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(54) **Titre : COMPOSITIONS POLYPEPTIDIQUES DE LIAISON A L'ALBUMINE**
(54) **Title: ALBUMIN BINDING POLYPEPTIDE COMPOSITIONS**

(57) **Abrégé/Abstract:**
The present invention relates to a class of engineered polypeptides having a binding affinity for albumin. It also relates to new methods and uses that exploit binding by these and other compounds to albumin in different contexts, some of which have significance for the treatment of disease in mammals including humans.

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FIGURE 8

MGSSHHHHHHLQVD | ABD VARIANT

(57) Abstract: The present invention relates to a class of engineered polypeptides having a binding affinity for albumin. It also relates to new methods and uses that exploit binding by these and other compounds to albumin in different contexts, some of which have significance for the treatment of disease in mammals including humans.

ALBUMIN BINDING POLYPEPTIDE COMPOSITIONS

Field of the invention

The present invention relates to a class of engineered polypeptides having a binding affinity for albumin. It also relates to new methods and uses that exploit binding by these and other compounds to albumin in different contexts, some of which have significance for the treatment of disease in mammals including humans.

Background

Serum albumin

Serum albumin is the most abundant protein in mammalian sera (40 g/l; approximately 0.7 mM in humans), and one of its functions is to bind molecules such as lipids and bilirubin (Peters T, Advances in Protein Chemistry 37:161, 1985). The half-life of serum albumin is directly proportional to the size of the animal, where for example human serum albumin (HSA) has a half-life of 19 days and rabbit serum albumin has a half-life of about 5 days (McCurdy TR *et al*, J Lab Clin Med 143:115, 2004). Human serum albumin is widely distributed throughout the body, in particular in the intestinal and blood compartments, where it is mainly involved in the maintenance of osmolarity. Structurally, albumins are single-chain proteins comprising three homologous domains and totaling 584 or 585 amino acids (Dugaiczyk L *et al*, Proc Natl Acad Sci USA 79:71, 1982). Albumins contain 17 disulfide bridges and a single reactive thiol, C34, but lack N-linked and O-linked carbohydrate moieties (Peters, 1985, *supra*; Nicholson JP *et al*, Br J Anaesth 85:599, 2000). The lack of glycosylation simplifies recombinant expression of albumin. This property of albumin, together with the fact that its three-dimensional structure is known (He XM and Carter DC, Nature 358:209 1992), has made it an attractive candidate for use in recombinant fusion proteins. Such fusion proteins generally combine a therapeutic protein (which would be rapidly cleared from the body upon administration of the protein per se) and a plasma protein (which exhibits a natural slow clearance) in a single polypeptide chain (Sheffield WP, Curr Drug Targets Cardiovacs Haematol Disord 1:1, 2001). Such fusion proteins may provide clinical benefits in requiring less frequent injection and higher levels of therapeutic protein *in vivo*.

Fusion or association with HSA results in increased in vivo half-life of proteins

Serum albumin is devoid of any enzymatic or immunological function and, thus, should not exhibit undesired side effects upon coupling to a bioactive polypeptide. Furthermore, HSA is a natural carrier involved in the endogenous transport and delivery of numerous natural as well as therapeutic molecules (Sellers EM and Koch-Weser MD, Albumin Structure, Function and Uses, eds Rosenoer VM *et al*, Pergamon, Oxford, p 159, 1977). Several strategies have been reported to either covalently couple proteins directly to serum albumins or to a peptide or protein that will allow *in vivo* association to serum albumins. Examples of the latter approach have been described e.g. in WO91/01743, in WO01/45746 and in Dennis *et al*, J Biol Chem 277:35035-43 (2002). The first document describes *inter alia* the use of albumin binding peptides or proteins derived from streptococcal protein G (SpG) for increasing the half-life of other proteins. The idea is to fuse the bacterially derived, albumin binding peptide/protein to a therapeutically interesting peptide/protein, which has been shown to have a rapid clearance in blood. The thus generated fusion protein binds to serum albumin *in vivo*, and benefits from its longer half-life, which increases the net half-life of the fused therapeutically interesting peptide/protein. WO01/45746 and Dennis *et al* relate to the same concept, but here, the authors utilize relatively short peptides to bind serum albumin. The peptides were selected from a phage displayed peptide library. In Dennis *et al*, earlier work is mentioned in which the enhancement of an immunological response to a recombinant fusion of the albumin binding domain of streptococcal protein G to human complement receptor Type 1 was found. US patent application published as US2004/0001827 (Dennis) also discloses the use of constructs comprising peptide ligands, again identified by phage display technology, which bind to serum albumin and which are conjugated to bioactive compounds for tumor targeting.

Association with HSA results in decreased immunogenicity

In addition to the effect on the *in vivo* half-life of a biologically active protein, it has been proposed that the non-covalent association with albumin of a fusion between a biologically active protein and an albumin binding protein acts to reduce the immune response to the biologically active protein.

Thus, in WO2005/097202, there is described the use of this principle to reduce or eliminate the immune response to a biologically active protein.

Albumin binding domains of bacterial receptor proteins

Streptococcal protein G (SpG) is a bi-functional receptor present on the surface of certain strains of streptococci and is capable of binding to both IgG and serum albumin (Björck *et al*, Mol Immunol 24:1113, 1987). The structure is highly repetitive with several structurally and functionally different domains (Guss *et al*, EMBO J 5:1567, 1986), more precisely three Ig-binding motifs and three serum albumin binding domains (Olsson *et al*, Eur J Biochem 168:319, 1987). The structure of one of the three serum albumin binding domains has been determined, showing a three-helix bundle domain (Kraulis *et al*, FEBS Lett 378:190, 1996). This motif was named ABD (*a*lbumin *b*inding *d*omain) and is 46 amino acid residues in size. In the literature, it has subsequently also been designated G148-GA3.

Other bacterial albumin binding proteins than protein G from *Streptococcus* have also been identified, which contain domains similar to the albumin binding three-helix domains of protein G. Examples of such proteins are the PAB, PPL, MAG and ZAG proteins. Studies of structure and function of such albumin binding proteins have been carried out and reported e.g. by Johansson and co-workers (Johansson *et al*, J Mol Biol 266:859-865, 1997; Johansson *et al*, J Biol Chem 277:8114-8120, 2002), who introduced the designation “GA module” (protein *G*-related *a*lbumin binding module) for the three-helix protein domain responsible for albumin binding. Furthermore, Rozak *et al* have reported on the creation of artificial variants of the GA module, which were selected and studied with regard to different species specificity and stability (Rozak *et al*, Biochemistry 45:3263-3271, 2006). In the present disclosure, the terminology with regard to GA modules from different bacterial species established in the articles by Johansson *et al* and by Rozak *et al* will be followed.

In addition to the three-helix containing proteins described above, other bacterial proteins exist that bind albumin. For example, the family of streptococcal proteins designated the “M proteins” comprises members that bind albumin (see e.g. Table 2 in Navarre & Schneewind, MMBR 63:174-229, 1999). Non-limiting examples are proteins M1/Emm1, M3/Emm3, M12/Emm12, EmmL55/Emm55, Emm49/EmmL49, and H.

Neonatal Fc receptor (FcRn) mediated transcytosis of HSA

The MHC class I-related neonatal Fc receptor (FcRn) mediates cellular trafficking and recycling of albumin and IgG (Brambell *et al*, Nature 203:1352, 1964; Chaudhury *et al*, J Exp Med 197:315, 2003). The FcRn, also known as the Brambell receptor, specifically binds albumin and IgG at low endosomal pH and thus protect pinocytosed proteins from lysosomal degradation by transportation to the cell surface and release at neutral pH. The FcRn has a good affinity for both albumin and IgG at pH 5-6, while showing from poor to no affinity at neutral pH. In this manner, the concentrations and the half-lives of albumin and IgG are regulated. Furthermore, the FcRn is responsible for actively transporting albumin and IgG over cellular barriers, e.g. the epithelium of the airways and the endothelium covering the intestines and the placenta.

As evident from the different sections of this background description, the provision of a selection of polypeptide molecules with a high affinity for albumin is a key factor in the development of various biomedical, biotechnological and other applications, and there is therefore a need in the art of additional such polypeptide molecules.

<u>Disclosure of the invention</u>

The first aspect of the invention meets the need for novel polypeptides with a comparably high albumin affinity, through the provision of an albumin binding polypeptide comprising an albumin binding motif, which motif consists of the amino acid sequence:

$GVSDX_5YKX_8X_9I$ $X_{11}X_{12}AX_{14}TVEGVX_{20}$ $ALX_{23}X_{24}X_{25}I$

wherein, independently of each other,

X_5 is selected from Y and F;
X_8 is selected from N, R and S;
X_9 is selected from V, I, L, M, F and Y;
X_{11} is selected from N, S, E and D;
X_{12} is selected from R, K and N;
X_{14} is selected from K and R;
X_{20} is selected from D, N, Q, E, H, S, R and K;

X_{23} is selected from K, I and T;
X_{24} is selected from A, S, T, G, H, L and D; and
X_{25} is selected from H, E and D;

with the proviso that the amino acid sequence is not

GVSDYYKNLI NNAKTVEGVK ALIDEI;

the albumin binding polypeptide binding to albumin such that the K_D value of the interaction is at most 1×10^{-9} M.

