Ser Gly Val
50 55 60

Pro Asp Arg Phe Thr Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr
65 70 75 80

Ile Ser Ser Val Arg Ala Glu Asp Leu Ala Val Tyr Phe Cys Gln Gln
85 90 95

Tyr Leu Ser Ser Leu Thr Phe Gly Ala Gly Thr Lys Leu Glu Leu Lys
100 105 110

<210> 111
<211> 120
<212> PRT
<213> Mus musculus

<400> 111

Glu Val Lys Leu Glu Glu Ser Gly Gly Gly Leu Val Gln Pro Gly Gly
1 5 10 15

Ser Met Lys Leu Ser Cys Val Ala Ser Gly Phe Thr Phe Ser Asn Tyr
20 25 30

Trp Met Asn Trp Val Arg Gln Ser Pro Glu Lys Gly Leu Glu Trp Val
35 40 45

Gly Glu Ile Lys Leu Lys Ser Asn Asn Tyr Gly Thr His Tyr Ala Glu
50 55 60

Ser Val Lys Gly Arg Phe Thr Ile Ser Arg Asp Asp Ser Lys Ser Ser
65 70 75 80

Val Tyr Leu Gln Met Asn Asn Leu Arg Ala Glu Asp Thr Gly Ile Tyr
85 90 95

Tyr Cys Thr Arg Asp Thr Glu Val Val Ala Gly Ala His Trp Gly Gln
100 105 110

Gly Thr Leu Val Thr Val Ser Ala
115 120

<210> 112
<211> 112
<212> PRT
<213> Mus musculus

<400> 112

Asn Ile Met Met Thr Gln Ser Pro Ser Ser Leu Ala Val Ser Ala Gly
1 5 10 15

Glu Lys Val Thr Met Ser Cys Lys Ser Ser Gln Ser Val Leu Tyr Ser
20 25 30

Ser Asn Gln Lys Asn Tyr Leu Ala Trp Tyr Gln Gln Lys Pro Gly Gln
35 40 45

Ser Pro Lys Leu Leu Ile Tyr Trp Ala Ser Thr Arg Glu Ser Gly Val
50 55 60

Pro Asp Arg Phe Thr Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr
65 70 75 80

Ile Ser Ser Val Gln Ala Glu Asp Leu Ala Val Tyr Phe Cys Gln Gln
85 90 95

Tyr Leu Ser Ser Leu Thr Phe Gly Ala Gly Thr Lys Leu Glu Leu Lys
100 105 110

<210> 113
<211> 118
<212> PRT
<213> Mus musculus

<400> 113

Gln Val Gln Leu Gln Gln Pro Gly Thr Glu Leu Val Lys Pro Gly Ala
1 5 10 15

Ser Val Lys Leu Ser Cys Lys Ala Ser Gly Tyr Thr Phe Thr Asn Tyr
20 25 30

Trp Met His Trp Val Lys Gln Arg Pro Gly Gln Gly Leu Glu Trp Ile
35 40 45

Gly Asn Ile Asn Pro Arg Asn Gly Gly Thr Asn Tyr Asn Glu Lys Phe
50 55 60

Lys Ser Lys Ala Thr Leu Thr Val Asp Lys Ser Ser Ser Thr Asp Tyr
65 70 75 80

Met Gln Leu Ser Ser Leu Thr Ser Glu Asp Ser Ala Val Tyr Tyr Cys
85 90 95

Ala Arg Gly Leu Tyr Asp Tyr Asp Phe Asp Tyr Trp Gly Gln Gly Thr
100 105 110

Thr Leu Thr Val Ser Ser
115

<210> 114
<211> 112
<212> PRT
<213> Mus musculus

<400> 114

Asp Val Leu Met Thr Gln Thr Pro Leu Ser Leu Pro Val Ser Leu Gly
1 5 10 15

Asp Gln Ala Ser Ile Ser Cys Arg Ser Ser Gln Ser Ile Val His Gly
20 25 30

Asn Gly Asn Thr Tyr Leu Gln Trp Phe Leu Gln Lys Pro Gly Gln Ser
35 40 45

Pro Lys Leu Leu Ile Tyr Lys Val Ser Ser Arg Phe Ser Gly Val Pro
50 55 60

Asp Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Lys Ile
65 70 75 80

Ser Arg Val Glu Ala Glu Asp Leu Gly Val Tyr Tyr Cys Phe Gln Gly
85 90 95

Ser His Val Pro Phe Thr Phe Gly Ser Gly Thr Lys Leu Glu Ile Lys
100 105 110

<210> 115
<211> 118
<212> PRT
<213> Mus musculus

<400> 115

Gln Val Gln Leu Gln Gln Pro Gly Thr Glu Leu Val Lys Pro Gly Ala

1	5	10	15
Ser Val Lys Leu Ser Cys Lys Ala Ser Gly Tyr Thr Phe Thr Arg Tyr	20	25	30
Trp Leu His Trp Val Lys Gln Arg Pro Gly Gln Gly Leu Glu Trp Ile	35	40	45
Gly Asn Ile Asn Pro Thr Asn Gly Gly Ala Asn Tyr Asn Glu Lys Phe	50	55	60
Lys Asn Lys Ala Thr Leu Thr Val Asp Lys Ser Ser Ser Thr Ala Tyr	65	70	75
Met Gln Leu Ser Ser Leu Thr Ser Glu Asp Ser Ala Val Tyr Tyr Cys	85	90	95
Ser Arg Gly Ala Tyr Asp Tyr Asp Cys Asp Tyr Trp Gly Gln Gly Thr	100	105	110
Thr Leu Thr Val Ser Ser	115		
<210> 116			
<211> 112			
<212> PRT			
<213> Mus musculus			
<400> 116			
Asp Val Leu Met Thr Gln Thr Pro Leu Ser Leu Pro Val Ser Leu Gly	1	5	10
Asp Gln Ala Ser Ile Ser Cys Arg Ser Ser Gln Arg Ile Val His Ser	20	25	30
Asn Gly Asn Ile Tyr Leu Gln Trp Tyr Leu Gln Lys Pro Gly Gln Ser	35	40	45
Pro Lys Leu Leu Ile Tyr Lys Val Ser Asn Arg Phe Ser Gly Val Pro	50	55	60
Asp Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Lys Ile	65	70	75
Ser Arg Val Glu Ala Glu Asp Leu Gly Val Tyr Tyr Cys Phe Gln Gly	85	90	95
Ser His Val Pro Tyr Thr Phe Gly Gly Gly Thr Lys Leu Glu Ile Lys	100	105	110