



(86) Date de dépôt PCT/PCT Filing Date: 1996/02/14  
 (87) Date publication PCT/PCT Publication Date: 1996/08/22  
 (45) Date de délivrance/Issue Date: 2006/10/10  
 (85) Entrée phase nationale/National Entry: 1997/08/13  
 (86) N° demande PCT/PCT Application No.: US 1996/002491  
 (87) N° publication PCT/PCT Publication No.: 1996/025435  
 (30) Priorité/Priority: 1995/02/14 (US08/388,463)

(51) Cl.Int./Int.Cl. *C07K 16/18* (2006.01),  
*A61K 39/395* (2006.01), *C07K 14/47* (2006.01),  
*C12N 5/20* (2006.01), *G01N 33/577* (2006.01),  
*A61K 38/00* (2006.01)  
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(54) Titre : ANTICORPS MONOCLONAL SPECIFIQUE DU PEPTIDE  $\beta$ A4  
 (54) Title: MONOCLONAL ANTIBODY SPECIFIC FOR  $\beta$ A4 PEPTIDE

(57) **Abrégé/Abstract:**

The instant invention provides for monoclonal antibody which is specific for the  $\beta$ A4 peptide, and in particular the free C-terminus of  $\beta$ A4 "1-42" but not "1-43", and stains diffuse and fibrillar amyloid, vascular amyloid, and neurofibrillary tangles. The instant invention further provides for antibody fragments and constructs thereof which have the same binding specificity. The instant invention also provides for methods of diagnosis, screening and therapeutics for treating unique forms of  $\beta$ A4 peptide, using the antibodies of the instant invention.



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## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>6</sup> :</b> <b>C07K 16/18</b>	<b>A1</b>	<b>(11) International Publication Number:</b> <b>WO 96/25435</b> <b>(43) International Publication Date:</b> 22 August 1996 (22.08.96)
<b>(21) International Application Number:</b> PCT/US96/02491 <b>(22) International Filing Date:</b> 14 February 1996 (14.02.96) <b>(30) Priority Data:</b> 08/388,463                      14 February 1995 (14.02.95)                      US <b>(71) Applicant:</b> BAYER CORPORATION [US/US]; 400 Morgan Lane, West Haven, CT 06512-4175 (US). <b>(72) Inventors:</b> KONIG, Gerhard; 10 Three Elms Road, Branford, CT 06405 (US). GRAHAM, Paul; 43 Hubinger Street, New Haven, CT 06511 (US). <b>(74) Agent:</b> CHAO, Mark; Banner & Allegretti, Ltd., Ten South Wacker Drive, Chicago, IL 60606 (US).		<b>(81) Designated States:</b> CA, JP, MX, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).  <b>Published</b> <i>With international search report.  Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
<b>(54) Title:</b> MONOCLONAL ANTIBODY SPECIFIC FOR $\beta$ A4 PEPTIDE		
<b>(57) Abstract</b>  The instant invention provides for monoclonal antibody which is specific for the $\beta$ A4 peptide, and in particular the free C-terminus of $\beta$ A4 "1-42" but not "1-43", and stains diffuse and fibrillar amyloid, vascular amyloid, and neurofibrillary tangles. The instant invention further provides for antibody fragments and constructs thereof which have the same binding specificity. The instant invention also provides for methods of diagnosis, screening and therapeutics for treating unique forms of $\beta$ A4 peptide, using the antibodies of the instant invention.		

## **Monoclonal Antibody Specific for BA4 Peptide**

### **Field of the Invention**

5 This application relates to Alzheimer's disease, particularly, to a monoclonal antibody specific for the BA4 peptide derived from Amyloid Precursor Protein, cells which produce such antibody, methods of generating such monoclonal antibodies, and to methods for using such antibodies in diagnostics and therapy.

### **Background**

10 Alzheimer's Disease (AD) is an irreversible progressive neurodegenerative brain disorder. Over the course of several years the progression of AD leads to memory loss, dementia, and finally death. Currently, it is the fourth leading cause of death in the United States, accounting for approximately 100,000 deaths annually. Typically, AD affects primarily the elderly and is therefore, with the aging of modern society,  
15 expected to be an increasing health concern in the near future. Soon after the onset of the disease, patients require assistance around the clock. This represents a tremendous psychological as well as financial problem for our society. At present, no proven means for diagnosis, prevention, treatment, or cure of AD exist.

20 Neuropathologically, AD is characterized by massive neuronal cell loss in certain brain areas, and by the deposition of proteinaceous material in the brains of AD patients. These deposits are the intracellular neurofibrillary tangles and the extracellular  $\beta$ -amyloid plaques. The major protein component of the  $\beta$ -amyloid plaque is the BA4 peptide. Sequence analysis of purified  $\beta$ -amyloid plaque material and mass spectrometry showed that the maximum length of the BA4 peptide is 43 amino acids.  
25 Typically, however, species of the peptide can also end either at position 40 or position 42 (Miller *et al.*, 1993, *Arch. Biochem. Biophys.* 301:41-52). Similarly, at the N-terminus, a certain raggedness can be observed, leading to several different forms of

the peptide, starting mainly at position 1, 4 or 11 (Miller *et al.*, 1993).

Molecular cloning revealed that the  $\beta$ A4 peptide is derived from a much larger precursor protein termed the "Amyloid Precursor Protein" (APP) (Kang *et al.*, 1987, *Nature* 325:733-736) (Figure 1). Figure 1 illustrates the Amyloid Precursor Protein (APP) which is a transmembrane (T<sub>m</sub> = membrane region) protein where the N-terminus is located extracellular and the C-terminus is located intracellular (cytoplasmic).  $\beta$ A4 is partially embedded into the membrane. Several alternatively spliced isoforms have been described, which undergo extensive post-translational modifications (Selkoe, 1994, *Ann. Rev. Neurosci.* 17:489-517). The  $\beta$ A4 sequence itself is located partially on the extracellular side and extends partially into the transmembrane region (Figure 2). Figure 2 (SEQ ID NO:3) illustrates the  $\beta$ A4 sequence, shown (encircled area) extending with its C-terminal end into the transmembrane region (T<sub>m</sub>, boxed area) and the N-terminal end located in the extracellular part. Asterisks indicate the location of familial mutations in the APP gene; they are either flanking the  $\beta$ A4 sequence, or are centered in the middle portion of the  $\beta$ A4 sequence. The three major cleavage sites ( $\alpha$ ,  $\beta$  and  $\gamma$ ) in APP are indicated. Release of  $\beta$ A4 was therefore postulated to occur through the proteolytic action of one or more proteases on the N-terminus ( $\beta$ -cut) and on the C-terminus ( $\gamma$ -cut) of the peptide (Figure 2) (Selkoe, 1994). The main event during the secretion of APP is at the  $\alpha$ -cut (position 16/17 of  $\beta$ A4 "1-42"). This secreted APP molecule ( $\alpha$ APPs) contains the first 16 amino acids of the  $\beta$ A4 sequence at its carboxyl end. The remaining cell-associated APP fragments (so called C-terminal fragments (CTF's)) contain the C-terminal portion of the  $\beta$ A4 sequence and extend to the cytoplasmic region of APP. Therefore, this proteolytic cut results in fragments which may not be processed in such a way that directly or indirectly leads to amyloidogenic fragments (non-amyloidogenic processing) (Selkoe, 1994).

Recently, it was demonstrated that cell lines which express large amounts of

APP through a stably transfected APP cDNA construct produce high picomolar to low nanomolar amounts of BA4 and release it rapidly into the medium (Shoji *et al.*, 1992, *Science* 258:126-129). It has also been found that virtually every primary cell culture and cell line releases BA4 constitutively (Busciglio *et al.*, 1993, *PNAS USA* 90:2092-2096). Additionally, healthy controls as well as Alzheimer patients have been shown to have low nanomolar amounts of BA4 in sera and CSF (Seubert *et al.*, 1992, *Nature* 359:325-327). The majority of the detected soluble BA4 species in these body fluids and conditioned media was BA4 "1-40", which is not truly reflective of the overall composition found in  $\beta$ -amyloid plaque depositions. The notion that the production and subsequent release of BA4 is sufficient and therefore responsible for the buildup of  $\beta$ -amyloid plaques in the brains of AD patients could therefore no longer be maintained; other factors must contribute to the deposition of  $\beta$ -amyloid plaques. One straightforward hypothesis is that acute or chronic overproduction of BA4 causes the increased amyloid load observed in AD

The finding that specific point mutations in and around the BA4 region of the APP gene are linked with certain familial Alzheimer's disease (FAD) cases showed unequivocally that the APP gene is a "disease gene" (Goate *et al.*, 1991, *Nature* 349:704-706; Murrell *et al.*, 1991, *Science* 254:97-99; Levy *et al.*, 1990, *Science* 248:1124-1126; Carter *et al.*, 1992, *Nature Genetics* 2:255-256). In families in which AD is inherited dominantly with a specific age of onset, the point mutations in the APP gene are necessary and sufficient to cause AD. Although the vast majority of Alzheimer disease cases are sporadic and probably multifactorial, these familial APP mutations can teach us a great deal about amyloidogenesis, i.e., the generation of the small BA4 peptide from the larger precursor and its subsequent deposition in  $\beta$ -amyloid plaques.

The double mutation at APP codon 670/671 (the "Swedish variant", at the N-terminus of BA4 in APP) causes a 5 to 8 fold higher release of BA4 in cell cultures

stably transfected with that mutated APP cDNA (Figure 2) (Citron *et al.*, 1992; Cai *et al.*, 1993). It is conceivable that this double point mutation leads to an increased turnover of APP due to increased proteolysis at the  $\beta$ -cut, which in turn leads to a higher level of released BA4. Increased amounts of BA4 monomers, as demonstrated by transfection studies with the "Swedish mutation", can explain the faster kinetics of BA4 aggregation to  $\beta$ -amyloid plaques in these families.

Another FAD mutation lies C-terminal of BA4 at position 717 ("London variant") and does not affect the level of released BA4 in tissue culture (Figure 2). It was recently demonstrated that this 717 mutation changes the "1-40/1-42" BA4 ratio (Suzuki *et al.*, 1994, *Science* 264:1336-1340). Although it is not clear at the moment how the generation of the C-terminus of BA4 occurs, since this part is embedded in the transmembrane region, it is tempting to hypothesize that the "London mutation" affects the proteolytic breakdown of APP to BA4. Possibly, this point mutation interferes with the cleavage fidelity of the responsible protease at the  $\gamma$ -site. BA4 1-40 exhibits among other things a dramatic difference in its solubility in aqueous solutions when compared to BA4 1-42 (Burdick *et al.*, 1992, *JBC* 267:546-554). The latter is virtually insoluble in water, whereas 1-40 is water soluble up to several mg/ml in vitro. Minor amounts of the longer 1-42 form can enhance precipitation of 1-40 in vitro dramatically. A slightly higher proportion of the longer 1-42 BA4 species would explain the early onset deposition of BA4 to  $\beta$ -amyloid plaques in patients with this "London mutation". The proportion of the 1-42 species to the shorter more soluble 1-40 species may also be one of the critical factors in the sporadic AD cases (i.e. cases where no genetic predisposition was identified). Monoclonal antibodies which specifically bind to the 1-42 species, are therefore useful to investigate the production and presence of BA4 species ending at amino acid position 42, and can be used as a diagnostic indicator of abnormal species present in AD.

Recent biochemical analyses with one antibody which recognizes BA4 ending at

position 40, and one antibody recognizing  $\beta$ A4 species extending to position 42 or further, showed that the contribution of the longer  $\beta$ A4 species might be critical for the onset of the disease (Suzuki *et al.*, 1994). However, the monoclonal antibody of Suzuki does not distinguish between  $\beta$ A4 1-42, 1-43, and longer  $\beta$ A4 species. This is also the case for another reported monoclonal antibody 2G9 (Yang *et al.*, 1994, *Neuro Report* 5:2117-2120). Therefore, in order to avoid this crossreactivity, antibodies which are specific for  $\beta$ A4 species ending at position 42 to the exclusion of the other forms would be very useful in order to avoid crossreactivity with membrane associated C-terminal APP fragments, which are typical cellular products not necessarily associated with the  $\beta$  amyloid plaques.

One monoclonal antibody recognizing  $\beta$ A4 1-42 has been described (Murphy *et al.*, 1994, *Am. J. Path.* 144:1082-1088). However, the  $\beta$ A4 1-43 peptide species was not used in these studies, thus it is not known what the exact specificity of this monoclonal antibody would be in response to the 1-43 peptide. Competition studies were only performed with  $\beta$ A4 peptides ending at position 40 ("1-40"), and position 44 ("1-44") and beyond with this antibody. Importantly, the antibody was reported to stain diffuse amyloid, fibrillar amyloid, intraneuronal and extraneuronal neurofibrillary tangles, but *not* vascular amyloid.

An *in vitro* biochemical diagnostic test for Alzheimer's disease in its early stages as well as a means of screening for at-risk AD individuals is not available. The current diagnosis of AD requires a detailed clinical evaluation which cannot give clear answers until significant symptoms of dementia and memory loss are manifested. In view of the research referred to above,  $\beta$ A4 1-42 represents a preclinical marker for AD. Thus, identifying the level of or the buildup of  $\beta$ A4 1-42, or other residue 42 terminating species, and how this may progress during the course of the disease, and how it is distributed in the brain, will provide valuable insights into monitoring the course of, as well as for specific diagnosis and possible treatment of AD.