The above definition of a class of sequence related, albumin binding polypeptides according to the invention is based on a statistical analysis of a large number of albumin binding polypeptides identified and characterized as detailed in the experimental section below. The variants were selected from a large pool of random variants of a parent polypeptide sequence or "scaffold", said selection being based on an interaction with albumin in e.g. phage display or other selection experiments. The identified albumin binding motif, or "*ABM*", corresponds to the albumin binding region of the parent scaffold, which region constitutes two alpha helices within a three-helical bundle protein domain. While the original amino acid residues of the two *ABM* helices in the parent scaffold already constitute a binding surface for interaction with albumin, that binding surface is modified by the substitutions according to the invention to provide an alternative albumin binding ability.

As the skilled person will realize, the function of any polypeptide, such as the albumin binding capacity of the polypeptides according to the invention, is dependent on the tertiary structure of the polypeptide. It is therefore possible to make minor changes to the sequence of amino acids in a polypeptide without affecting the function thereof. Thus, the invention encompasses modified variants of the *ABM*, which are such that the albumin binding characteristics are retained. For example, it is possible that an amino acid residue belonging to a certain functional grouping of amino acid residues (e.g. hydrophobic, hydrophilic, polar etc) could be exchanged for another amino acid residue from the same functional group.

In one embodiment of the polypeptide according to this aspect of the invention, X_5 is Y.

In one embodiment of the polypeptide according to this aspect of the invention, X_8 is selected from N and R, and may in particular be R.

In one embodiment of the polypeptide according to this aspect of the invention, X_9 is L.

In one embodiment of the polypeptide according to this aspect of the invention, X_{11} is selected from N and S, and may in particular be N.

In one embodiment of the polypeptide according to this aspect of the invention, X_{12} is selected from R and K, such as X_{12} being R or X_{12} being K.

In one embodiment of the polypeptide according to this aspect of the invention, X_{14} is K.

In one embodiment of the polypeptide according to this aspect of the invention, X_{20} is selected from D, N, Q, E, H, S and R, and may in particular be E.

In one embodiment of the polypeptide according to this aspect of the invention, X_{23} is selected from K and I, and may in particular be K.

In one embodiment of the polypeptide according to this aspect of the invention, X_{24} is selected from A, S, T, G, H and L.

In a more specific embodiment of the polypeptide according to this aspect of the invention, X_{24} is L.

In an even more specific embodiment of the polypeptide according to this aspect of the invention, $X_{23}X_{24}$ is KL.

In another even more specific embodiment of the polypeptide according to this aspect of the invention, $X_{23}X_{24}$ is TL.

In one embodiment of the polypeptide according to this aspect of the invention, X_{24} is selected from A, S, T, G and H.

In a more specific embodiment of the polypeptide according to this aspect of the invention, X_{24} is selected from A, S, T, G and H and X_{23} is I.

In one embodiment of the polypeptide according to this aspect of the invention, X_{25} is H.

As described in detail in the experimental section to follow, the selection of albumin binding variants led to the identification of a substantial amount of individual albumin binding motif (*ABM*) sequences. These sequences constitute individual embodiments of the *ABM* sequence in the definition of albumin binding polypeptides according to this aspect of the present invention. The sequences of individual albumin binding motifs are presented in Figure 1 and as SEQ ID NO:1-257. In certain embodiments of the albumin binding polypeptide according to the invention, the *ABM* consists

of an amino acid sequence selected from SEQ ID NO:1-257. In a more specific embodiment of this aspect of the invention, the *ABM* sequence is selected from SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:9, SEQ ID NO:15, SEQ ID NO:25, SEQ ID NO:27, SEQ ID NO:46, SEQ ID NO:49, SEQ ID NO:53, SEQ ID NO:54, SEQ ID NO:55, SEQ ID NO:155, SEQ ID NO:239, SEQ ID NO:240, SEQ ID NO:241, SEQ ID NO:242, SEQ ID NO:243, SEQ ID NO:244 and SEQ ID NO:245. In yet more specific embodiments of this aspect of the invention, the *ABM* sequence is selected from SEQ ID NO:3, SEQ ID NO:53 and SEQ ID NO:239.

In embodiments of the present invention, the *ABM* may form part of a three-helix bundle protein domain. For example, the *ABM* may essentially constitute or form part of two alpha helices with an interconnecting loop, within said three-helix bundle protein domain.

In particular embodiments of the invention, such a three-helix bundle protein domain is selected from the group consisting of three-helix domains of bacterial receptor proteins. Non-limiting examples of such bacterial receptor proteins may be selected from the group consisting of albumin binding receptor proteins from species of *Streptococcus*, *Peptostreptococcus* and *Finegoldia*, such as for example selected from the group consisting of proteins G, MAG, ZAG, PPL and PAB. In a specific embodiment of the invention, the *ABM* forms part of protein G, such as for example protein G from *Streptococcus* strain G148. In different variants of this embodiment, the three-helix bundle protein domain of which the *ABM* forms a part is selected from the group consisting of domain GA1, domain GA2 and domain GA3 of protein G from *Streptococcus* strain G148, in particular domain GA3.

In alternative embodiments, the *ABM* forms part of one or more of the five three-helix domains of the bacterial receptor protein protein A from *Staphylococcus aureus*; i.e. the three-helix bundle protein domain is selected from the group consisting of protein A domains A, B, C, D and E. In other similar embodiments, the *ABM* forms part of protein Z, derived from domain B of protein A from *Staphylococcus aureus*.

In embodiments of the present invention wherein the *ABM* "forms part of" a three-helix bundle protein domain, this is understood to mean that the sequence of the *ABM* is "inserted" into or "grafted" onto the sequence of the naturally occurring (or otherwise original) three-helix bundle domain, such that the *ABM* replaces a similar structural motif in the original domain. For example, without wishing to be bound by theory, the *ABM* is thought to

constitute two of the three helices of a three-helix bundle, and can therefore replace such a two-helix motif within any three-helix bundle. As the skilled person will realize, the replacement of two helices of the three-helix bundle domain by the two *ABM* helices has to be performed so as not to affect the basic structure of the polypeptide. That is, the overall folding of the Cα backbone of the polypeptide according to this embodiment of the invention will be substantially the same as that of the three-helix bundle protein domain of which it forms a part, e.g. having the same elements of secondary structure in the same order etc. Thus, an *ABM* according to the invention "forms part" of a three-helix bundle domain if the polypeptide according to this embodiment of the invention has the same fold as the original domain, implying that the basic structural properties are shared, those properties e.g. resulting in similar CD spectra. The skilled person is aware of other parameters that are relevant.

In one embodiment of the invention, the albumin binding polypeptide is a three-helix bundle protein domain, which comprises the albumin binding motif as defined above and additional sequences making up the remainder of the three-helix configuration. Thus, the invention provides an albumin binding polypeptide, which comprises the amino acid sequence:

$$LAEAKX_aX_bAX_cX_d\ ELX_eKY\text{-}[ABM]\text{-}LAALP$$

wherein

[*ABM*] is an albumin binding motif as defined above,

and, independently of each other,

X_a is selected from V and E;
X_b is selected from L, E and D;
X_c is selected from N, L and I;
X_d is selected from R and K; and
X_e is selected from D and K.

In one embodiment of this polypeptide, X_a is V.
In one embodiment of this polypeptide, X_b is L.
In one embodiment of this polypeptide, X_c is N.
In one embodiment of this polypeptide, X_d is R.
In one embodiment of this polypeptide, X_e is D.

Again, as described in detail in the experimental section to follow, the selection and sequencing of a number of albumin binding variants led to the identification of individual albumin binding polypeptide sequences. These sequences constitute individual embodiments of the albumin binding polypeptide according to the above embodiment of the first aspect of the present invention. The sequences of these individual albumin binding polypeptides are presented in Figure 1 and as SEQ ID NO:257-514. Also encompassed by the present invention is an albumin binding polypeptide having an amino acid sequence with 85 % or greater identity to a sequence selected from SEQ ID NO:257-514. In particular embodiments, the sequence of the albumin binding polypeptide is selected from SEQ ID NO:247, SEQ ID NO:248, SEQ ID NO:254, SEQ ID NO:260, SEQ ID NO:270, SEQ ID NO:272, SEQ ID NO:291, SEQ ID NO:294, SEQ ID NO:298, SEQ ID NO:299, SEQ ID NO:300, SEQ ID NO:400, SEQ ID NO:484, SEQ ID NO:485, SEQ ID NO:486, SEQ ID NO:487, SEQ ID NO:488, SEQ ID NO:489 and SEQ ID NO:490 and sequences having 85 % or greater identity thereto. In more specific embodiments of this aspect of the invention, the sequence of the albumin binding polypeptide is selected from SEQ ID NO:248, SEQ ID NO:298 and SEQ ID NO:484 and sequences having 85 % or greater identity thereto.

As is evident from the above, in addition to a polypeptide whose amino acid sequence is selected from SEQ ID NO:257-514 or a subset thereof, the present invention also encompasses variants thereof. The amino acid sequences of such encompassed variants exhibit small differences only in comparison with SEQ ID NO:257-514. One definition of such variants is given above, i.e. an albumin binding polypeptide with an amino acid sequence having at least 85 % identity to a sequence selected from SEQ ID NO:257-514. In some embodiments, the inventive polypeptide may have a sequence which is at least 86 %, at least 87 %, at least 88 %, at least 89 %, at least 90 %, at least 91 %, at least 92 %, at least 93 %, at least 94 %, at least 95 %, at least 96 %, at least 97 %, at least 98 % or at least 99 % identical to the sequence selected from SEQ ID NO:257-514. The comparison may be performed over a window corresponding to the shortest of the sequences being compared, or over a window corresponding to an albumin binding motif in at least one of the sequences being compared.