It would be useful for preparing diagnostic tests, therapeutics and for monitoring assays of AD, to have a monoclonal antibody which, in contrast to the specificity of presently available antibodies (crossreactive with 1-43; reported not to stain vascular amyloid), does stain vascular amyloid and is specific for  $\beta$ A4 peptide ending at residue 42, and therefore extends the diagnostic capabilities of the art, i.e. one that recognizes the free C-terminus of  $\beta$ A4 1-42 and stains diffuse and fibrillar amyloid, neurofibrillary tangles, and vascular amyloid. Such an antibody is the subject of the present application.

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### Summary of the Invention

The instant invention provides for monoclonal antibody which is specific for the  $\beta$ A4 peptide, and in particular, the C-terminus of  $\beta$ A4 "1-42" and stains diffuse and fibrillar amyloid, neurofibrillary tangles, and vascular amyloid. In particular, the instant invention provides for monoclonal antibody which is specific for all  $\beta$ A4 peptides in which the C-terminus is residue 42 of the  $\beta$ A4 amino acid sequence. The instant invention further encompasses antibody fragments and constructs thereof which have the same binding specificity. The instant invention encompasses in particular the monoclonal antibody known as "Mab 369.2B" and is produced by the cell line "369.2B" which has been deposited under the Budapest Treaty with the American Type Culture Collection (ATCC) on January 26, 1995, and has been assigned the ascension number HB11829. The instant invention encompasses the use of the monoclonal antibody of the instant invention in diagnostic, purification, and therapeutic uses.

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Thus one embodiment of the instant invention encompasses a monoclonal antibody specific for  $\beta$ A4 peptide ending at position 42, wherein said antibody binds to diffuse amyloid, fibrillar amyloid, vascular amyloid, and neurofibrillary tangles. In one particular embodiment, the instant invention provides for a monoclonal antibody which is specific for the C-terminal amino acids of the  $\beta$ A4 1-42 peptide. In the most

preferred embodiment the instant invention encompasses a monoclonal antibody that is identified as 369.2B, and is produced by the cell line deposited with the American Type Culture Collection (ATCC) as ascension number HB 11829. A preferred embodiment of the instant invention also encompasses a cell which is identified by the ATCC ascension number HB 11829. In a further embodiment of the instant invention, an immunologically reactive fragment of the monoclonal antibody of the instant invention which is capable of the same binding as the monoclonal antibody of the instant invention, is encompassed.

The instant invention also provides for methods of generating  $\beta$ A4 specific antibodies which recognize the free C-terminal residue 42. The instant invention also provides for methods for detecting the presence of  $\beta$ A4 peptides ending at position 42, in tissue comprising contacting a tissue sample with monoclonal antibody of the instant invention, by detecting the presence of monoclonal antibody in a selective fashion. The instant invention also provides for methods for selective purification of  $\beta$ A4 peptides ending at position 42, comprising contacting a sample to be purified with monoclonal antibody of the instant invention, separating the  $\beta$ A4 peptide from the sample to be purified, and isolating the  $\beta$ A4 peptide. In a further embodiment, the instant invention provides for methods for detection of  $\beta$ A4 peptide associated with Alzheimer's Disease, comprising contacting a sample to be tested with monoclonal antibody of the instant invention, and detecting the presence of  $\beta$ A4 peptides.

Thus the instant invention also provides for methods for the prevention of aggregation of  $\beta$ A4 peptide by administering monoclonal antibody of the instant invention. In a preferred embodiment the monoclonal antibody is identical to 369.2B, or is an immunologically active fragment with equivalent binding specificity thereof. The instant invention thus provides a means for detecting the presence of  $\beta$ A4 peptide comprising an immunologically reactive fragment of the monoclonal antibody of the instant invention. As well as a means for preventing the aggregation of  $\beta$ A4 peptide

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comprising an immunologically reactive fragment of the monoclonal antibody of the instant invention. The instant invention provides a means for detecting and monitoring the level of  $\beta$ A4 peptide in tissue or fluid samples (e.g. blood, other body fluids, tissue sections, biopsy tissues etc.). In a preferred embodiment, the  $\beta$ A4 peptide being detected, monitored, inhibited or purified is a  $\beta$ A4 peptide with a free carboxy terminal amino acid residue being residue number 42 of the  $\beta$ A4 peptide amino acid sequence.

10           Specific preferred embodiments of the present invention will become more evident from the following more detailed description of certain preferred embodiments and the claims.

          According to one aspect of the present invention, 15 there is provided a monoclonal antibody that is identified as 369.2B, and is produced by the cell line deposited with the American Type Culture Collection (ATCC) as ATCC number HB 11829.

          According to another aspect of the present invention, 20 there is provided a cell line which is identified by ATCC number HB 11829.

          According to still another aspect of the present invention, there is provided a method for generating an antibody for harvesting from a non-human mammal being immunized 25 that specifically binds  $\beta$ A4 (1-42) peptide and not  $\beta$ A4 (1-40) and  $\beta$ A4 (1-43) peptides, the method comprising immunization of the non-human mammal with a peptide CDGDGDMVGGVVIA (SEQ ID NO: 1) conjugated to a suitable immunological carrier, and harvesting the antibody from the non-human mammal.

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According to yet another aspect of the present invention, there is provided a method of specifically detecting the presence of  $\beta$ A4 peptide species ending at carboxy-terminus residue 42 (alanine) in tissue, comprising contacting a tissue sample with monoclonal antibody 369.2B or binding fragment thereof to specifically bind said peptide, and detecting the presence of specifically bound monoclonal antibody 369.2B or binding fragment thereof, wherein the monoclonal antibody is produced by ATCC hybridoma 11829.

According to a further aspect of the present invention, there is provided a method of specifically detecting in a sample, the presence of  $\beta$ A4 peptide species ending at carboxy-terminus residue 42 (alanine) in diffuse amyloid, fibrillar amyloid, neurofibrillary tangles, and vascular amyloid deposits, comprising contacting a sample to be tested with monoclonal antibody 369.2B or binding fragment thereof to specifically bind said peptide, and detecting the presence of specifically bound monoclonal antibody 369.2B or binding fragment thereof, wherein the monoclonal antibody is produced by ATCC hybridoma 11829.

According to yet a further aspect of the present invention, there is provided a kit comprising a means for selectively detecting the presence of  $\beta$ A4 peptide species ending at carboxy-terminus residue 42 (alanine) comprising an active  $\beta$ A4 peptide-binding fragment of monoclonal antibody 369.2B, wherein the monoclonal antibody is produced by ATCC hybridoma 11829, and a suitable detectable signal.

According to still a further aspect of the present invention, there is provided a method for selectively isolating  $\beta$ A4 (1-42) peptide ending at carboxy-terminus residue 42

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(alanine) from a sample, comprising contacting a sample with monoclonal antibody 369.2B or peptide binding fragment thereof to specifically bind said peptide, and separating the bound  $\beta$ A4 (1-42) peptide ending at residue 42 (alanine) from the  
5 sample.

According to another aspect of the present invention, there is provided a method for generating a monoclonal antibody that specifically binds to  $\beta$ A4 (1-42) peptide, and not to  $\beta$ A4 (1-40) and  $\beta$ A4 (1-43) peptides, the method comprising  
10 immunizing a non-human mammal with a peptide CDGDGDMVGGVVIA (SEQ ID NO: 1) conjugated to a suitable immunological carrier and harvesting antibody-producing cells from the non-human mammal.

According to still another aspect of the present  
15 invention, there is provided a pharmaceutical composition comprising a monoclonal antibody that specifically binds to  $\beta$ A4 (1-42) peptide, and not to  $\beta$ A4 (1-40) and  $\beta$ A4 (1-43) peptides, and a pharmaceutically acceptable carrier or diluent, for therapeutic use.

20 According to yet another aspect of the present invention, there is provided a monoclonal antibody that specifically binds to  $\beta$ A4 (1-42) peptide, and not to  $\beta$ A4 (1-40) and  $\beta$ A4 (1-43) peptides, for use in preventing or treating Alzheimer's Disease.

25 According to a further aspect of the present invention, there is provided a monoclonal antibody that specifically binds to  $\beta$ A4 (1-42) peptide, and not to  $\beta$ A4 (1-40) and  $\beta$ A4 (1-43) peptides, for use in preventing the aggregation of  $\beta$ A4 peptide.

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According to yet a further aspect of the present invention, there is provided use of a monoclonal antibody that specifically binds to  $\beta$ A4 (1-42) peptide, and not to  $\beta$ A4 (1-40) and  $\beta$ A4 (1-43) peptides, in the manufacture of a medicament for the prevention or treatment of Alzheimer's Disease.

#### Brief Description of the Drawings

The invention will be more completely understood from a consideration of the following detailed description, taken in conjunction with the drawings, in which:

10 Fig. 1 is a schematic showing the  $\beta$ A4 portion of the Amyloid Precursor Protein (APP), its location relative to the cell membrane, and the  $\alpha$ ,  $\beta$  and  $\gamma$  cleavage sites;

15 Fig. 2 shows the  $\beta$ A4 portion of APP, its position relative to the transmembrane region of a cell, and the three major cleavage site ( $\alpha$ ,  $\beta$  and  $\gamma$ ) in APP;

Fig. 3 is a diagram of the clone pGK003 which was used to generate the  $\beta$ A4 1-42 peptide;

20 Fig. 4A shows SDS-PAGE on a 16% Tris/Tricine gel, of in vitro translated radioactively labelled  $\beta$ A4 in a wheat germ system;

Fig. 4B shows SDS-PAGE on a 16% Tris/Tricine gel, of in vitro translated radioactively labelled  $\beta$ A4 from wheat germ system, immunoprecipitated with MAb 286.8A;

25 Fig. 5 is a graph showing immunoprecipitation of in vitro translated  $\beta$ A4 (IVT  $\beta$ A4) with 286.8A;

Fig. 6 is a diagram of the peptides used to generate the immune response (immunogen) and to screen the sera of mice;

Fig. 7 is a graph showing immunoprecipitation of in vitro translated BA4 vs. antibody concentration; -○- 286.8A, -△- 369.2B, -□- 369.6;

5 Fig. 8 is a graph showing the % of various BA4 sequences immunoprecipitated by MAb 369.2;

Fig. 9 is a graph showing epitope mapping of MAb 369.2 by competitive assay, with -■- being 35-42(OVA) (Ovalbumin coupled 35-42 BA4 peptide), -□- being 1-42 BA4 peptide, and -◆- being 1-40 BA4 peptide;

10 Fig. 10 is a photograph showing the binding of MAb 369.2B to vascular amyloid and other plaques with various morphologies.

#### Detailed Description of the Invention

15 The  $\beta$ -amyloid depositions in Alzheimer's disease brains are made up mainly of BA4 peptides which show both N- as well as C-terminal heterogeneity. The major C-terminal species, identified by peptide sequencing of  $\beta$ -amyloid purified from postmortem brain material, end either at position 40 or 42 of the BA4 peptide which is at most 43 residues long. In vitro experiments with synthetic BA4 peptides ending either at position 40 or 42 indicate profound physico-chemical differences. Previously, 20 the distribution of these two BA4 species in postmortem tissue as well as their generation in vitro could not be addressed due to the lack of specific antibodies against the carboxy end capable of distinguishing between subspecies of BA4 peptide.

25 Recent evidence suggests that release of BA4 is a normal event in virtually every cell culture. Typically high picomolar to low nanomolar concentrations of BA4 can be measured in serum and cerebral spinal fluid (Seubert *et al.*, 1993). This finding was surprising because it had been assumed that the production of BA4 is a pathological event since BA4 is massively deposited as  $\beta$ -amyloid plaques in the

cortical and hippocampal brain regions of Alzheimer disease patients. Detailed sequence analysis of the released BA4 from cell culture revealed that the major species end at position 40 (Selkoe, 1994). Amyloid plaques purified from postmortem brain show a slightly different picture: amyloid deposits of the congophilic amyloid angiopathy (CAA) are  $\beta$ A4 aggregates surrounding blood vessels and are predominantly BA4 1-40, whereas in contrast amyloid plaques cores (APC) which are present in the brain parenchyma and are not associated with blood vessels exhibit an N-terminal raggedness (starting most commonly at residues 1, 4 and 11) and end mainly either at position 40 or 42 (Glennner and Wong, 1984, *Biochem. Biophys. Res. Comm.* 120:885-890; Masters *et al.*, 1985, *PNAS USA* 82:4245-4249; Miller *et al.*, 1993). Occasionally, longer species ending at 43 or extending even further have been described (Miller *et al.*, 1993). Because the length of the hydrophobic C-terminus is critical for the ability of the peptide to self-aggregate in vitro (Burdick *et al.*, 1992; Jarrett *et al.*, 1993, *Biochem.* 32:4693-4697), it is entirely possible that the two distinct pathological aggregates, APC and CAA and other vascular  $\beta$ -amyloid plaques, can be explained by the differing properties of the two species 1-40 and 1-42. This could also be the case for the so called "diffuse plaques" (Selkoe, 1994) which are seen frequently in brains of aged humans and are not associated with AD, however, have been proposed to be precursors of fibrillar  $\beta$ -amyloid deposits. A non-fibrillar aggregation of  $\beta$ A4 has been suggested for these structures. It is therefore of primary importance to determine the tissue specific production of these longer BA4 species (i.e., those ending at position 42) and their pathological appearance in brains of AD patients. Recently three reports have been published where antibodies have been described, which distinguish 1-42 and 1-40 species of  $\beta$ A4 (Suzuki *et al.*, 1994; Murphy *et al.*, 1994; Yang *et al.*, 1994). Unlike the antibodies of the instant invention, the antibodies reported by Suzuki *et al.*, and Yang *et al.*, crossreact in a significant degree with both the 1-43 and 1-42 species of  $\beta$ A4 peptide. The antibody of Murphy *et al.*, while not

tested for binding with the 1-43 species of  $\beta$ A4 peptide, exhibits a different tissue binding pattern than the antibodies of the instant invention, and thus must recognize a different, or modified epitope from that recognized by the antibodies of the instant invention.

5           Positions 29 through 42 of the  $\beta$ A4 peptide lie entirely within the putative transmembrane region of the Amyloid Precursor Protein and are hydrophobic in nature (Miller *et al.*, 1993). Synthetic peptides to the C-terminal sequences in this region must overcome the ability of the 34-42 sequence to form an unusually stable  $\beta$ -structure  
10           which is virtually insoluble in water and strong denaturants (Halverson *et al.*, 1990, *Biochem.* 29:2639-2644) if they are to be used to elicit good immune responses against soluble  $\beta$ A4. We designed a hydrophilic spacer five residues long which would overcome those insolubility problems and also extend the presumed epitope away from the proximity of the carrier. To reduce the likelihood of cross-reactivity with the shorter but major  $\beta$ A4 species, 1-40, we chose a minimal peptidyl epitope of 8 residues  
15           corresponding to positions 35-42 of the  $\beta$ A4 sequence. The entire synthetic sequence designed in this way was coupled by a free sulfhydryl group on a N-terminal cysteine residue to KLH (keyhole limpet hemocyanin).

          Successful use of spacers and hydrophilic residues in the production of antipeptide antibodies is well documented as is the use of hydrophilic structures to  
20           bring insoluble haptens into solution for conjugation (McMillan *et al.*, 1983, *Cell* 35:859-863; Makela and Seppala, 1986, in Handbook of Experimental Immunology, Volume 1: Immunochemistry, Wier, D.M., editor. Blackwell Scientific Publications, Oxford. pp 3.1-3.13). The success of this method in producing specific antibodies may at least in part be attributed to the presence of a free charged carboxyl terminal,  
25           especially in context of a hydrophobic sequence, as terminal residues on peptide antigens give rise to significant proportions of antipeptide antibodies (Gras-Masse *et al.*, 1985, in Synthetic Peptides in Biology and Medicine, Alitalo, K. *et al.*, editors,

Elsevier, Amsterdam, p 105). This, along with the selective and novel use of a minimal BA4 sequence used as an immunogen maximized the probability of producing an antibody which could distinguish between BA4 species ending at positions 42 with those that do not. Although peptide competition studies did not fine map the antigenic determinant, BA4 sequences other than 1-42 were not effective in inhibiting binding. The fact that 1-42 did not totally compete with <sup>35</sup>S-methionine-labelled in vitro-translated BA4 may be due either to the particular properties of the molecule itself or to the fact that the 35-42 peptide immunogen was presented in the context of a specific spacer and/or carrier, or that a 200-1000 fold excess of unlabelled peptide is not enough to quench the signal. Non-specific effects of N-terminal residues on antigenic activity are also well documented (Benjamini *et al.*, 1968, *Biochem.* 7:1261-1264).

The intriguing finding that 25-35 actually enhances the ability of 369.2B and other antibodies to bind to BA4 may be due to a peculiar interaction between the abstracted peptide and the full length BA4 sequence itself. Residues 26-33 are believed to exist as a random coil in aqueous solution (Halverson *et al.*, 1990) and may be able to interact with soluble BA4 in such a way that makes the C- terminus more accessible to the binding sites on antibodies.

The highly specific antibody of the instant invention, of which 369.2B is a particular example was raised against a synthetic BA4 peptide having residues 35-42, and does not recognize the shorter BA4 species 1-40 in solution or on a solid phase. Furthermore, both 1-40 and 1-43 were unable to absorb out the antibody when used immunohistochemically. A secondary screening method with medium capacity through-put for the screening of hybridoma supernatants using radioactively labelled in vitro translated BA4 was applied so that antibodies culled from the primary screening could be further selected for their ability to immunoprecipitate soluble BA4. This method can be easily adapted to other proteins/antibodies of interest. The resulting

MAB 369.2B represents a superior tool to investigate the role of  $\beta$ A4 peptides ending at position 42 in situ, postmortem tissue, transgenic animals, and the in vitro generation of  $\beta$ A4 peptides in established cellular  $\beta$ A4 production models, for diagnostic use, and for therapeutics.

5           The monoclonal antibody of the present invention represents an important tool needed to establish a diagnostic test kit. It permits one to measure/quantify the amount of the  $\beta$ A4 1-42 or derivatives thereof (e.g. 4-42 species, and other truncated forms with the "42" carboxy end) in human body fluids (CSF, serum, urine etc.) or tissues. It can also be used to study the distribution pattern of 1-42 or  $\beta$ A4 species ending in  
10 residue 42, in AD brains compared to healthy controls. Its exceptional high avidity makes it a superior and novel tool for such testing. The monoclonal antibody here disclosed can also be used in biological model systems such as transfected cell cultures or animal models (transgenic mice), designed to measure and/or influence the presence and/or production of  $\beta$ A4 species ending in amino acid 42. These model systems  
15 represent means to identify selective modulators of the production of  $\beta$ A4 ending in amino acid position 42 of the  $\beta$ A4 in biological systems. The antibodies of the instant invention provide for methods of preventing aggregation of  $\beta$ A4 peptide because the specificity of the antibody will allow for the specific interference with the free C-terminal residue, thereby interfering with and disrupting aggregation that may be  
20 pathogenic in AD.

          Surprisingly, the antibody of the present invention differs from that of the prior art in that it stains diffuse and fibrillar amyloid, neurofibrillary tangles, and vascular amyloid while being specific for the  $\beta$ A4 peptide free C-terminal residue 42. This  
25 unique binding pattern shows that the antibody of the instant invention recognizes a different epitope from that of the prior art, and that the tissue distribution or accessibility of the  $\beta$ A4 peptide recognized by the antibody of the instant invention is also different. Further, the instant invention provides for monoclonal antibody which

can precipitate the BA4 peptide out of solution, which was not demonstrated by the antibodies of the prior art. Thus the instant invention provides for unique monoclonal antibodies which recognize a unique subset of BA4 species which has a distinct tissue distribution that is most likely a better diagnostic indicator than what was previously available, and a unique target for therapeutic intervention.

Thus the instant invention provides for antibodies, antibody fragments and constructs thereof which are specific for the BA4 species of peptide where the C-terminal ends at residue 42. The instant invention also provides for the use of such antibodies, binding fragments and constructs thereof in diagnostic, analytic, therapeutic, and biochemical purification methods which employ the binding specificity of the instant monoclonal antibodies and their use within pharmaceutical formulations.

The following examples will further explain the instant invention and are shown by way of illustration, and not by way of limitation. The following examples illustrate certain aspects of the above-identified methods and compositions as well as advantageous results.

#### **Example 1: BA4 Peptide Expression System**

##### **Preparation of plasmid pGK002**

General cloning and molecular biology procedures are found for example in Sambrook, Fritsch, and Maniatis, 1989, Molecular Cloning 2nd edition, Cold Spring Harbor Lab Press. Plasmid pMTI-26, which is a Bluescript KS clone containing 2.415 kb (kilobase pairs) of the APP sequence with a TAG stop codon followed by a BamHI site placed in frame by site-directed mutagenesis after the 42nd amino acid codon of the BA4 region, was modified by excising a 1.8 kb Xba I/Bgl II fragment and religating the plasmid after filling in the ends. The resulting construct, designated pGK002, places the consensus containing initiation codon of the BA4 sequence immediately downstream of the Bluescript<sup>\*</sup> T7 promotor.

**Preparation of plasmid pGK003**

Plasmid pGK003 (Fig. 3), used in all of the wheat germ in vitro translations of BA4 to be described below, was made by subcloning a 590 bp (base pair) NotI/Xho I fragment from pGK002 containing the entire human BA4 sequence with the mutagenized stop/Bam HI into a pSP64 polyA vector (Promega Corp.). In preparing this plasmid, pGK002 was digested with Not I and Xho I and the resulting 590 bp fragment was filled in with Klenow, isolated, and ligated with pSP64polyA linearized with Sma I. Figure 3 is a diagram of Clone pGK003. The open reading frame of BA4 1-42 is expressed in vitro from the bacterial SP6 promoter. The 3' untranslated (3'-UT) region of APP is shown in black.

**Example 2: In Vitro Transcription and Translation of pGK003**

Plasmid pGK003 was linearized with EcoRV and complete digestion was confirmed by agarose gel electrophoresis. The sample was extracted twice with phenol/chloroform, followed by two chloroform extractions and ethanol precipitation. The resulting pellet was washed once in 70% ethanol, partially dried under vacuum, and resuspended in TE at a concentration of 1 µg/µl.

In vitro transcripts using linearized templates at 30 µg/ml were prepared in 80 mM HEPES-KOH (pH 7.5) buffer containing 16 mM MgCl<sub>2</sub>, 2 mM spermidine, 40 mM DTT, 3 mM ATP/CTP/GTP/UTP, 800 units/ml RNAsin Ribonuclease Inhibitor (Promega Corp.), 5 units/ml Yeast Inorganic pyrophosphatase (Sigma Corp.), and 1800 units/ml SP6 RNA polymerase (Promega Corp.). The reaction mixture was kept at 37°C for 4 hrs. The resultant transcript was verified by electrophoresis through a 1.2% agarose/TBE/EtBr gel with denatured samples (65°C x 10 min).

Transcripts were translated using Wheat germ extract (Sigma Corp.). Briefly, transcripts were heated (65°C x 10 min), mixed with the wheat germ extract containing KAc, RNAsin, and a methionine-minus amino acid mixture, and translated at 25°C for

1 hr in the presence of  $^{35}\text{S}$ -labelled methionine (Amersham). Translation of a 4 kD (kilo dalton) BA4 protein was verified by SDS-PAGE using 16% Tris/Tricine gel (Novex). Gels were fixed and proteins visualized fluorographically using a commercial system, "Amplify" (Amersham).

5           The incorporation of label into in vitro translated BA4, which contains one methionine residue per molecule, was determined by gel slicing. 2 mm slices were solubilized in 1 ml of 30% hydrogen peroxide, 0.75M  $\text{NH}_4\text{OH}$  overnight at 37°C. Next a 10 ml volume of "Ready Value" scintillation cocktail (Beckman) was added and DPMs (Decay per minute) determined using a Beckman LS6000IC scintillation counter  
10 in the auto DPM mode. Typical reactions produce ~250 ng BA4/ml, or ~56 nM.

          In vitro transcription followed by translation of the BA4 clone, pGK003, in a wheat germ system resulted in a single 4 kD protein product when visualized by fluorography on a 16% Tris-Tricine SDS polyacrylamide gel (Figure 4A). Figure 4A shows the results of SDS-PAGE on a 16 % Tris/Tricine gel. Lane 1: High MW  
15 markers. Lane 2: Low MW markers. Lane 3: In vitro translated BA4 in a wheat germ system. The identity of this 4 kD product was confirmed by immunoprecipitation with BA4 specific antibodies (Figure 4B). Figure 4B shows results of SDS-PAGE on a 16 % Tris/Tricine gel. Lane 1: High MW markers. Lane 2: Low MW markers. Lane 3:  
20 In vitro translated BA4 from wheat germ system immunoprecipitated with MAb 286.8A. Transcription and translation of this as well as other BA4 clones in a combined reticulocyte lysate system (TnT) did not result in the same yield or purity of radioactively labelled BA4 (data not shown). This could be due to the short transcript or to the peculiar nature of the BA4 peptide itself.

          The monoclonal antibody 286.8A, which was raised against crude peptide 1-42  
25 and maps to region 3-8 of BA4, was able to precipitate this protein in a concentration-dependent manner (Figure 5). Figure 5 graphs this immunoprecipitation of in vitro translated BA4 (IVT BA4). Increasing amounts of IVT BA4 were

immunoprecipitated with a fixed amount of 286.8A (7.4  $\mu$ g) in 100  $\mu$ l in RIPA buffer.

### **Example 3: Immunogen and Screening Peptide Preparation**

Peptides were prepared by standard Fmoc solid-phase procedures. (see for  
5 example *Gras-Masse et al.*, 1985).

Peptide #959, a 14 residue synthetic peptide having an N-terminal cysteine  
attached to a hydrophilic DGDGD spacer and residues 35-42 of human  $\beta$ A4 (resulting  
complete sequence: CDGDGDMVGGVVIA (SEQ ID NO: 1)), was coupled to a  
maleimide-activated KLH (Keyhole Limpet Hemocyanin) carrier using the  
10 commercially available "Imject" Activated Immunogen conjugation kit (Pierce).  
Briefly, 2 mg of peptide were dissolved in 200  $\mu$ l of conjugation buffer and allowed to  
react at room temperature for 2 hrs with 2 mg of reconstituted maleimide-activated  
KLH. The conjugate was purified by gel filtration and used as an immunogen for  
monoclonal antibody production using standard protocols as described in Example 4.