The terms “albumin binding” and ”binding affinity for albumin” as used in this specification refer to a property of a polypeptide which may be tested for example by the use of surface plasmon resonance technology, such as in

a Biacore instrument. For example as described in the examples below, albumin binding affinity may be tested in an experiment in which albumin, or a fragment thereof, is immobilized on a sensor chip of the instrument, and the sample containing the polypeptide to be tested is passed over the chip. Alternatively, the polypeptide to be tested is immobilized on a sensor chip of the instrument, and a sample containing albumin, or a fragment thereof, is passed over the chip. Albumin may, in this regard, be a serum albumin from a mammal, such as human serum albumin. 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 polypeptide for albumin. If a qualitative measure is desired, for example to determine a K_D value for the interaction, surface plasmon resonance methods may also be used. Binding values may for example be defined in a Biacore2000 instrument (Biacore AB). Albumin is suitably immobilized on a sensor chip of the measurement, and samples of the polypeptide whose affinity is to be determined are prepared by serial dilution and injected in random order. K_D values may then be calculated from the results using for example the 1:1 Langmuir binding model of the BIAevaluation 4.1 software provided by the instrument manufacturer (Biacore AB).

The albumin binding polypeptide according to this first aspect of the present invention binds to albumin such that the K_D value of the interaction is at most 1×10^{-9} M, i.e. 1 nM. In some embodiments, the K_D value of the interaction is at most 1×10^{-10} M, such as at most 1×10^{-11} M, for example at most 1×10^{-12} M.

In one embodiment of the invention, the albumin to which the albumin binding polypeptide binds is human serum albumin.

The invention also encompasses an albumin binding polypeptide as described above, which further comprises one or more additional amino acid(s) positioned on one or both sides of the albumin binding motif. These additional amino acid residues may play a role in enhancing the binding of albumin by the polypeptide, but may equally well serve other purposes, related for example to one or more of the production, purification, stabilization *in vivo* or *in vitro*, coupling or detection of the polypeptide, as well as any combination thereof. Such additional amino acid residues may comprise one or more amino acid residue(s) added for purposes of chemical coupling, e.g. to a chromatographic resin to obtain an affinity matrix or to a chelating moiety for complexing with a metal radionuclide. An example of this is the addition of

a cysteine residue at the very first or very last position in the polypeptide chain, i.e. at the N or C terminus. Such additional amino acid residues may also comprise a ”tag” for purification or detection of the polypeptide, such as a hexahistidyl (His_6) tag, or a ”myc” (“c-Myc”) tag or a ”FLAG” tag for interaction with antibodies specific to the tag. The skilled person is aware of other alternatives.

The ”additional amino acid residues” discussed above may also constitute one or more polypeptide domain(s) with any desired function, such as the same binding function as the first, albumin binding domain, or another binding function, or a therapeutic function, or an enzymatic function, or a fluorescent function, or mixtures thereof. Linked polypeptide ”units” in a such a polypeptide according to the invention may be connected by covalent coupling using known organic chemistry methods, or expressed as one or more fusion polypeptides in a system for recombinant expression of polypeptides, or joined in any other fashion, directly or mediated by a linker comprising a number of amino acids.

Furthermore, this aspect of the invention also encompasses fragments of albumin binding polypeptides that retain albumin binding. The possibility of creating fragments of a wild-type three-helix domain with retained binding specificity was shown by Braisted AC *et al* in Proc Natl Acad Sci USA 93:5688-5692 (1996). In the experiments described in that paper, using a structure-based design and phage display methods, the binding domain of a three-helix bundle of 59 residues was reduced to a resulting two-helix derivative of 33 residues. This was achieved by stepwise selection of random mutations from different regions, which caused the stability and binding affinity to be iteratively improved. Following the same reasoning, with the polypeptides of the present invention, the skilled addressee will be able to obtain a ”minimized” albumin binding polypeptide with the same binding properties as that of the ”parent” albumin binding polypeptide. Thus, a polypeptide constituting a fragment of a polypeptide according to the invention and substantially retaining albumin binding is within the scope of the invention. As a non-limiting example, the fragment may correspond to an albumin binding polypeptide according to the description above which has been N-terminally truncated. Such a truncation may for example be by from 1 to 3 amino acids.

As outlined above, the invention also encompasses multimers of the polypeptide with affinity for albumin, i.e. polypeptide chains comprising at

least two albumin binding polypeptides or fragments thereof as monomer units. It may be of interest, e.g. in a method of purification of albumin or in a therapeutic method exploiting the albumin binding function, to obtain even stronger binding of albumin than is possible with one polypeptide according to the invention. In this case, the provision of a multimer, such as a dimer, trimer or tetramer, of the polypeptide may provide the necessary avidity effects. The multimer may consist of a suitable number of polypeptides according to the invention. These polypeptide domains according to the invention, forming monomers in such a multimer, may all have the same amino acid sequence, but it is equally possible that they have different amino acid sequences. As described above, the linked polypeptide "units" in a multimer according to the invention may be connected by covalent coupling using known organic chemistry methods, or expressed as one or more fusion polypeptides in a system for recombinant expression of polypeptides, or joined in any other fashion, directly or mediated by a linker comprising a number of amino acids.

Additionally, "heterogenic" fusion polypeptides or proteins, or conjugates, in which an albumin binding polypeptide according to the invention, or fragment or multimer thereof, constitutes a first domain, or first moiety, and the second and further moieties have other functions than binding albumin, are also contemplated and fall within the ambit of the present invention. The second and further moiety/moieties of the fusion polypeptide or conjugate in such a protein suitably has a desired biological activity. Non-limiting examples of such a desired biological activity comprise a therapeutic activity, a binding activity, and an enzymatic activity. In some embodiments of this aspect of the invention, the second moiety and any further moieties are selected from the group consisting of GLP-1 (glucagon-like peptide 1); HGH (human growth hormone); G-CSF (granulocyte colony-stimulating factor); IL-1 receptor agonist (interleukin 1 receptor agonist); TNF-α (tumor necrosis factor alpha); and blood clotting factors VII, VIII, IX and X. In other embodiments, said second and any further moieties are selected from binding moieties capable of selective interaction (binding) with a target molecule, typically a target molecule other than albumin even though albumin is not excluded. Such a binding moiety is suitably selected from the group consisting of antibodies and fragments and domains thereof substantially retaining antibody binding activity; microbodies, maxybodies, avimers and other small disulfide-bonded proteins; and binding proteins derived from a scaffold selected from the group consisting of staphylococcal protein A and domains

thereof, lipocalins, ankyrin repeat domains, cellulose binding domains, γ crystallines, green fluorescent protein, human cytotoxic T lymphocyte-associated antigen 4, protease inhibitors, PDZ domains, peptide aptamers, staphylococcal nuclease, tendamistats, fibronectin type III domain, zinc fingers, conotoxins, and Kunitz domains. In some embodiments of the invention, the target molecule for binding of said target binding moiety is selected from the group consisting of Aß peptide; other disease-associated amyloid peptides; toxins, such as bacterial toxins and snake venoms; blood clotting factors, such as von Willebrand factor; interleukins, such as IL-13; myostatin; pro-inflammatory factors, such as TNF-α, TNF-α receptor and IL-8; complement factors, such as C3a and C5a; hypersensitivity mediators, such as histamine and IgE; tumor-related antigens, such as CD19, CD20, CD22, CD30, CD33, CD40, CD52, CD70, cMet, HER1, HER2, HER3, HER4, CA9, CEA, IL-2 receptor, MUC1, PSMA, TAG-72.

Other possibilities for the creation of fusion polypeptides or conjugates are also contemplated. Thus, an albumin binding polypeptide according to the first aspect of the invention may be covalently coupled to a second or further moiety or moieties, which in addition to or instead of target binding exhibit other functions. One example is a fusion between one or more albumin binding polypeptide(s) and an enzymatically active polypeptide serving as a reporter or effector moiety. Examples of reporter enzymes, which may be coupled to the albumin binding polypeptide to form a fusion protein, are known to the skilled person and include enzymes such as β-galactosidase, alkaline phosphatase, horseradish peroxidase, carboxypeptidase. Other options for the second and further moiety or moieties of a fusion polypeptide or conjugate according to the invention include, also without limitation, fluorescent polypeptides, such as green fluorescent protein, red fluorescent protein, luciferase and variants thereof.

With regard to the description above of fusion proteins or conjugates incorporating an albumin binding polypeptide according to the invention, it is to be noted that the designation of first, second and further moieties is made for clarity reasons to distinguish between albumin binding polypeptide or polypeptides according to the invention on the one hand, and moieties exhibiting other functions on the other hand. These designations are not intended to refer to the actual order of the different domains in the polypeptide chain of the fusion protein or conjugate. Thus, for example, said first moiety

may without restriction appear at the N-terminal end, in the middle, or at the C-terminal end of the fusion protein or conjugate.

The invention also encompasses polypeptides in which an albumin binding polypeptide as described above has been provided with a label, such as selected from the group consisting of fluorescent dyes and metals, chromophoric dyes, chemiluminescent compounds and bioluminescent proteins, enzymes, radionuclides and particles, for example for purposes of detection of the polypeptide *in vitro* or *in vivo*.