15 Peptide #958, a 14 residue synthetic peptide having an N-terminal cysteine  
attached to a GGGGG spacer and residues 35-42 of the human  $\beta$ A4, (resulting  
complete sequence: CGGGGGMVGGVVIA (SEQ ID NO: 2)), was coupled to  
ovalbumin by dissolving 2 mg of peptide in 200  $\mu$ l 6M guanidine, 0.01M phosphate pH  
7.0 and conjugated as above to 2 mg of a reconstituted maleimide activated ovalbumin.  
20 The purified conjugate was used in ELISA-screening of monoclonal fusion products.  
Antibodies screened in this way are specific for the "35-42" determinant rather than the  
spacer, cysteine bridge or carrier portions of the immunogen.

25 Figure 6: Illustrates the peptide used to generate the immunresponse (the  
immunogen) and the peptide used to screen the sera of mice, as well as fusions, in the  
enzyme immunoassay plate (EIA) are shown.  $\beta$ A4 sequence 35-42 was synthesized  
together with a spacer and a C-terminal Cysteine, which was then used to couple it  
covalently via maleimide bridge to a large carrier molecule. Both, the spacer and the

carrier molecule in immunogen and screening peptide are different in order to select for BA4 sequence specific antibodies.

### **ELISA (Enzyme Linked Immunosorbant Assay)**

#### **5 Biotinylation of MAb**

The N-hydroxysuccinimide ester of biotin is used to biotinylate monoclonal antibody 286.8A. The integrity of the reagent is first verified by watching it's spontaneous hydrolysis in the absence of primary amines: an 0.2 mg/ml solution of NHS-LC-Biotin (Vector Labs, Burlingame, CA) in PBS is monitored at 260nm over  
10 time. An OD260 of 1.0 after approximately 2 hrs (rising from an initial OD260 - 0.55) indicates the expected hydrolysis.

In the biotinylation reaction a 66:1 molar ratio of Biotin to monoclonal 286.8A at neutral pH has been found to give optimal results when the biotinylated 286.8A was tested in an Elisa format. NHS-LC-Biotin 0.6 mg in H<sub>2</sub>O at a concentration of 0.1  
15 mg/ml is added (within 5 min of dissolving) to 1 ml (2 mg) of 286.8A in PBS. Nucleophilic attack of the NHS ester is allowed to occur at 25°C for 4 hrs after which 10 mg of glycine in 50 µl H<sub>2</sub>O is added to stop the reaction. The reaction is then placed over a 10 ml cross-linked dextran desalting column equilibrated to PBS and 0.5 ml aliquots are collected. The first peak representing the IgG peak is pooled and stored  
20 at 4°C until used.

#### **Elisa procedures**

Corning 25801 96-well Elisa plates are coated overnight at 4°C with 100 µl monoclonal 4G8 or other capture antibody at 5 µg/ml typically in H<sub>2</sub>O or  
25 buffer. The plate is then washed with PBS containing 1% Triton X-100 in a Dynatech Ultrawash<sup>\*</sup> plus. Wells are then blocked for 90 min with 300 µl PBS containing 1% Triton X-100<sup>\*</sup> and 1% Elisa grade BSA (Blocking Buffer). After washing antigen or

unknown diluted in blocking buffer is added to the wells in triplicates and incubated at room temperature for 2 hrs. The plate is washed 2 times and 400 ng biotinylated 286.8A or other detecting antibody is added. After 30 min the plate is more extensively washed (2 times wash, 2 min soak, 2 times wash) and 100  $\mu$ l preformed Avidin-Biotin-Alkaline Phosphatase Complexes (Vector Labs) are added. The plate is washed (2 times wash, 2 min soak, 2 times wash, 5 min soak, 4 washes) and MUP substrate added at 0.06 mg/ml 1x diethanolamine buffer. Plates are read in a Millipore Cytoflour after 15 min using a 360 nm excitation filter and a 460 nm emission filter.

#### Example 4: Generation of Monoclonal Antibodies

Balb/c mice were immunized with multiple I.P. inoculations of KLH conjugated peptide #959. Splenocytes from immunized animals were fused with the mouse myeloma AG8 using standard protocols (Wunderlich et al., 1992, J. Immunol. Methods 147:1-11). Supernatants from resultant hybridomas were screened for immunoreactivity to ovalbumin-coupled peptide #958 using standard Elisa protocols as described above. Hybridomas positive for the expression of immunoreactive MAbs were cloned at least twice by limiting dilution and MAb isotype analysis was performed. Purified MAb IgG was prepared from ascites fluid using protein-A affinity chromatography.

After fusion screening showed that immunization of mice with peptide #959 conjugated to KLH and screened in a solid-phase ELISA format with peptide #958 coupled to ovalbumin resulted in six positive parental signals (identified as 369.1 through 369.6). Both peptides have amino acids 35-42 of the  $\beta$ A4 region, different N-terminal spacers, and a cysteine for covalent coupling to carrier proteins (Figure 6). The free C-terminus with the charged carboxy group and a limited length of only 8 amino acids favors the generation of antibodies which are specifically directed against longer forms of  $\beta$ A4 peptides; shorter  $\beta$ A4 peptides ending before amino acid 42 would

thus not be recognized.

Figure 6 diagrams the structure of the immunogen (carrier-peptide) and the screening peptide (carrier-screening peptide) used.

Two of the six parental signals were ultimately not clonable. Of the remaining four, two gave immunoprecipitation/scintillation signals only a few percent above normal non-immune controls; the other two (identified as 369.6 and 369.2) showed signals of 18% and 19% respectively. Production of monoclonal antibodies from ascities fluid and subsequent immunopurification of these clones was done. Table I compares the data obtained with the IPSA for hybridoma supernatants and purified antibodies.

**Table I**

<u>Cell Line</u>	<u>Isotype</u>	<u>IPSA (supernatant)</u>	<u>IPSA (purified)</u>
369.1	IgG1/IgG2b	3%	N.D.
369.2	IgG1	19%	25% (with 5 $\mu$ g)
369.3	IgG1	2%	N.D.
369.6	IgG2b	18%	7% (with 10 $\mu$ g)

Table 1. Comparison of antibody activities in hybridoma cell lines. IPSA data represents the percent of in vitro translated  $\beta$ A4 which could be immunoprecipitated by either hybridoma supernatants or purified antibody.

**Example 5: Immunoprecipitation/Scintillation Assay for Hybridoma Screening**

To develop and screen for monoclonal antibodies which recognize the  $\beta$ A4 peptide in solution rather than when attached to a solid phase, an assay was developed in which immunoprecipitation of an  $^{35}$ S-methionine-labelled in vitro-translated  $\beta$ A4 (IVT  $\beta$ A4) is measured. A standard amount of in vitro translated  $\beta$ A4 is allowed to form antibody/antigen complexes in a solution which can be optimized for ionic

strength, pH, and detergent composition. After the immune complexes are precipitated with Protein G (Omnisorb cells) and washed extensively, bound radioactivity is counted in a liquid scintillation counter, background is subtracted and the efficiency of precipitation calculated. This Immunoprecipitation/Scintillation assay (IPSA) allows for both the rapid identification and characterization of antibodies, and has been used to test a variety of BA4 antibodies. The assay can be applied in general to monoclonal hybridoma supernatants as well as polyclonal sera to identify antibodies which can be used for immunoprecipitations. Typically 18 IPSAs can be performed in one day. This is therefore a fast and informative secondary hybridoma screening method

Briefly, approximately  $1.5 \times 10^3$  DPMs of  $^{35}\text{S}$ -methionine-labeled in vitro-translated BA4 (TVT BA4) were added to 10  $\mu\text{l}$  of a 10x immunoprecipitation buffer (150mM NaCl, 10%NP-40, 5% deoxycholic acid, 1% SDS, 500mM Tris pH8). To this, 90  $\mu\text{l}$  of monoclonal cell supernatant from the monoclonal fusion of interest (our designation # 369) were added and allowed to react for 2 hrs at 4°C. Background was determined using 90  $\mu\text{l}$  of supernatant of a non-reactive clone; the positive control was 90  $\mu\text{l}$  of supernatant containing monoclonal antibody 286.8A which was made previously against a crude synthetic BA4(1-42) preparation. After 2 hrs, 40  $\mu\text{l}$  of a 10% solution of Omnisorb\* cells (Calbiochem) equilibrated in 1x immunoprecipitation buffer (RIPA buffer, 150mM NaCl, 1%NP-40, 0.5% deoxycholic acid, 0.1% SDS, 50mM Tris pH8) were added and allowed to react for an additional 2 hrs at 4°C with rocking. The cells were pelleted by centrifugation for 5 min at 4500 g and 4°C, and washed 3x with 800  $\mu\text{l}$  cold 1x immunoprecipitation buffer. Pellets were quantitatively transferred to scintillation vials and counted in a Beckman LS6000 scintillation counter in the Auto DPM mode. The percentage of BA4 immunoprecipitated was calculated.

Immunoprecipitation/Scintillation assays were performed with 1  $\mu\text{g}$  of purified monoclonal antibody 369.2B in a total volume of 100  $\mu\text{l}$  1x immunoprecipitation buffer to which 5  $\mu\text{g}$  of competing peptide were added. Incubations and precipitations

were as described above.

Figure 7 depicts the percentage of immunoprecipitated IVT BA4 as a function of antibody concentration for MAbs 369.2, 369.6 and MAb 286.8A. Under the conditions of this assay, 369.2 (and further subclone 369.2B) is approximately four times better than 369.6 in immunoprecipitating soluble IVT BA4, but precipitates a little less than half as much as 286.8A. Figure 7 shows the results of Immunoprecipitation of in vitro translated BA4 vs. antibody concentration ( $\mu\text{g}$  antibody/100  $\mu\text{l}$  RIPA buffer) where;

$$\text{Percent BA4 immunoprecipitated} = \frac{(\text{dpm's with MAb}) - (\text{dpm's with non or preimmune control})}{(\text{total BA4 dpm's/reaction})}$$

Percents with a given MAb concentration varied only a few percentage points between and within experiments.

#### **IPSA for Monoclonal Characterization**

Approximately  $1.5 \times 10^5$  DPMs of  $^{35}\text{S}$ -methionine-labeled in vitro-translated BA4 were added to various amounts of purified monoclonal antibody, either 369.2B, 369.6, or 286.8A, in a total volume of 100  $\mu\text{l}$  1x immunoprecipitation buffer, and allowed to react as described above. Immune complexes were precipitated with Omnisorb, washed, and counted as described above.

#### **Example 6: Characterization of MAb 369.2B**

To further characterize the best cell line, 369.2B, a competition immunoprecipitation/scintillation assay (Competition IPSA) was performed. In this variation 369.2B was added to an approximate 200 fold molar excess of unlabelled competitor peptide at the same time as labelled in vitro translated BA4 1-42. As expected, peptides to the human BA4 region, 1-40, 1-11, 1-28, 12-28, as well as the reverse peptide 40-1 did not compete with the  $^{35}\text{S}$ -methionine-labeled in vitro-translated BA4 for immunoprecipitation, whereas the complete 1-42 peptide did

(Figure 8).

These results were corroborated in a solid-phase ELISA format: the absorbed ovalbumin-coupled screening peptide which contains the BA4 region 35-42, as well as the 1-42 peptide, compete whereas 1-40 did not (Figure 9). The decreased competitiveness of the 1-42 peptide compared to the ovalbumin coupled 35-42 may be due to conformational and/or solubility factors involving the antigenic determinant, or perhaps more simply to the particular stoichiometry of the conjugation (ovalbumin, a carrier with molecular weight of 45 kD compared to 4 kD for the 1-42 peptide, and having somewhere between 5-15 conjugatable maleimide groups per mole of carrier).

Figure 8 shows Immunoprecipitation Peptide competition/Scintillation Assay for epitope determination of MAb 369.2. Peptide competitor (5  $\mu$ g) was mixed with in vitro translated BA4 ( $\sim 1.5 \times 10^5$  DPMs or  $\sim 200$  pg) then immunoprecipitated with 2  $\mu$ g 369.2, where;

$$\text{Percent BA4 immunoprecipitated} = \frac{(\text{dpm's with MAb}) - (\text{dpm's with non or preimmune control})}{(\text{total BA4 dpm's/reaction})}$$

Percents with a given MAb concentration varied typically only a few percentage points between and within experiments.

Figure 9 shows epitope mapping of MAb 369.2 by competitive assay. C369.2 (50ng IgG/100  $\mu$ l) was preincubated with or without synthetic competitor peptides (22°C, 1 hr), then subjected to Elisa against plate-bound Ovalbumin-coupled 35 - 42 (200ng/well). Percent competition was calculated relative to MAb binding in the absence of competitor, i.e. where;

$$\% \text{ competitor} = \frac{(\text{signal w/o competitor}) - [(\text{signal w competitor}) - (\text{background})]}{(\text{signal w/o competitor})}$$

The solid square is 35-42(OVA) peptide conjugate; the open square is 1-42 peptide; and the solid diamond is 1-40 peptide.

From this data we conclude that monoclonal 369.2B is specific for the C-terminal end of the full length (1-42) BA4 peptide. Although the exact antigenic

determinant has not been fine mapped, it clearly involves residues beyond position 40 and, since the antibody was made to a short synthetic peptide the determinant is unlikely to involve other residues of  $\beta$ A4 which may be conformationally juxtaposed. Specifically, 369.2B is a very important tool in recognizing  $\beta$ A4 species ending at position 42.