Related aspects of the present invention provide a polynucleotide encoding a polypeptide as described above, as well as an expression vector comprising the polynucleotide and a host cell comprising the expression vector. The latter three aspects of the invention are tools for the production of a polypeptide according to the invention, and the skilled person will be able to obtain them and put them into practical use without undue burden, given the information herein concerning the polypeptide that is to be expressed and given the current level of skill in the art of recombinant expression of proteins. Thus, other related aspects of the invention are methods of producing a polypeptide according to the first aspect of the invention, comprising expressing a polynucleotide as herein described, for example via the culturing of a host cell as herein defined under conditions permitting expression of the polypeptide from the expression vector, and isolating the polypeptide.

As described in the background section and as is well known to the person skilled in the art, the possible applications of a polypeptide molecule with a binding affinity for albumin are several. The albumin binding polypeptide, as well as a fragment, multimer and fusion protein or conjugate thereof, of the invention may find use in any one or more of these applications.

As a non-limiting example of applications of the albumin binding polypeptides described above, the present invention provides, in another of its aspects, the use of a fusion protein or conjugate of an albumin binding polypeptide according to the first aspect of the invention with a polypeptide having a desired biological activity (as defined above) for the preparation of a medicament which exhibits a half-life *in vivo* which is longer than the half-life *in vivo* of the polypeptide having a desired biological activity *per se*. Alternatively speaking, the invention provides a method for prolonging the half-life *in vivo* of a polypeptide having a desired biological activity, through the fusion or conjugation of such a polypeptide to an albumin binding

polypeptide according to the first aspect of the invention. For details of this application of albumin binding molecules, reference is made e.g. to the teachings of the PCT applications published as WO91/01743 and WO01/45746.

As another non-limiting example of applications, the present invention provides, in another of its aspects, the use of a fusion protein or conjugate of an albumin binding polypeptide according to the first aspect of the invention with a polypeptide having a desired biological activity (as defined above) for the preparation of a medicament which elicits no or a reduced immune response upon administration to the mammal, as compared to the immune response elicited upon administration to the mammal of the polypeptide having a desired biological activity *per se*. Alternatively speaking, the invention provides a method for decreasing the immunogenicity of a polypeptide having a desired biological activity, through the fusion or conjugation of such a polypeptide to an albumin binding polypeptide according to the first aspect of the invention. For details of this application of albumin binding molecules, reference is made to the teachings of the PCT application published as WO2005/097202.

Another set of aspects of the present invention concern the provision of new means to increase the solubility in water of a poorly soluble compound, through coupling thereof to an albumin binding polypeptide. The ensuing complex of poorly soluble compound and albumin binding polypeptide is able to associate with albumin *in vivo* or *in vitro*, which association increases the solubility in water. Examples of compounds whose solubility in water may be thus increased through use of the present invention may typically include poorly soluble cytotoxic agents useful for cancer chemotherapy. Using this approach, e.g. in the formulation of drug compositions, enables lyophilization of the resulting preparation, which may then subsequently be reconstituted in aqueous solution. Also, the invention in these aspects provides preparations that have a reduced tendency for aggregation as compared to the compound *per se*.

Thus, yet another aspect of the present invention provides a composition comprising

a compound which *per se* has a solubility in water of no more than 100 µg/ml; coupled to

an albumin binding polypeptide, which has an affinity for albumin such that the K_D of the interaction is no more than 1 x 10^{-6} M.

In one embodiment, the compound *per se* has a solubility in water of no more than 10 µg/ml, such as no more than 1 µg/ml.

In one embodiment, the albumin binding polypeptide has an affinity for albumin such that the K_D of the interaction is no more than 1 x 10^{-7} M, such as no more than 1 x 10^{-8} M, for example no more than 1 x 10^{-9} M, such as no more than 1 x 10^{-10} M, such as no more than 1 x 10^{-11} M, for example no more than 1 x 10^{-12} M.

In some embodiments, the compound may be a pharmaceutically active compound, for example a cytotoxic agent. Non-limiting examples of cytotoxic agents are those selected from calicheamycin, auristatin, doxorubicin, maytansinoid, taxol, ecteinascidin, geldanamycin and their derivatives, and combinations thereof. Alternatively, the cytotoxic agent may be a synthetic chemotoxin not derived from a naturally occurring compound.

The compound and albumin binding polypeptide may be non-covalently associated, but it is currently preferred that they be covalently coupled together.

The composition according to this aspect of the present invention comprises an albumin binding polypeptide. In one embodiment, the albumin binding polypeptide is a naturally occurring polypeptide or an albumin binding fragment or derivative thereof. The albumin binding polypeptide may, as non-limiting examples, be selected from the group consisting of albumin binding proteins M1/Emm1, M3/Emm3, M12/Emm12, EmmL55/Emm55, Emm49/EmmL49, H, G, MAG, ZAG, PPL and PAB. In a more specific embodiment, the albumin binding polypeptide is streptococcal protein G or an albumin binding fragment or derivative thereof. In an even more specific embodiment, the polypeptide capable of binding to albumin is selected from the group consisting of domain GA1, domain GA2 and domain GA3 of protein G from *Streptococcus* strain G148, and may thus, for example, be the GA3 domain.

In one embodiment, the albumin binding polypeptide comprises from about 5 to about 214 amino acid residues, such as from about 5 to about 46 amino acid residues, for example from about 10 to about 20 amino acid residues.

In another embodiment of this aspect of the present invention, the albumin binding polypeptide comprises an amino acid sequence selected from DICLPRWGCLW, DLCLRDWGCLW and DICLARWGCLW.

In yet another embodiment of these aspects of the present invention, the albumin binding polypeptide comprises any albumin binding polypeptide according to the first aspect of the invention discussed extensively above, i.e. that aspect of the present invention which defines a class of novel albumin binding polypeptides via the sequence of the albumin binding motif thereof.

In another embodiment of these aspects of the present invention, the albumin binding polypeptide is capable of interacting with at least one of, and preferably all of, residues F228, A229, A322, V325, F326 and M329 in human serum albumin so as to enhance binding of the molecule to albumin. For example, the albumin binding polypeptide includes an amino acid residue which forms an interaction with the M329 residue in human serum albumin so as to enhance binding of the molecule to albumin. In addition, or alternatively, the albumin binding polypeptide may include an amino acid residue which forms an interaction with helix 7 in the human serum albumin domain IIB so as to enhance binding of the molecule to albumin. In addition, or alternatively, the albumin binding polypeptide includes an amino acid residue which forms an interaction with residues in human serum albumin domain IIA so as to enhance binding of the molecule to albumin. In addition, or alternatively, the albumin binding polypeptide includes an amino acid residue which forms an interaction with residues between helices 2 and 3 of human serum albumin so as to enhance binding of the molecule to albumin.

In addition to the poorly soluble compound and albumin binding polypeptide, the composition according to this aspect of the invention may, in some embodiments, also comprise a binding polypeptide with an affinity for a clinically relevant target. This binding polypeptide is suitably different from the albumin binding polypeptide, and may be non-covalently or covalently coupled to the other components of the inventive composition. As non-limiting examples, the binding polypeptide with an affinity for a clinically relevant target may be selected from the group consisting of antibodies and fragments and domains thereof substantially retaining antibody binding activity; microbodies, maxybodies, avimers and other small disulfide-bonded proteins; and binding proteins derived from a scaffold selected from the group consisting of staphylococcal protein A and domains thereof, lipocalins, ankyrin repeat domains, cellulose binding domains, γ crystallines, green

fluorescent protein, human cytotoxic T lymphocyte-associated antigen 4, protease inhibitors, PDZ domains, peptide aptamers, staphylococcal nuclease, tendamistats, fibronectin type III domain, zinc fingers, conotoxins, and Kunitz domains.

The composition according to the above aspect of the present invention has an ability to associate with albumin *in vivo* or *in vitro*, through the provision in the composition of an albumin binding polypeptide. In certain cases, it may be of benefit to form a complex of the composition with albumin outside of a living organism, i.e. to add exogenous albumin to the composition. Thus, the present invention also provides a composition as defined above which further comprises albumin, such as human serum albumin.

The present invention also provides the composition according to the above aspect for use as a medicament, in cases where the compound is a therapeutically active compound. Suitably, the provision of an albumin binding polypeptide and optionally albumin does not deleteriously affect the therapeutic efficacy of the active compound, so the inventive composition will be useful in those therapeutic or prophylactic settings where the compound *per se* is indicated.

A related aspect of the present invention provides a method of preparation of a composition as described immediately above. The method comprises

providing a compound which *per se* has a solubility in water of no more than 100 µg/ml; and

covalently coupling the compound to an albumin binding polypeptide, which has an affinity for albumin such that the K_D of the interaction is no more than 1×10^{-6} M, thus forming a covalent complex of compound and albumin binding polypeptide.

In embodiments of the invention where albumin is included into the composition, the method may beneficially comprise the additional step of mixing said complex of compound and albumin binding polypeptide with albumin, thus forming a composition comprising a non-covalent complex of i) the covalent complex of compound and albumin binding polypeptide and ii) albumin. The relative proportions of the two components of this non-covalent complex may for example be 1:1, so that one unit of the complex of poorly soluble compound and albumin binding polypeptide is associated with one

molecule of albumin. In one embodiment, the method additionally comprises lyophilizing the non-covalent complex to obtain a lyophilized composition.

In another closely related aspect, the present invention provides a method of increasing the aqueous solubility of a compound, comprising

providing a compound which *per se* has a solubility in water of no more than 100 µg/ml M;

covalently coupling the compound to an albumin binding polypeptide, which has an affinity for albumin such that the K_D of the interaction is no more than 1 x 10^{-6} M, thus forming a covalent complex of compound and albumin binding polypeptide; and

mixing said complex of compound and albumin binding polypeptide with albumin under conditions that promote the non-covalent association of the albumin binding polypeptide with albumin;

whereby the solubility in water of the compound in said complex is greater than the solubility in water of the compound *per se*.

In these method aspects concerning the solubility of a poorly soluble compound, the optional features of the various components are as described in connection with the immediately preceding composition aspect.

As described above, embodiments of these aspects of the present invention relate *inter alia* to the combination of a targeting polypeptide with an albumin-binding polypeptide, conjugation of this molecule with e.g. a chemotoxin, and the formulation and administration of the resulting chemotoxin conjugate with albumin to avoid problems with low solubility.

Chemotoxins are generally hydrophobic compounds. Therefore, poor solubility is one of the challenges with handling and formulating chemotoxin conjugates, including antibodies conjugated with chemotoxins. The problem is accentuated when trying to couple clusters of toxin molecules to one carrier protein. In contrast, a chemotoxin conjugated albumin-binding fusion protein in complex with a molecule of albumin has a superior solubility stemming from the solubilizing properties of albumin, as reflected by its role as a carrier of many small molecules in plasma. One aspect of these embodiments of the invention is a strong association between the albumin-binding domain and albumin to prevent other interactions that could result in precipitation of the non-associated albumin-binding protein conjugate.

A slow extravasation of monoclonal antibodies from blood has been raised as one of the biological barriers that limit the efficacy of antibody mediated therapy (Wu and Senter, Nature Biotechnology 23:1137-46, 2005).

Interestingly, at equilibrium, approximately 60 % of the serum albumin in a human being is found in the interstitial space, whereas only 40 % is found in the blood stream. Thus, the association with albumin as provided by the present invention is considered a superior means of obtaining a wide distribution outside of the blood stream. The affinity of the association with serum albumin is suitably characterized by an off-rate (decomposition of the complex) that is sufficiently slow, such that only a minute fraction of the complex dissociates during transition from the blood stream to the interstitium. However, the interaction does not have to be covalent, since some rebinding is possible during the transition.

One possible contributing mechanism for extravasation and wide distribution is active transport following binding of serum albumin to the FcRn receptor. Consequently, there are certain requirements on the albumin binding moiety in an albumin binding fusion protein to obtain a similar distribution. For example, the affinity may be very tight also in the acidic environment encountered during receptor transport in the cell, probably down to a pH below 6.

The present invention as claimed relates to:

- albumin binding polypeptide comprising an albumin binding motif, which motif consists of the amino acid residues according to the sequence: $GVSDX_5YKX_8X_9I$ $X_{11}X_{12}AX_{14}TVEGVX_{20}$ $ALX_{23}X_{24}X_{25}I$ wherein, independently of each other, X_5 is selected from Y and F; X_8 is selected from N, R and S; X_9 is selected from V, I, L, M, F and Y; X_{11} is selected from N, S, E and D; X_{12} is selected from R, K and N; X_{14} is selected from K and R; X_{20} is selected from D, N, Q, E, H, S, R and K; X_{23} is selected from K, I and T; X_{24} is selected from A, S, T, G, H, L and D; and X_{25} is selected from H, E and D; with the proviso that the amino acid sequence is not GVSDYYKNLI NNAKTVEGVK ALIDEI; the albumin binding polypeptide binding to albumin such that the KD value of the interaction is at most 1×10^{-9} M;

- albumin binding polypeptide, whose amino acid sequence comprises a sequence selected from SEQ ID NOs:258-513 and 514;

- multimer of albumin binding polypeptides as described herein, comprising at least two albumin binding polypeptides as monomer units; and

- fusion protein or conjugate comprising i) a first moiety consisting of an albumin binding polypeptide as described herein or multimer as described herein; and ii) a second moiety consisting of a second polypeptide having a desired biological activity.

Brief description of the figures

Figure 1 is a listing of the amino acid sequences of examples of albumin binding motifs comprised in albumin binding polypeptides of the invention (SEQ ID NO:1-257), examples of albumin binding polypeptides according to the invention (SEQ ID NO:257-514), and the GA3 domain from protein G of *Streptococcus* strain G148 (SEQ ID NO:515).

Figure 2 is an illustration of the main features of the coding insert in expression vector pAY1075 without (A) and with (B) a cassette encoding helices 2 and 3 of variant ABD molecules.

Figure 3A shows the strategy for amplification of DNA fragments encoding dummy, Zwt and GIII in the preparation of the coding insert of expression vector pAY1075. Figure 3B shows the overlap of these fragments for the creation of the entire coding insert.

Figure 4 is a vector map of the expression vector pAY1075, prepared as described in Example 1.

Figure 5 is a vector map of the expression vector pAY1075-ABD, prepared as described in Example 1.

Figure 6 is a table showing the theoretical (shaded columns) and experimental (clear columns) values for the amino acid variation at each

varied position in the ABD variant sub-library created using the AFFI-793 mixture of oligonucleotides, as described in Example 1.

Figure 7 is a table showing the theoretical (shaded columns) and experimental (clear columns) values for the amino acid variation at each varied position in the ABD variant sub-library created using the AFFI-794 mixture of oligonucleotides, as described in Example 1.

Figure 8 is a schematic illustration of the amino acid sequence of an ABD variant as expressed in the pAY442 vector according to the description in Example 2.

Figures 9A-9C show ELISA titration curves for serum obtained at days 0-45 from primates injected with Z00342 as described in Example 4, when analyzed on ELISA plates coated with Z00342.

Figures 10A-10C show ELISA titration curves for serum obtained at days 0-45 from primates injected with Z00342-ABD00003 as described in Example 4, when analyzed on ELISA plates coated with Z00342-ABD00003.

Figure 11 shows the median concentration of IgG specific for Z variants in serum obtained at days 0-45 from primates injected with Z00342 and Z00342-ABD00003 as described in Example 4.

Figure 12 shows the amount of A) Z00342 and B) Z00342-ABD00003 in blood circulation over time as analyzed by sandwich ELISA as described in Example 4.

Figures 13A-13B show ELISA titration curves for serum obtained at days 0-45 from primates injected with $(Z01154)_2$ as described in Example 5, when analyzed on ELISA plates coated with $(Z01154)_2$.

Figures 14A-14B show ELISA titration curves for serum obtained at days 0-45 from primates injected with $(Z01154)_2$-ABD00239 as described in Example 5, when analyzed on ELISA plates coated with $(Z01154)_2$-ABD00239.

Figure 15 shows normalized values of the A) $(Z01154)_2$ and B) $(Z01154)_2$-ABD00239 samples analyzed in Figures 13 and 14 respectively. The sample absorbances were normalized against the positive control at 1600x dilution.

The invention will now be illustrated further through the non-limiting description of experiments conducted in accordance therewith. Unless otherwise specified, conventional chemistry and molecular biology methods were used throughout.

Examples

Example 1

Construction of phage display library of variants of an albumin binding polypeptide

Summary

In this example, a phage display library of polypeptide variants was created, through variation of 16 positions in helices 2 and 3 of the albumin binding domain GA3 of *Streptococcus* strain G148 (in the following referred to as "ABD"). The wildtype sequence of ABD ("ABDwt") is provided as SEQ ID NO:491 in Figure 1 and in the appended sequence listing. A new phage display vector (pAY1075) based on the previously described pAffi1 vector (Grönwall *et al*, J Biotechnol 128:162-183, 2007) was constructed for this new library. The varied ABD fragment (helices 2-3) was cloned into pAY1075 with restriction enzymes *Sac*I and *Nhe*I. Ligations were purified and electroporated to *E. coli* RR1ΔM15 cells (Rüther, Nucleic Acids Res 10:5765–5772, 1982). The newly constructed library was designated LibABDmat2005 and consisted of two sub-libraries, depending on which oligonucleotides had been used for creation of the varied sequence of helices 2 and 3. One was built on the ABD molecule and the other had an extra amino acid inserted between positions 17 and 18 of ABD, which some of the proteins homologous to ABD have (see e.g. Rozak *et al*, Biochemistry 45:3263-3271, 2006). The size of LibABDmat2005 was 1 x 10^9 members (5 x 10^8 for each sub-library). The quality of the new library was satisfying, in that DNA sequencing showed that about 87 % of the clones were functional and in that the measured values of relative frequencies of amino acids agreed well with the theoretical values.

Construction of phagemide vector pAY1075

A new phage display vector (pAY1075) was constructed for the new library. pAY1075 was based on the phagemid vector pAffi1 (Grönwall *et al*, *supra*). For creation of pAY1075, pAffi1 was digested with *Xho*I and *Xma*I (10 units/µl; New England Biolabs), and a new insert or cloning cassette was created and ligated into the vector. The new insert contained DNA encoding helix 1 of ABDwt, a dummy sequence, a thrombin site, Zwt (an engineered IgG binding domain based on domain B of staphylococcal protein A, see

Nilsson *et al*, Prot Eng 1:107-113, 1987), truncated GIII (residues 249-406), the termination domain TT and some additional restriction enzyme sites. For a schematic representation of the elements encoded by this insert, see Figure 2A. Figure 2B shows the insert of the expression vector when the dummy sequence has been replaced by a sequence encoding the remaining ABD variant polypeptide (see below). The sequences of the various DNA oligonucleotides used as primers and templates in the cloning experiment and library construction are provided below in Table 1.