One additional and interesting observation from the peptide competition assay is the enhanced immunoprecipitability of in vitro-translated  $\beta$ A4 by the decapeptide 25-35. This phenomena has also been seen in assays using one other monoclonal antibody (i.e., 286.8A) as well as one rabbit polyclonal antisera (data not shown). We also know from other experiments using varying amounts of detergent, specifically SDS, in IPISA assays with MAb 286.8A, that more  $\beta$ A4 can be immunoprecipitated with increasing amounts of detergent (data not shown). SDS, interestingly, has been shown to be ineffective in solubilizing  $\beta$ A4 aggregates, at least as shown by SDS-PAGE (Burdick et al., 1992). However it is not immediately clear why SDS should enhance the immunoprecipitability of  $\beta$ A4.

#### **Example 7: Immunohistochemical Studies**

We have undertaken immunohistochemistry studies with 369.2B. The staining pattern of 369.2B (1/10,000 dilution of a 22 mg/ml ascities purified antibody solution) when compared to the monoclonal antibody 286.8A which we have shown recognizes epitope 3-8 of  $\beta$ A4 and is human specific (data not shown) showed interesting differences. Results obtained from immunohistochemistry demonstrated that 369.2B is an excellent antibody (at 1/10,000 dilution) to specifically label amyloid plaques cores, diffuse as well as fibrillar amyloid deposits and vascular amyloid deposits (Figure 10).

Figure 10 is a photomicrograph showing  $\beta$ -amyloid plaques, blood vessels, and perivascular deposits of  $\beta$ A4 in a paraffin embedded 10  $\mu$ m thick section from the brain of a 76 year old female patient with Alzheimer's disease. Tissue sections were

pretreated with 88% formic acid (30 min), then labeled using an  
avidin-biotin-peroxidase kit (Vector Laboratories, Burlington, CA) with  
nickel-diaminobenzidine as the chromagen. Monoclonal antibody 369.2B labels  
plaques with a variety of morphologies, including cored, perivascular, and diffuse  
5 (non-amyloidotic) plaques. It also labels a subset of amyloidotic blood vessels.

Further studies also showed that  $\beta$ A4 1-43 peptide was not able to compete for  
staining (more than a 1000 fold excess peptide), whereas  $\beta$ A4 1-42 completely  
abolished the signal (Table 2). Again as expected, 1-40 or 40-1 did not compete for  
staining. From these studies we can already conclude that this antibody is an excellent  
10 tool for immunohistochemistry. As suggested by in vitro studies which show  
physico-chemical differences between 1-40 and 1-42, it is possible that these two  $\beta$ A4  
species show distinct patterns in Alzheimer brains. With the monoclonal antibody of  
the instant invention, we are now able to begin addressing this question. Thus the  
monoclonal antibody and methods of the instant invention are useful for diagnostic and  
15 therapeutic uses in all immunological and related methodologies which can be applied  
to the detection, monitoring, extraction, inhibition and modification of unique  $\beta$ A4  
species, in the diagnosis and treatment of AD.

**Table 2**

		<b>Monoclonal Antibody used for Staining</b>	
<b><u>Competing peptide</u></b>		<b><u>N-terminal Mab 286.8A</u></b>	<b><u>C-terminal Mab 369.2B</u></b>
	None/buffer	+++	+++
5	None/DMSO	+++	+++
	Human "40-1"	+++	+++
	Human "1-16"	-	+++
	Mouse "1-16"	+++	+++
	Human "1-40"	-	+++
10	Human "1-42"	-	-
	Human "1-43"	-	+++
	Human "35-42" with spacer	+++	-

**Table 2. Results from competition binding experiments and inhibition of staining, a +++ indicates strong staining, - indicates no detectable staining.**

15

It should be understood that the foregoing disclosure emphasizes certain specific embodiments of the invention and that all modifications or alternatives equivalent thereto are within the spirit and scope of the invention as set forth in the appended claims.

20

## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

(i) APPLICANT: König, Gerhard  
Graham, Paul

5 (ii) TITLE OF INVENTION: Monoclonal Antibody Specific for BA4  
Peptide

(iii) NUMBER OF SEQUENCES: 3

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15 (v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk  
(B) COMPUTER: IBM PC compatible  
(C) OPERATING SYSTEM: PC-DOS/MS-DOS  
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

20 (vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:  
(B) FILING DATE:  
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:

25 (A) NAME: McDonnell, John J  
(B) REGISTRATION NUMBER: 26,949  
(C) REFERENCE/DOCKET NUMBER: 95,216

(ix) TELECOMMUNICATION INFORMATION:

30 (A) TELEPHONE: 312-715-1000  
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## (2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

35 (A) LENGTH: 14 amino acids  
(B) TYPE: amino acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Cys Asp Gly Asp Gly Asp Met Val Gly Gly Val Val Ile Ala  
 1 5 10

5

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 14 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

10

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Cys Gly Gly Gly Gly Gly Met Val Gly Gly Val Val Ile Ala  
 1 5 10

15

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 59 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

20

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:

(A) NAME/KEY: Cleavage-site

(B) LOCATION: 4..5

(D) OTHER INFORMATION: /label= Beta

/note= "Beta cleavage site in APP"

25

(ix) FEATURE:

(A) NAME/KEY: Cleavage-site

(B) LOCATION: 20..21

(D) OTHER INFORMATION: /label= Alpha

/note= "Alpha cleavage site in APP, residues 16/17  
 of BA4."

30

(ix) FEATURE:

(A) NAME/KEY: Cleavage-site

(B) LOCATION: 46..47

35



70850-123

CLAIMS:

1. A monoclonal antibody that is identified as 369.2B, and is produced by the cell line deposited with the American Type Culture Collection (ATCC) as ATCC number HB 11829.
- 5 2. A cell line which is identified by ATCC number HB 11829.
3. A method for generating an antibody for harvesting from a non-human mammal being immunized that specifically binds  $\beta$ A4 (1-42) peptide and not  $\beta$ A4 (1-40) and  $\beta$ A4 (1-43) peptides,  
10 the method comprising immunizing the non-human mammal with a peptide CDGDGDMVGGVVIA (SEQ ID NO: 1) conjugated to a suitable immunological carrier, and harvesting the antibody from the non-human mammal.
4. A method of specifically detecting the presence of  
15  $\beta$ A4 peptide species ending at carboxy-terminus residue 42 (alanine) in tissue, comprising contacting a tissue sample with monoclonal antibody 369.2B or binding fragment thereof to specifically bind said peptide, and detecting the presence of specifically bound monoclonal antibody 369.2B or binding  
20 fragment thereof, wherein the monoclonal antibody is produced by ATCC hybridoma 11829.
5. A method of specifically detecting in a sample, the presence of  $\beta$ A4 peptide species ending at carboxy-terminus residue 42 (alanine) in diffuse amyloid, fibrillar amyloid,  
25 neurofibrillary tangles, and vascular amyloid deposits, comprising contacting a sample to be tested with monoclonal antibody 369.2B or binding fragment thereof to specifically bind said peptide, and detecting the presence of specifically bound monoclonal antibody 369.2B or binding fragment thereof,

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wherein the monoclonal antibody is produced by ATCC hybridoma 11829.

6. A kit comprising a means for selectively detecting the presence of  $\beta$ A4 peptide species ending at carboxy-terminus residue 42 (alanine) comprising an active  $\beta$ A4 peptide-binding fragment of monoclonal antibody 369.2B, wherein the monoclonal antibody is produced by ATCC hybridoma 11829, and a suitable detectable signal.
7. A method for selectively isolating  $\beta$ A4 (1-42) peptide ending at carboxy-terminus residue 42 (alanine) from a sample, comprising contacting a sample with monoclonal antibody 369.2B or peptide binding fragment thereof to specifically bind said peptide, and separating the bound  $\beta$ A4 (1-42) peptide ending at residue 42 (alanine) from the sample.
8. A method for generating a monoclonal antibody that specifically binds to  $\beta$ A4 (1-42) peptide, and not to  $\beta$ A4 (1-40) and  $\beta$ A4 (1-43) peptides, the method comprising immunizing a non-human mammal with a peptide CDGDGDMVGGVVIA (SEQ ID NO:1) conjugated to a suitable immunological carrier and harvesting antibody-producing cells from the non-human mammal.
9. A pharmaceutical composition comprising a monoclonal antibody that specifically binds to  $\beta$ A4 (1-42) peptide, and not to  $\beta$ A4 (1-40) and  $\beta$ A4 (1-43) peptides, and a pharmaceutically acceptable carrier or diluent, for therapeutic use.
10. A monoclonal antibody that specifically binds to  $\beta$ A4 (1-42) peptide, and not to  $\beta$ A4 (1-40) and  $\beta$ A4 (1-43) peptides, for use in preventing or treating Alzheimer's Disease.

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11. A monoclonal antibody that specifically binds to  $\beta$ A4 (1-42) peptide, and not to  $\beta$ A4 (1-40) and  $\beta$ A4 (1-43) peptides, for use in preventing the aggregation of  $\beta$ A4 peptide.

12. Use of a monoclonal antibody that specifically binds  
5 to  $\beta$ A4 (1-42) peptide, and not to  $\beta$ A4 (1-40) and  $\beta$ A4 (1-43) peptides, in the manufacture of a medicament for the prevention or treatment of Alzheimer's Disease.