Table 1: Oligonucleotide primers and templates

Oligo	Sequence
AFFI-21	5'-tgcttccggctcgtatgttgtgtg-3'
AFFI-22	5'-cggaaccagagccaccaccgg-3'
AFFI-40	5'-tccccccgggttaagactccttattacgcag-3'
AFFI-72	5'-*biotin*-cggaaccagagccaccaccgg-3'
AFFI-772	5'-gaagccctcgagttagctgaagctaaag-3'
AFFI-773	5'-gttagctgaagctaaagtcttagctaacagagagctctgaaagcttggcttatgc-3'
AFFI-774	5'-cgcgcggaaagctagccaaacttcggatag-3'
AFFI-775	5'-ctagctttccgcgcgtagacaacaaattcaac-3'
AFFI-776	5'-ccggactatacgtatttcggcgcctgagc-3'
AFFI-777	5'-gaaatacgtatagtccggtggtggctc-3'
AFFI-791	5'-acagagagctcgacaaatatggag-3'
AFFI-792	5'-cggaaagctagcaggtaatgcagc-3'

In order to create the new cloning cassette for pAY1075, the dummy fragment and GIII were PCR amplified from pAffi1, and Zwt was amplified from plasmid pEZZ18 (Löwenadler *et al*, Gene 58:87-97, 1987), using primers according to Figure 3A. The newly generated fragments had overlapping segments to each other, as shown in Figure 3B. The PCR fragments were gel purified with QIAquick gel extraction kit (Qiagen) according to the manufacturer's recommendations and thereafter assembled together with the oligonucleotide AFFI-772 (Figure 3B) in an assembly PCR. A further PCR reaction using external primers AFFI-772 and AFFI-40 was performed to amplify the entire fragment. PCR products were purified using QIAquick PCR purification kit (Qiagen) according to the manufacturer's recommendations.

The plasmid pAffi1 was purified with QIAgen™ midi-prep kit (Qiagen), according to the manufacturer's recommendations. Thereafter, pAffi1 and the

amplified PCR fragment for the cloning cassette were digested with *Xho*I and *Xma*I (10 units/µl; New England Biolabs) in NEB4 buffer (20 mM Tris acetate, 10 mM magnesium acetate, 50 mM potassium acetate,1 mM dithiothreitol, pH 7.9; New England Biolabs) for 1 h at 37 °C and the vector was thereafter dephosphorylated using calf intestinal alkaline phosphatase (CIAP; Fermentas). The digestions were purified on a 1 % agarose gel using QIAquick gel extraction kit according to the manufacturer's recommendations. The new fragment was ligated into *Xho*I and *Xma*I cleaved pAffi1 for 1 h at room temperature using T4 DNA ligase (5 units/µl; Fermentas). Part of the ligation mixture was electroporated into *E. coli* TG1 cells (Stratagene) using 1 mm cuvettes. The cells were plated on tryptose blood agar base plates (TBAB plates; 30 g/l TBAB) supplemented with 200 µg/ml ampicillin. Clones having a correct insert were identified by PCR, using the three different primer pairs AFFI-21/AFFI-42, AFFI-47/AFFI-40 and AFFI-21/AFFI-40. PCR fragments were analyzed on a 1 % agarose gel, and positive clones were plasmid purified with QIAprep Miniprep kit (Qiagen), according to the manufacturer's recommendations. and thereafter sequenced with primers AFFI-38, 40, 71, 72, and 772 using ABI PRISM® BigDye™ Terminator Cycle Sequencing Ready Reaction kit 3.1 (Applied Biosystems). The sequence PCR reactions were purified on a Magnatrix 8000 instrument (Magnetic Biosolutions) and the nucleotide sequence was determined with an ABI PRISM® 3100 Genetic analyzer (Applied Biosystems). Sequencer™ v 4.0.5 (Gene Codes Corporation) was used to record and analyze the sequence data. Sequencing revealed that the new phagemid vector had been successfully created. Vector maps of the vector with dummy sequence (pAY1075) or with sequence encoding varied ABD helices 2 and 3 (pAY1075-ABD) are shown in Figure 4 and 5, respectively.

Design of a library of variant ABD sequences

A set of oligonucleotides having a randomized sequence for helices 2 and 3 of the ABD molecule were prepared as described below. These oligonucleotides were subsequently used for replacement of the dummy sequence in pAY1075, to create pAY1075-ABD (Figures 2B and 5). The pAY1075-ABD vector was subsequently used for the expression of the library of ABD variants on the surface of phages

The design was based on information from alanine mutations of ABD (Linhult *et al*, Protein Science 11:206-213, 2002), a study of the ALB8-HSA

complex (Lejon *et al*, J Biol Chem 279:42924-42928, 2004), sequence homologies with other known albumin binding domains and ease of oligonucleotide preparation. 16 amino acid positions in the ABDwt sequence represented by SEQ ID NO:491 were selected for some degree of randomization, and were grouped in 4 different groups depending on characteristic: (I) the hydrophobic core, (II) conserved positions, (III) electrostatic interactions and (IV) others:

(I) Positions Y20, L24, L27 and I41 are involved in creating the central hydrophobic core in the interaction with serum albumin. These positions are highly conserved among domains homologous to ABD and randomization in these positions tested whether another hydrophobic amino acid residue could improve the hydrophobic interaction.

(II) Positions 18', S18, T30, E32 and G33 are very conserved among the albumin binding domains. Positions S18 and T30 are involved in two intermolecular H-bonds, and the rationale for randomization was that similar polar amino acids like threonine (T) and asparagine (N) could also work. E32 and G33 do not interact with the binding surface to any great extent. However, they are likely to be important for protein structure, and it was of interest to see if another amino acid could work. The sequence of ABDwt does not comprise position 18' (i.e. 18' represents an added amino acid residue between positions 17 and 18 in ABDwt), but homologous domains have threonine or serine at that position. It was of interest to see if binding could be improved with this additional amino acid.

(III) Positions N23, N27, K29 and E40 are involved or could be involved in electrostatic interactions. The randomization at these positions was based on an interest to see whether or not it would be possible to enhance or suppress some of the attractive or repulsive interactions of these amino acid residues with albumin.

(IV) Positions A36, K35 and D39 were randomized due to other similar considerations.

In order to create the desired mix of amino acid residues at each position, the ABDwt sequence was varied in accordance with Table 2. Variations were categorized as "randomized" or "doped". In "randomized" positions, all chosen amino acids were represented in equal proportions. In "doped" positions, the original amino acid was more frequent than the others, i.e. the position was biased towards the original amino acid.

Table 2: Design strategy for variant ABD sequences

Randomized positions				
Position	Desired variation	Codon combinations[1]	# codons	# amino acids
20	F, Y	T(T/A)T	2	2
23	N, S, K, R	A(G/A)(A/C)	4	4
27	N, S, K, R	A(G/A)(A/C)	4	4
33	All except aromatic	(C/A/G)N(G/T)	24	16
35	All	NN(G/T)	32	20
36	S, T, A	(T/A/G)CC	3	3
39	All	NN(G/T)	32	20
40	H, E, D, Q	(G/C)A(G/T)	44	
Doped positions				
Position	Desired variation[2]	Codon combinations[1]	# codons	# amino acids
18'	-, S, T	no codon (50 %) + A(C/G)C	3	3
18	S, T, N	AGT (80 %) + A(A/G/C)C	3	3
24	F, L, I, M, V	CTG/T (70 %) + NT(G/T)	8	5
29	R, K	AAG (90 %) + A(G/A)A	2	2
30	T, S, N	ACT (80 %) + A(A/G/C)C	3	3
32	All (E)	GAG (72 %) + NN(G/T)	32	20
37	F, L, I, M, V	CTG/T (70 %) + NT(G/T)	8	5
41	F, L, I, M, V	ATT (84 %) + NT(G/T)	8	5

[1] N = any nucleotide

[2] "doped" or biased towards the underlined amino acid residue

Oligonucleotide mixtures AFFI-793 and AFFI-794 corresponding to DNA encoding residues 13-46 of the ABDwt sequence as modified according to Table 2 and including restriction sites were obtained from Scandinavian Gene Synthesis AB. AFFI-794 comprises the extra amino acid represented by position 18'.

AFFI-793:

```
                                41 40 39      37 36 35     33 32     30 29     27         24 23
5'-TTGCTAGCAGGTAATGCAGCTAAXXXXXXXXXXTATXXXXXXXXXXTACXXXXXXAACXXXXXXGGCXXXGTTGATXXXXXXCTT
        20     18
   GTAXXXGTCXXXTACTCCATATTTGTCGAG-3'      113 bp
```

AFFI-794:

```
                                41 40 39      37 36 35     33 32     30 29     27         24 23
5'-TTGCTAGCAGGTAATGCAGCTAAXXXXXXXXXXTATXXXXXXXXXXTACXXXXXXAACXXXXXXGGCXXXGTTGATXXXXXXCTT
       20     18 18'
  GTAXXXGTCXXXXXXTACTCCATATTTGTCGAG-3'    116 bp
```

Table 3 summarizes the required percentage distribution of nucleotides in the oligonucleotide mixtures necessary to achieve the library design described in Table 2.

Table 3: Distribution of nucleotides in AFFI-793 and AFFI-794 oligonucleotide mixtures

	Position																							
Nucleotide	18'			18			20			23			24			27			29			30		
	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
T	0	0	100	0	10	100	0	50	0	50	50	100	0	0	10	50	50	100	100	90	100	0	10	100
C	0	50	0	0	80	0	0	0	0	0	50	0	50	0	10	0	50	0	0	10	0	0	10	0
A	0	0	0	0	0	0	100	50	100	0	0	0	50	100	10	0	0	0	0	0	0	0	0	0
G	100	50	0	100	10	0	0	0	0	50	0	0	0	0	70	50	0	0	0	0	0	100	80	0
	32			33			35			36			37			39			40			41		
	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
T	0	79	7	0	25	33	0	25	25	0	0	33	0	0	10	0	25	25	0	100	0	0	0	88
C	90	7	79	50	25	34	50	25	25	0	0	34	50	0	10	50	25	25	50	0	50	10	0	4
A	10	7	7	50	25		50	25	25	0	0	33	50	100	10	50	25	25	50	0	0	90	100	4
G	0	7	7	0	25	33	0	25	25	100	100	0	0	0	70	0	25	25	0	0	50	0	0	4

Library construction

The following procedure was used to create the genetic library LibABDmat2005, encoding ABD variants. In an assembly reaction, the oligonucleotide AFFI-791 and oligonucleotide mixture AFFI-793 or AFFI-794 were annealed and extended with Taq DNA polymerase. A PCR reaction using the external primers AFFI-791 and AFFI-792 was performed to amplify the fragment. PCR products were purified using QIAquick PCR purification kit (Qiagen) according to the manufacturer's recommendations.

Phagemid pAY1075 was prepared from 250 ml overnight culture (tryptic soy broth, 2 % glucose, 100 µg/ml ampicillin) using Qiagen plasmid midi kit (Qiagen) following the manufacturer's recommendations. The phagemid was digested with *Sac*I and *Nhe*I (10 units/µl; New England Biolabs) in NEB4 buffer (20 mM Tris acetate, 10 mM magnesium acetate, 50 mM potassium acetate,1 mM dithiothreitol, pH 7.9; New England Biolabs) for 3 h at 37 °C. The solution was phenol/chloroform purified and EtOH precipitated, and the vector was then gel purified from a 1 % agarose gel using QIAquick gel extraction kit (Qiagen) according to the manufacturer's recommendations.

The PCR amplified fragments from the assembly reactions between AFFI-791 and AFFI-793 or AFFI-794 were digested with *Sac*I and *Nhe*I in

NEB4 buffer for 3 h at 37 °C. The DNA fragments were purified from a 1 % agarose gel using QIAquick gel extraction kit according to the manufacturer's recommendations. The resulting gene fragments encoding two sub-libraries of variants of ABD were ligated into *Sac*I and *Nhe*I cleaved pAY1075 for 1 h at room temperature using T4 DNA ligase (5 units/µl; Fermentas).

The ligations were then phenol/chloroform extracted, EtOH precipitated and resolved in a smaller volume of 10mM Tris. Electrocompetent *E. coli* RR1ΔM15 cells (Rüther, 1982, *supra*) were transformed with 60 aliquots of ligated material of each of the two sub-libraries using 0.2 cm gap size cuvettes in an ECM 630 set (BTX) using the parameters 2.5 kV, 125 Ω and 50 µF. Cells were grown in SOC medium (47 ml TSB+YE (30 g/l tryptic soy broth, 5 g/l yeast extract) supplemented with 1 % glucose, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 10 mM NaCl and 2.5 mM KCl) for 50 minutes and transferred to ten Erlenmayer flasks, each containing 1 l of TSB + YE (30 g/l tryptic soy broth, 5 g/l yeast extract) supplemented with 2 % glucose and 100 µg/ml ampicillin, and grown overnight at 37 °C. The cells were then centrifuged at 6000 *g* and re-suspended in PBS/glycerol solution (PBS: 2.68 mM KCl, 1.47 mM KH_2PO_4, 137 mM NaCl, 8.1 mM Na_2HPO_4, pH 7.4) to a final approximate concentration of 20 % glycerol. The cells were then aliquoted and stored at – 80 °C. The number of cells after electroporation, amplification and transfer to glycerol stocks was titrated on TBAB plates supplemented with 200 µg/ml ampicillin.

The size of each sub-library was 5×10^8, i.e. the total size of the library LibABDmat2005 was 1×10^9. The library was amplified about 50000 times and the glycerol stocks had a density of about 1×10^{11} cells/ml. In this context, the "size" of the library means the total number of members comprised in the library, without any regard to the number of unique variants encoded by the library.

Ninety-six colonies from each of the two sub-libraries were picked for DNA sequencing in order to verify the design and the frequency of clones with a correct reading frame. These randomly picked colonies, cultured from glycerol stocks and originating from each pool of the library were PCR amplified using oligonucleotides AFFI-21 and AFFI-22. Sequencing of the amplified fragments was performed using ABI PRISM® dGTP, BigDye™ Terminator v3.0 Ready Reaction Cycle Sequencing Kit (Applied Biosystems) according to the manufacturer's recommendations and with the biotinylated oligonucleotide AFFI-72. The sequencing reactions were purified by binding

to magnetic, streptavidin-coated beads using a Magnatrix 8000 (Magnetic Biosolutions), and analyzed on ABI PRISM® 3100 Genetic Analyser (Applied Biosystems).

In the sub-library created using AFFI-793, three clones were not readable, eleven were incorrect and seven clones were contaminations from the other sub-library. The amino acid distribution in this sub-library was deducted from the sequencing data, and compared to the theoretical values, and the results are presented in Figure 6.

Regarding the sub-library created using AFFI-794, three clones were not readable and 16 were incorrect. The amino acid distribution in this sub-library was deducted from the sequencing data, and compared to the theoretical values, and the results are presented in Figure 7.

The frequency of each amino acid agreed well with expected value, and about 87 % of the clones had a correct reading frame.

Example 2

Phage display selection and characterization of albumin binding polypeptide variants

Summary

Biotinylated human serum albumin (HSA) was used as target in phage display selections using the library constructed in Example 1. Selections were carried out using a variety of conditions in order to maximize the likelihood of obtaining ABD variants having a high affinity for albumin. After elution of selected phages, the corresponding expressed proteins were tested for affinity to albumin in an ELISA setup. Positive clones were identified and sequenced, and the predicted amino acid sequences of the corresponding polypeptides and their albumin binding motifs were deduced, which yielded a large number of sequences of albumin binding polypeptides according to the invention. The amino acid sequences of deduced albumin binding motifs are listed in Figure 1 and in the sequence listing as SEQ ID NO:1-257, whereas the amino acid sequences of the corresponding full-length ABD variants are listed in Figure 1 and in the sequence listing as SEQ ID NO:258-514.

Biotinylation of human serum albumin

Lyophilized human serum albumin (Sigma, cat. no. A3782-5G) was dissolved in PBS (2.68 mM KCl, 1.47 mM KH_2PO_4, 137 mM NaCl, 8.1 mM Na_2HPO_4, pH 7.4) to a final concentration of 10 mg/ml. EZ-link Sulfo-NHS-LC-Biotin (Pierce, cat. no. 21335) was dissolved in water to a final concentration of 1 mg/ml and a 5 and 10 fold molar excess was added to 500 mg of albumin in a total volume of 0.5 ml. The mixtures were incubated at room temperature for 30 min. Unbound biotin was removed by dialyzing against PBS using a dialysis cassette (Slide-A-Lyser, 10 kDa; Pierce).

Phage display selection

In total, five rounds of selection were carried out, using increasingly stringent conditions. After three initial rounds performed chiefly with a view to establish a suitable selection protocol, the resulting phage stocks were prepared from glycerol stock prepared as in Example 1. Selection was then carried out for two more cycles using the combinations of selection buffer, target concentration and solid support that are listed in Table 4.

Table 4: Selection conditions for HSA selection

	Sample name	Selection buffer	Target conc. (pM)	Beads (µg)
Cycle 4	A	Gelatin	1000	100
	B	Gelatin	200	100
	C	BSA	400	100
	D	BSA	100	100
Cycle 5	A	Gelatin	500	50
	B	Gelatin	50	50
	C	BSA	100	50
	D	BSA	10	50

All tubes and beads used in the selection procedure were pre-blocked in TPBSB (5 %) (0.05 % Tween20, 5 % bovine serum albumin (BSA), 0.02 % Na azide in PBS) or gelatin (0.5 %) for 30 min under gentle agitation at room temperature and subsequently left with no agitation over night at 4 °C.

Selection solutions (1 ml) contained biotinylated human serum albumin, phages, Na azide (0.02 %), Tween 20 (0.05 %) and either BSA (3 %) or gelatin (0.1 %) according to Table 4, and were prepared in PBS. The phages were incubated with biotinylated human serum albumin target at 4 °C during three days for Cycle 4 and during one day for Cycle 5, followed by 1 h incubation under agitation at room temperature. The selection samples were

transferred to blocked streptavidin beads for 15 min under agitation at room temperature. The beads were washed 10 times with 1 ml of selection buffer (i.e. TPBSB (3 %) (0.05 % Tween20, 3 % bovine serum albumin (BSA), 0.02 % Na azide in PBS) or GT (0.1 %) (0.1 % gelatin, 0.1 % Tween 20 and 0.02 % Na azide in PBS)), followed by 10 washes with PBS where the second to last wash lasted for 2 hours. Phages were either eluted with 1000 ml 0.05 M Glycine-HCl, pH 2.2, for 10 min at room temperature, followed by immediate neutralization with 900 ml PBS supplemented with 100 ml 1 M Tris-HCl, pH 8.0, or eluted with 1000 µl trypsin (2 mg/ml) for 30 min at room temperature followed by addition of 1000 µl aprotinin. The eluted phages (¾ of the volume) were used to infect 50 ml log phase *E. coli* RR1ΔM15 cells (Rüther, 1982, *supra*) after each cycle of selection. After 30 min incubation with gentle agitation and 30 min with vigorous agitation at 37 °C, the cells were centrifuged and the pellet was dissolved in a smaller volume and spread on TSB+YE plates (30 g/l tryptic soy broth, 5 g/l yeast extract) and finally incubated over night at 37 °C.