SMART & BIGGAR

OTTAWA, CANADA

PATENT AGENTS

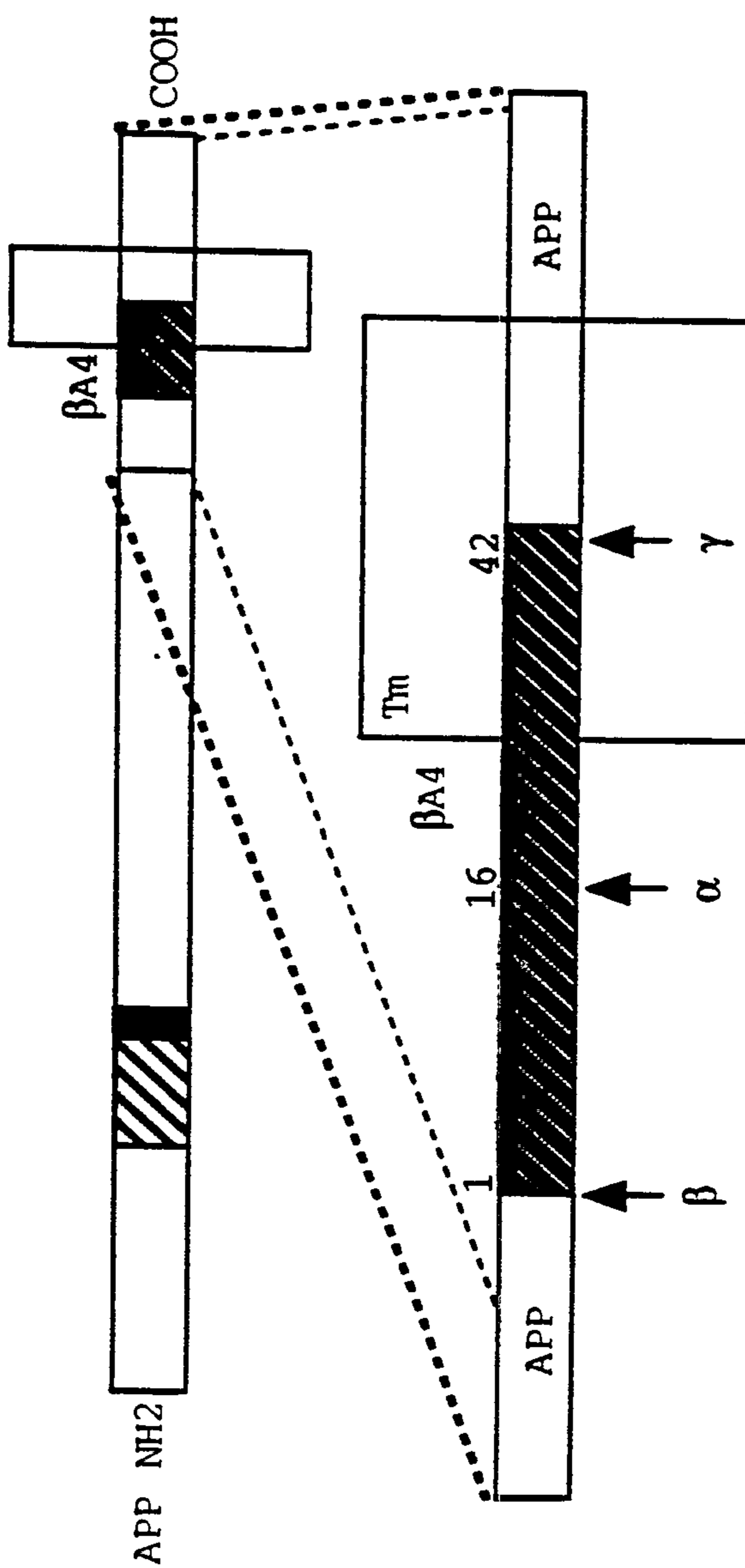


Figure 1

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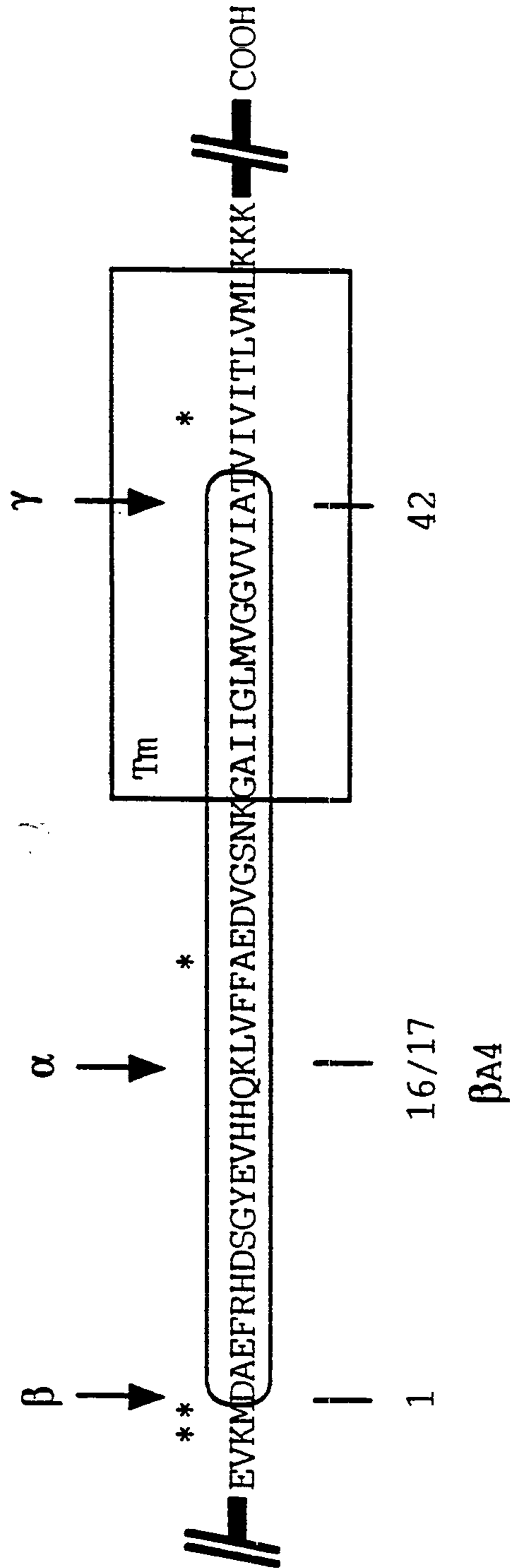


Figure 2

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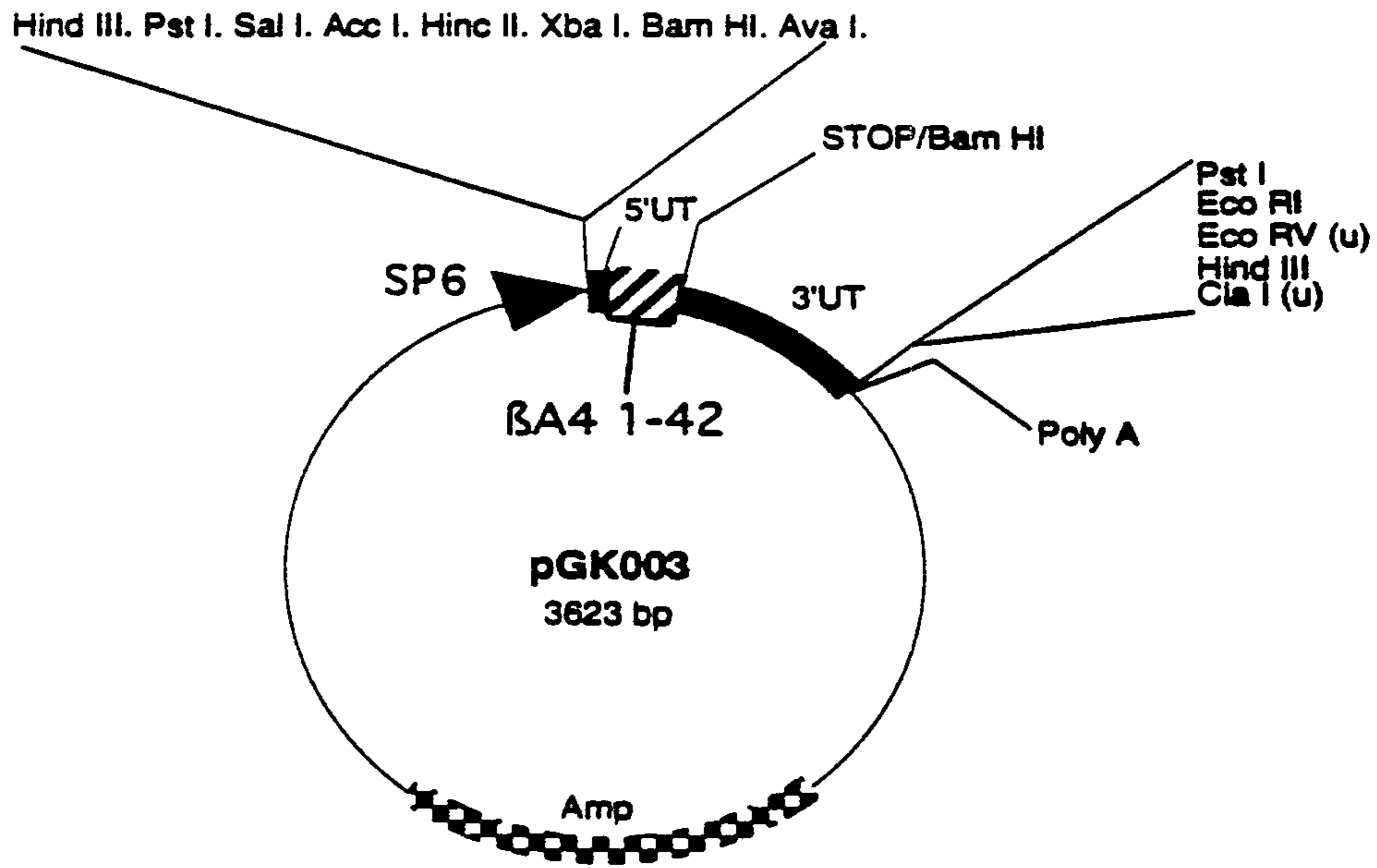


Fig. 3

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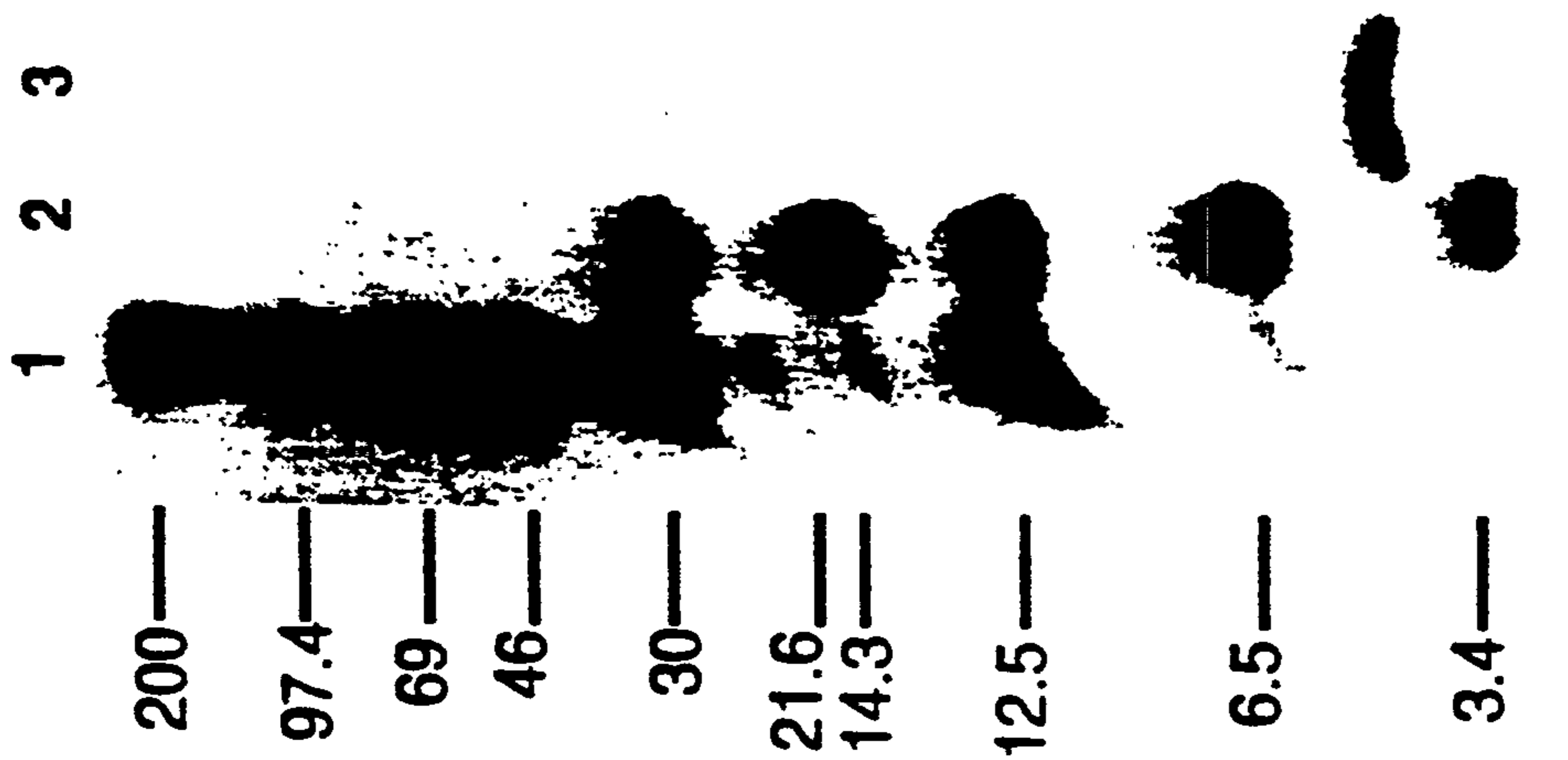


Fig. 4A.

e gel.

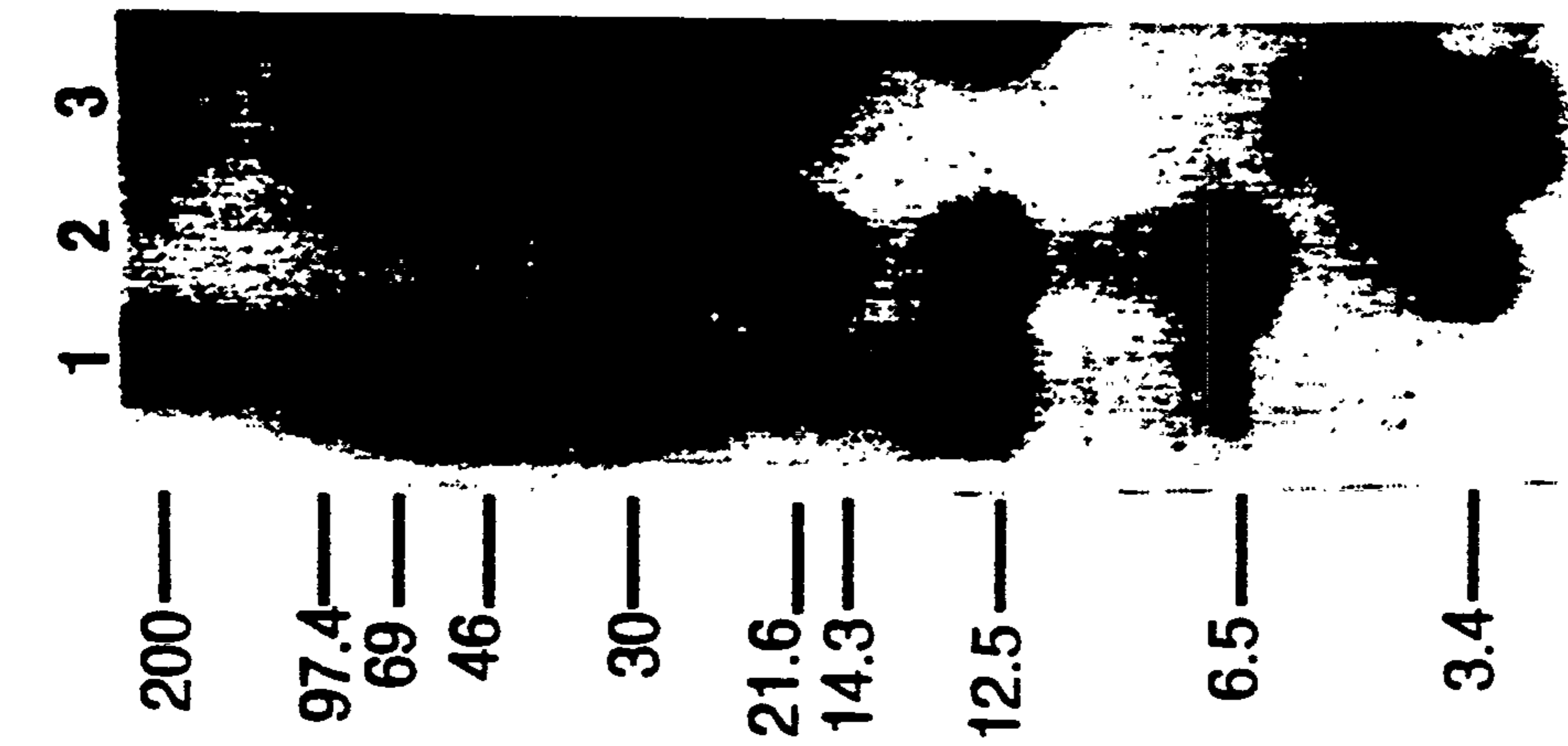


Fig 4B.

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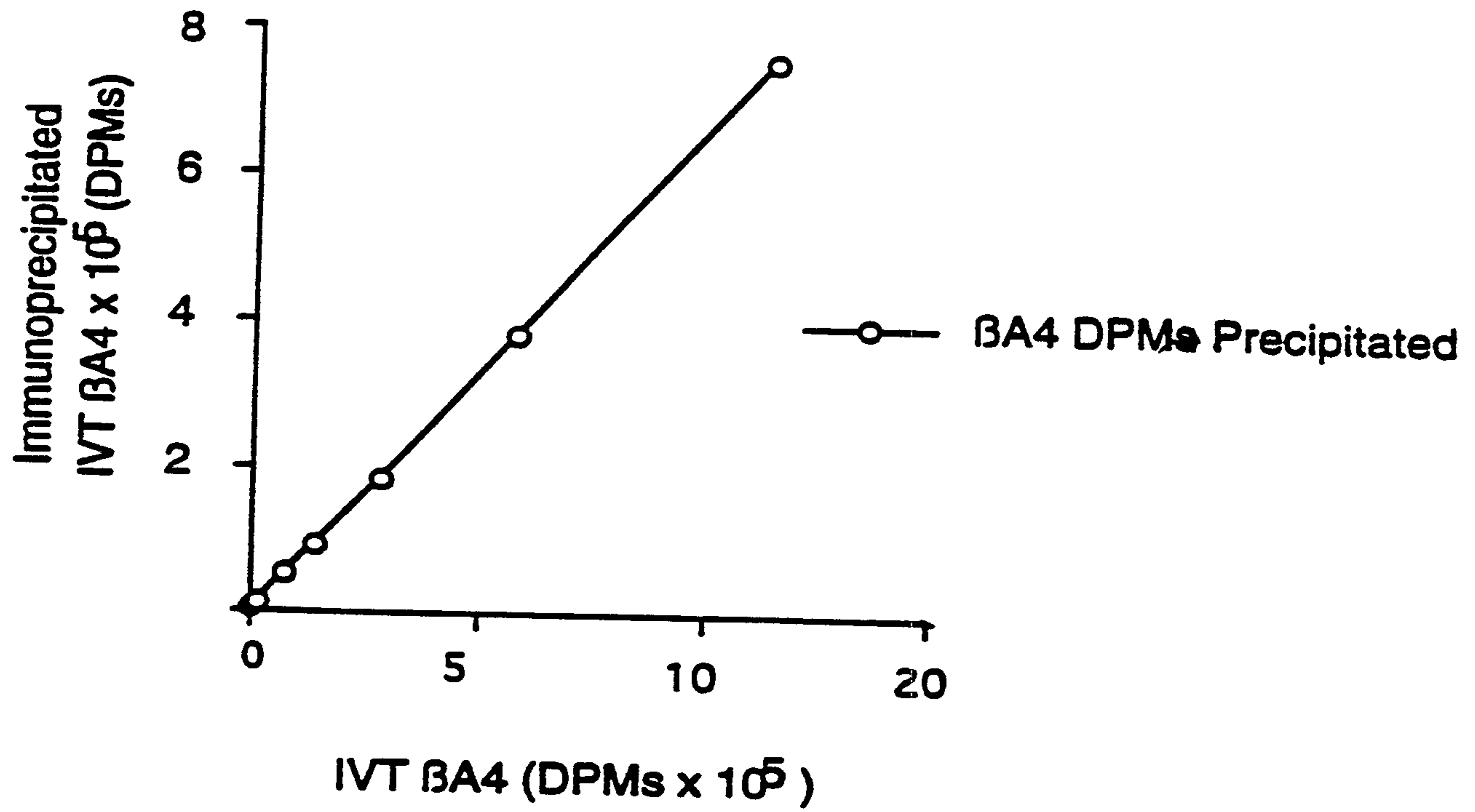


Fig. 5

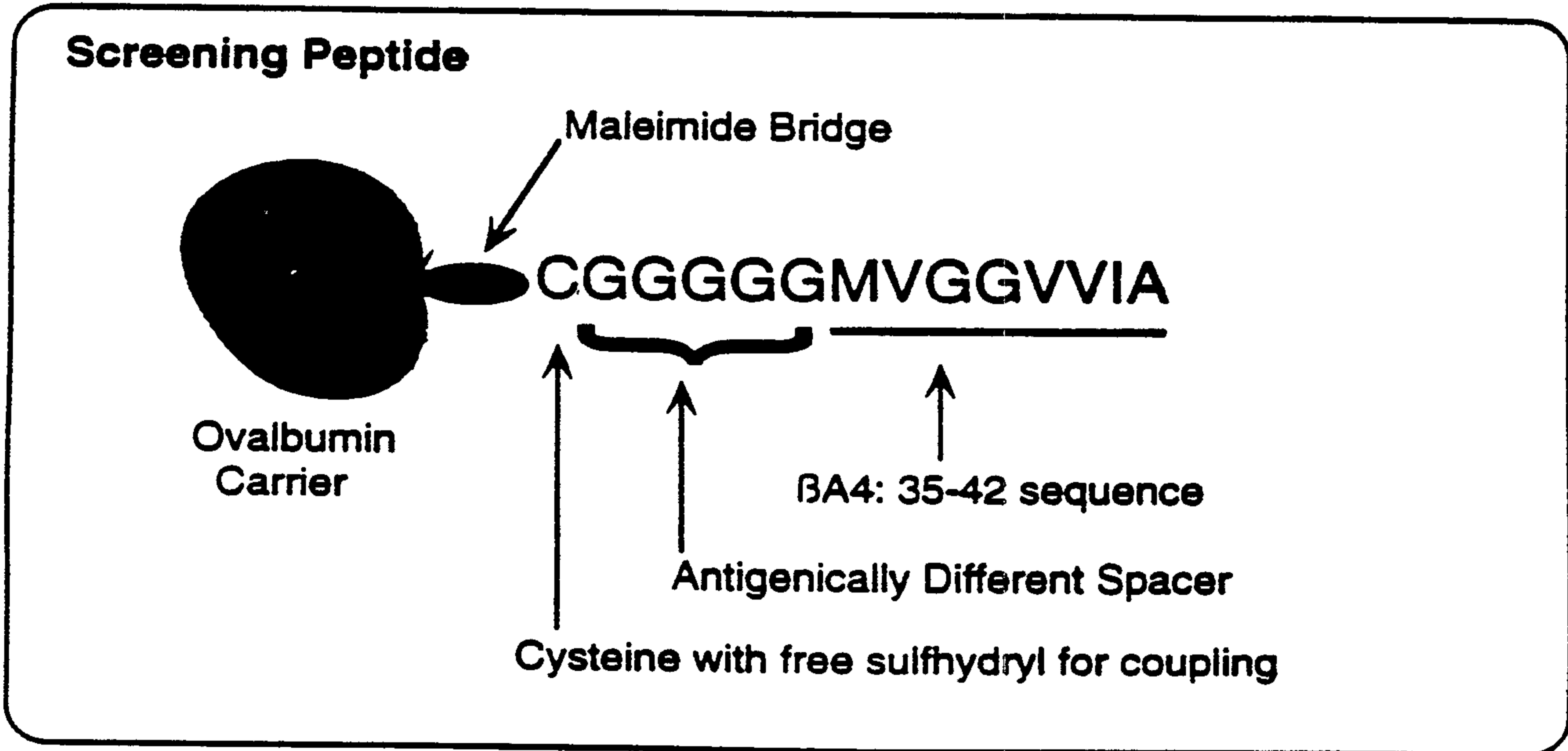
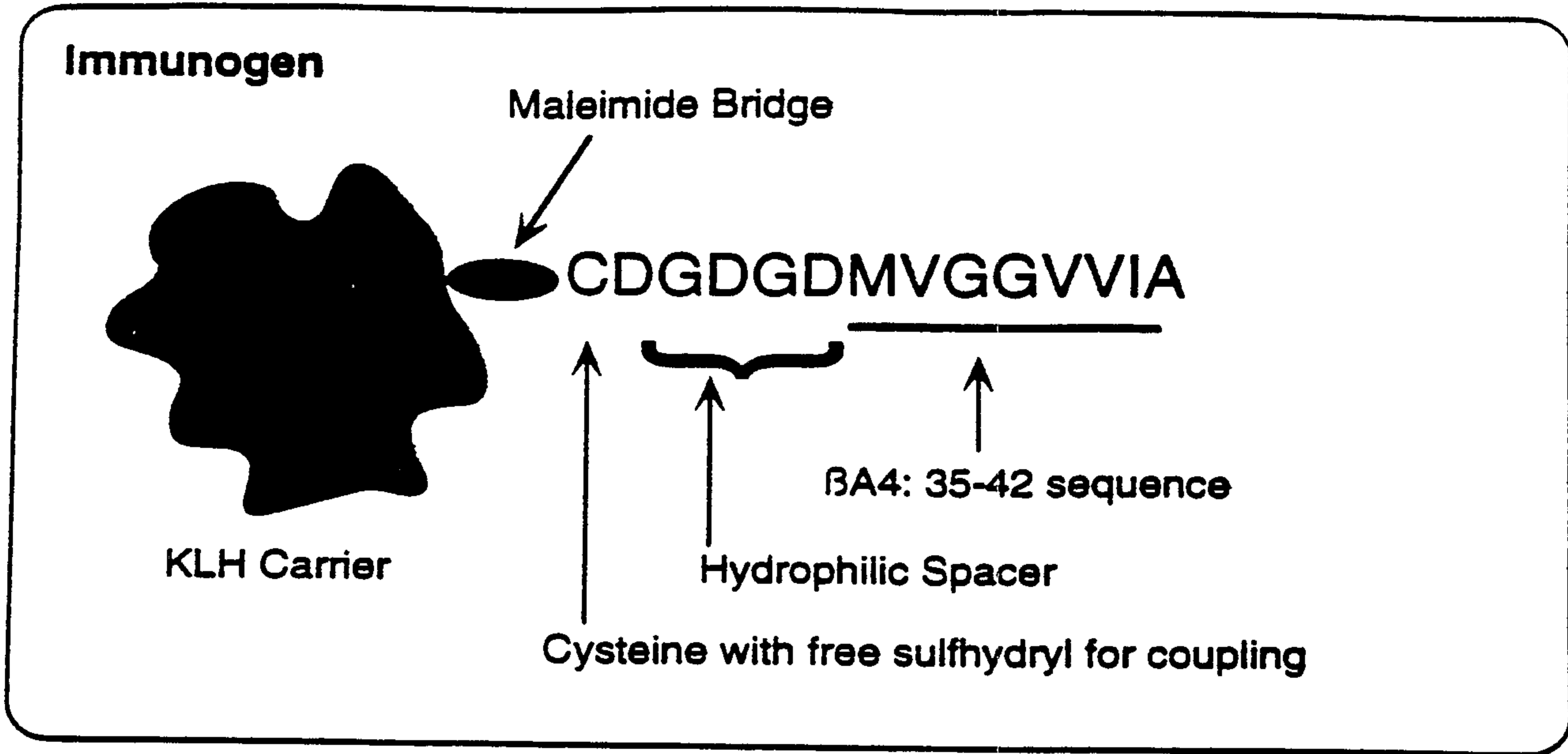


Fig 6

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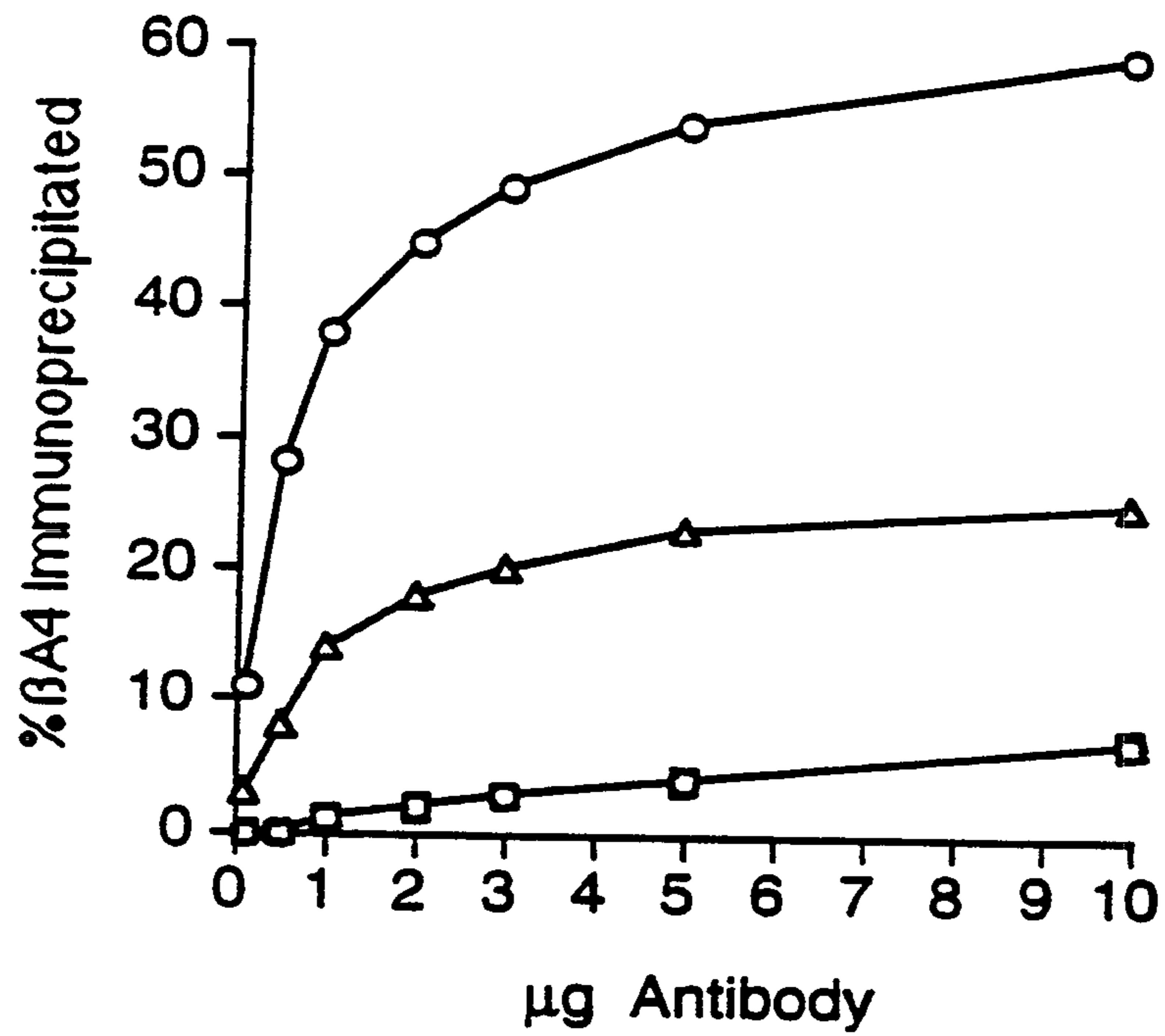


Fig. 7.

—○— 286.8A, —△— 369.2B, —□— 369.6

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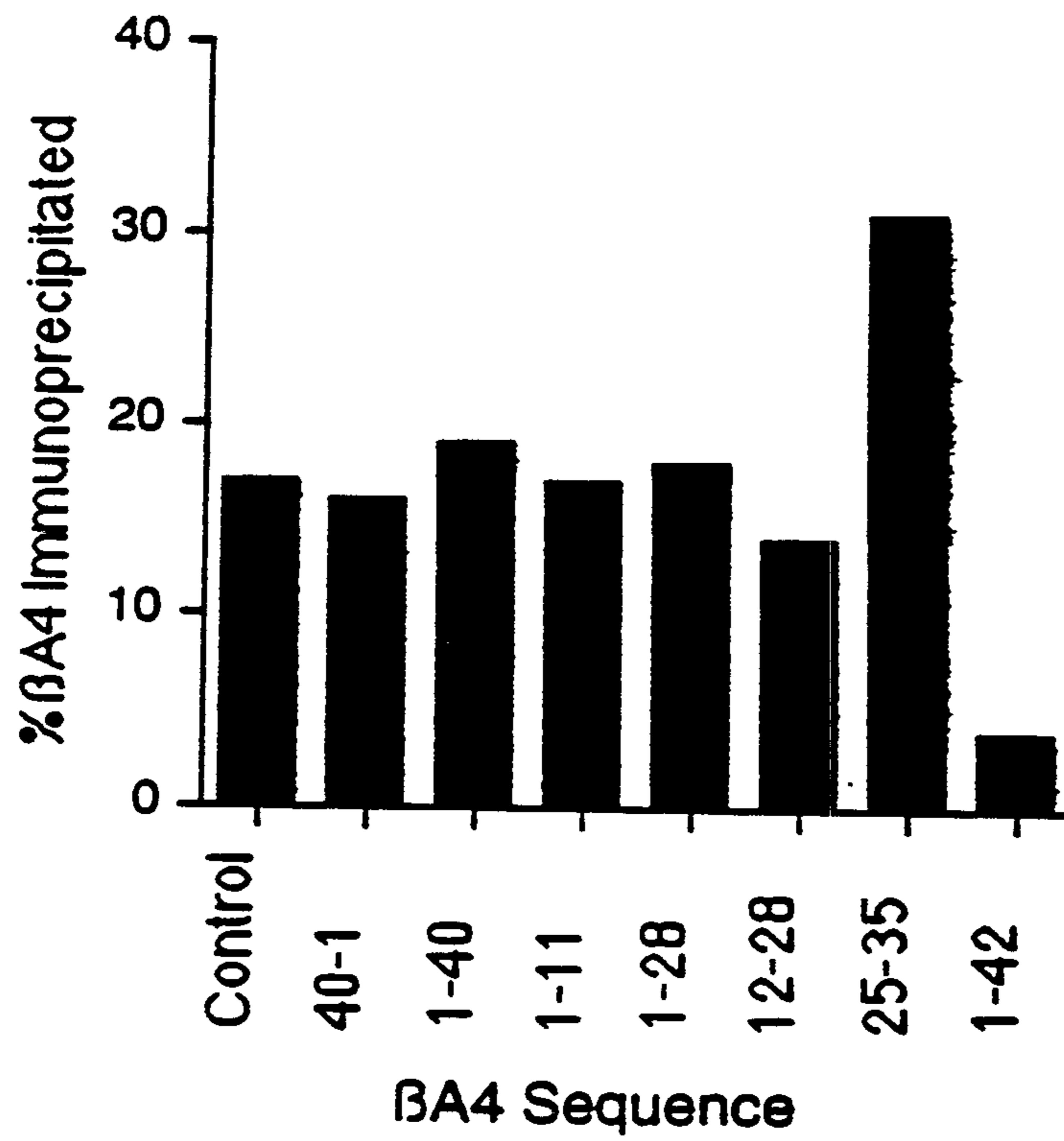


Fig. 8.

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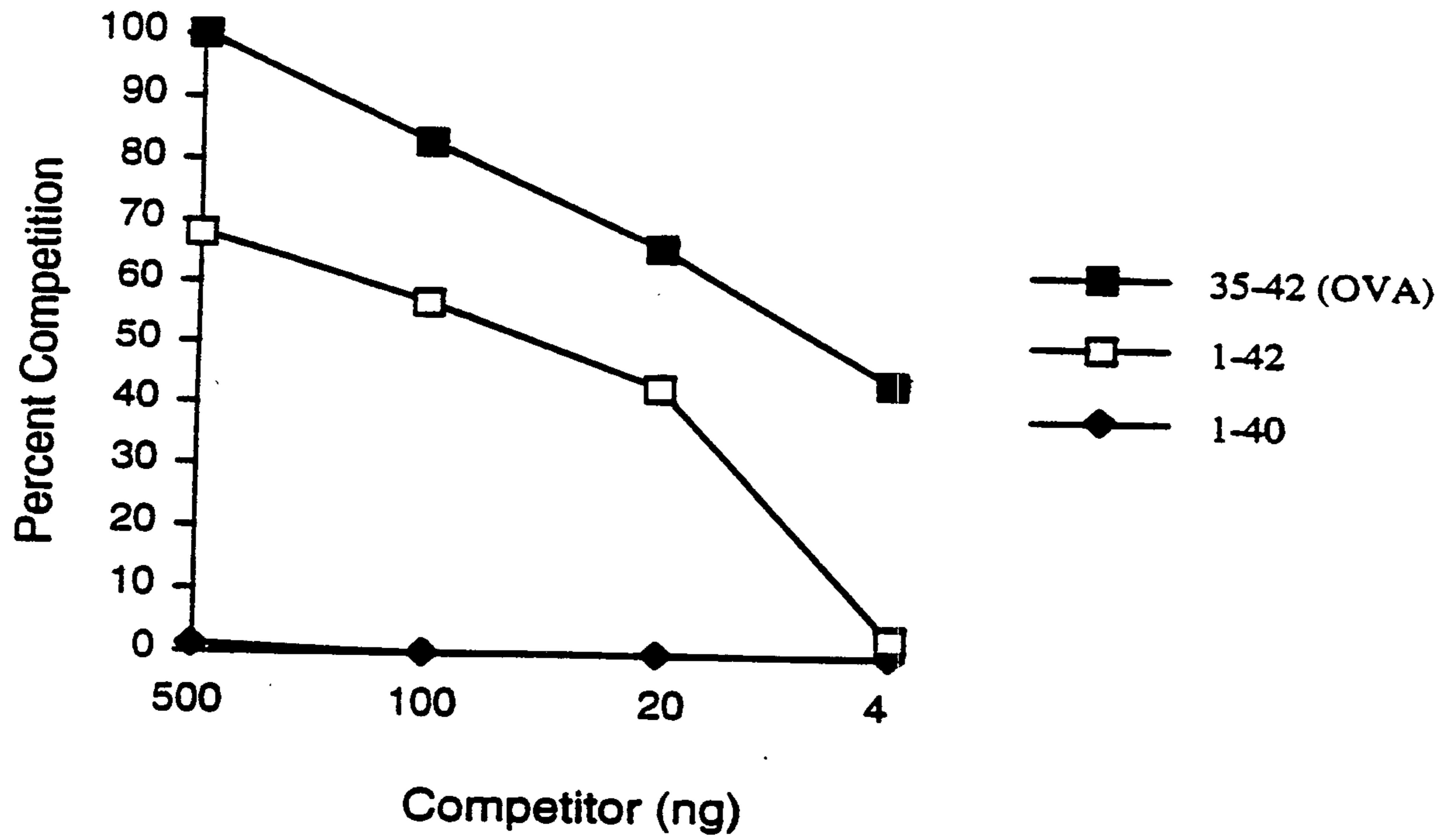


Fig. 9.

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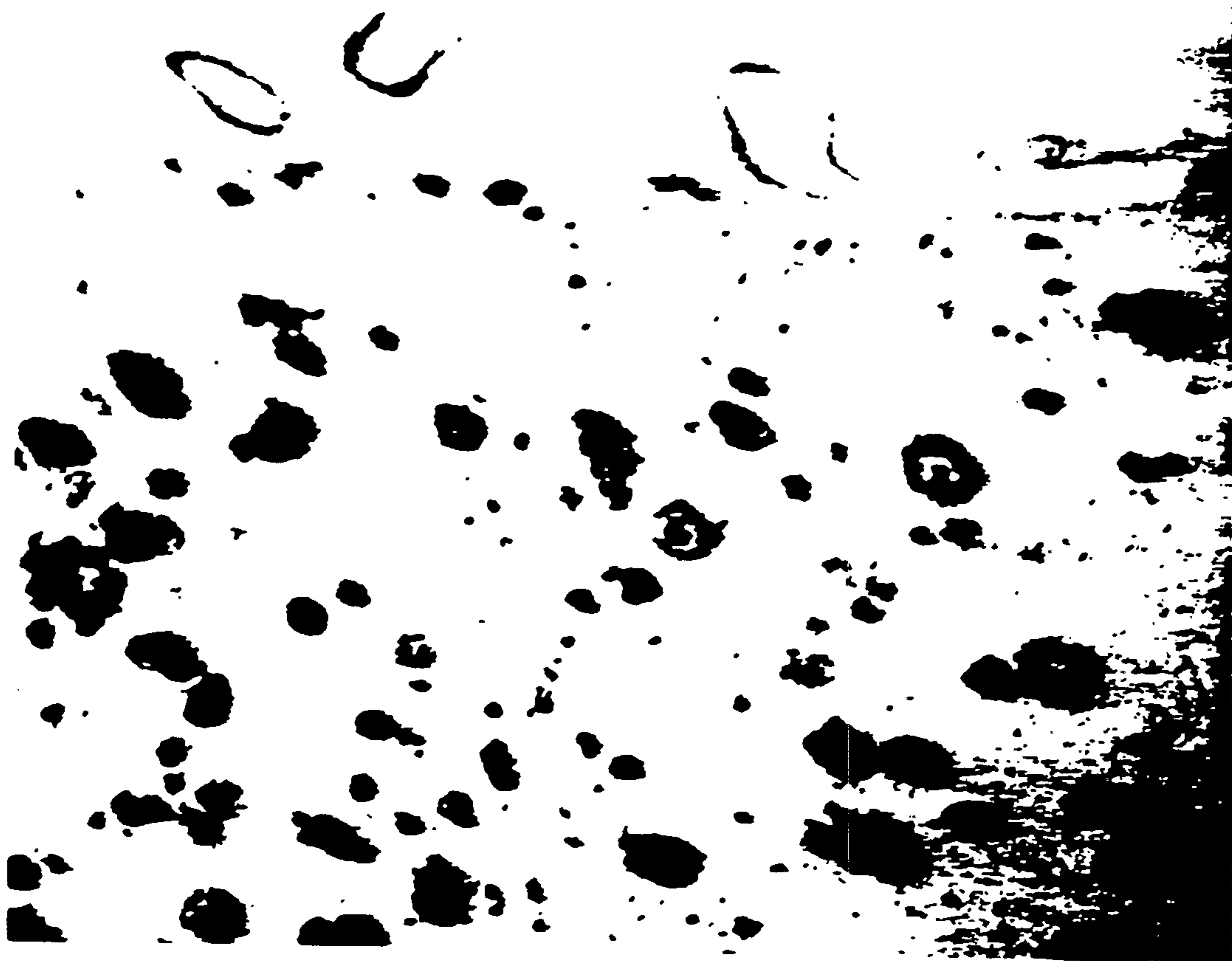


Fig. 10