The cycles of selection resulted in a satisfying number of eluted phages.

Phage stock preparation

Cells from plates were re-suspended in TSB medium (30 g/l tryptic soy broth) and the cell concentration was determined by measuring the optical density at 600 nm assuming that OD_{600} = 1 corresponds to 5 x 10^8 cells/ml. Cells were inoculated (approximately 100 times excess of cells compared to eluted phages) in 100 ml TSB+YE medium supplemented with 2 % glucose and 100 mg/ml ampicillin and grown at 37 °C to approximately OD_{600} = 0.5-0.7. Thereafter, 10 ml were transferred to a new flask and infected by 25 times excess of M13K07 helper phage (1 x 10^{12} cfu/ml; New England Biolabs, cat. no. NO315S) and incubated for 30 min with low agitation. Cells were centrifuged at 2000 *g* for 10 min and re-suspended in 100 ml TSB+YE medium supplemented with 100 mM isopropyl β-D-1-thiogalactopyranoside (IPTG), 50 mg/ml kanamycin and 100 mg/ml ampicillin and grown over night at 100 rpm and 25 °C. A portion of the re-suspended cells was stored at –80°C as a glycerol stock.

The induced culture was centrifuged at 2500 *g* for 10 min and phages in the supernatant were precipitated by adding ¼ of the volume of precipitation buffer (PEG/NaCl) and incubated on ice for 1 hour. Precipitated

phages were pelleted by centrifugation at 10000 *g* at 4 °C for 30 min, re-suspended in 20 ml PBS and thereafter the phage solution was filtered through a 0.45 µm filter. The precipitation procedure was repeated and the phages were finally re-suspended in 1 ml PBS.

Selection solution was titrated after the selection together with wash and elution solutions after each round of selection. Phage solutions were diluted in sterile water in a microtiter plate and 100 µl log phase *E. coli* RR1ΔM15 cells were added to each phage dilution. After 20 min incubation at room temperature, 5 µl from each titration were dropped on a TYE plate (15 g agar, 10 g tryptone, 5 g yeast extract, 3 g NaCl supplemented with 2 % glucose and 100 µg/ml ampicillin) and incubated over night at 37 °C. The resulting colonies were counted and the titers (cfu/ml) calculated.

ELISA analysis of albumin binding

Clones from each selection plus ABDwt were expressed and screened for HSA binding activity using an ELISA setup enabling detection of binders having a K_D value of from 10 nM down to low pM against serum albumin. Randomly picked colonies were expressed in 96 deep-well plates by inoculating each colony into 1 ml TSB+YE medium supplemented with 100 mg/ml ampicillin and 1 mM IPTG and grown over night at 37 °C. Cells were pelleted by centrifugation at 3000 *g* for 15 min, re-suspended in 400 µl PBS-T (0.5 % Tween 20 in PBS) and frozen at –80 °C. Frozen samples were thawed in a water bath and cell debris was pelleted at 3700 *g* for 40 min. Supernatants containing ABD variant-Zwt fusion proteins were collected and stored at 4 °C until used in ELISA as follows.

Microtiter wells (Costar) were coated over night at 4 °C with 100 µl of HSA and with the controls rat serum albumin (RSA), human serum albumin (HSA) and mouse serum albumin (MSA) in one well each, at a concentration of 0.4 µg/ml in ELISA coating buffer (0.1 M sodium carbonate, pH 9.5). The wells were blocked with blocking buffer (2 % milk in PBS) for 2 h at room temperature. A volume of 100 µl of the prepared ABD variant-Zwt fusion proteins was added to each well, and the plates were incubated for 1.5 h at room temperature. Biotinylated IgG at a concentration of 0.5 mg/ml in washing buffer (0.5 % Tween 20 in PBS) was added to the wells and incubated for 1.5 h, so that the Zwt moiety of any albumin binding fusion proteins could bind to IgG. Bound complexes were detected with horse radish peroxidase conjugated streptavidin (Dako, cat. no. P0397) diluted 1:5000 in

washing buffer, and incubated for 1 h at room temperature. Developing solution was prepared by mixing an equal volume of TMB substrates A and B (ImmunoPure TMB, Pierce), and 100 µl was added to each well. After 30 min incubation in darkness, 100 µl stop solution (2 M H_2SO_4) was added. The plates were read at A_{450} in an ELISA spectrophotometer (Basic Sunrise, Tecan). Prior to addition of each new reagent, four washes were done with washing buffer.

In total, 372 clones (93 clones from each selection denoted Sample A-D in Table 4) were randomly picked for analysis of their HSA binding activity using the ELISA set-up described above. The majority of the analyzed clones gave a higher signal to HSA as compared to the ABDwt interaction with rat serum albumin, which is a low pico molar binding (70 pM; unpublished results). Based on the result of this experiment, clones were picked for sequencing as described next.

Sequencing of ELISA positive clones

PCR fragments from selected colonies were amplified using oligonucleotides AFFI-69 (5'-gtgagcggataacaattcccctc-3') and AFFI-70 (5'-cagcaaaaaaccctcaagaccc-3'). Sequencing of amplified fragments was performed using ABI PRISM® dGTP, BigDye™ Terminator v3.0 Ready Reaction Cycle Sequencing Kit (Applied Biosystems) according to the manufacturer's recommendations and with the biotinylated oligonucleotide AFFI-202 (5'-*biotin*-gtgagcggataacaattcccctc-3'). The sequencing reactions were purified by binding to magnetic streptavidin-coated beads using a Magnatrix 8000 instrument (Magnetic Biosolutions), and finally analyzed on ABI PRISM® 3100 Genetic Analyser (Applied Biosystems).

The clones exhibiting the highest A_{450} value in the ELISA screening were subjected to sequencing of their ABD variant insert. 257 different identified ABD variants were given the designation ABD#####, wherein ##### is a five-digit unique label for the variant in question. The sequences of these identified ABD variants are listed in Figure 1 as SEQ ID NO:257-514. Based on the existing knowledge of the albumin binding properties of the wild-type or "parent" ABD, the albumin binding motifs of the identified ABD variants were deduced to reside in the two helices 2 and 3, corresponding to the stretch from amino acid position G16 to I41. The albumin binding motifs of the identified ABD variants were given the designation ABM#####, where ##### is a five-digit unique label for the motif in question. The sequences of

the identified albumin binding motifs are listed in Figure 1 as SEQ ID NO:1-257. Interestingly, a subset of the sequences identified comprised a spontaneous mutation at the position corresponding to position 38 in ABDwt, despite the fact that this position had not been randomized in the creation of the library of variants.

Sub-cloning of ABD variants into plasmid pAY442

DNA encoding ABDwt (SEQ ID NO:515) and twelve clones from the selection were selected for sub-cloning into the expression vector pAY442 (Grönwall *et al, supra*). With reference to Figure 1, the selected ABD variant clones were ABD00002, ABD00003, ABD00009, ABD00015, ABD00025, ABD00027, ABD00046, ABD00049, ABD00053, ABD00054, ABD00055 and ABD00245. Plasmids containing inserts encoding these ABD variant molecules were purified from 2 ml over night cultures (tryptic soy broth medium (30 g/l) supplemented with 2 % glucose and 100 µg/ml ampicillin) of *E. coli* RR1ΔM15 cells (Rüther, 1982, *supra*) using Qiagen Mini Kit (Qiagen) according to the manufacturer's recommendations.

DNA for ABDwt and ABD variant molecules was sub-cloned into the expression vector pAY442 by *Acc*I-*Not*I PCR sticky end cloning (10 units/µl of each enzyme; New England Biolabs) using the primer pairs AFFI-780, -898 and AFFI-782, -899 as listed in Table 5:

Table 5: Oligonucleotide primers

Oligo	Sequence
AFFI-780	5'-*P*-agacttagctgaagctaaagtcttagc-3'
AFFI-782	5'-acttagctgaagctaaagtcttagc-3'
AFFI-898	5'-gctttaaggtaatgcagctaaaat-3'
AFFI-899	5'-*P*-ggccgctttaaggtaatgcagctaaaat-3'

Two overlapping PCR products for each ABD variant molecule were generated from the library vector pAY1075, resulting in approximately 25 % correct fragments with an *Acc*I-*Not*I site. The expression vector pAY442 was digested in two steps at 37°C for 4 h using *Acc*I and *Not*I in NEB4 buffer (20 mM Tris acetate, 10 mM magnesium acetate, 50 mM potassium acetate, 1 mM dithiothreitol, pH 7.9; New England Biolabs) and NEB3 buffer (50 mM Tris-HCl, 10 mM $MgCl_2$, 100 mM NaCl, 1 mM dithiothreitol, pH 7.9; New England Biolabs), respectively, and dephosphorylated with calf intestinal

alkaline phosphatase (CIAP; Fermentas) for 1 h at 37 °C. The cleaved plasmid and fragments were purified by QIAquick PCR purification kit (Qiagen) according to the manufacturer's recommendations.

The PCR products were hybridized and ligated into *Acc*I-*Not*I digested and dephosphorylated pAY442 for 1 h at room temperature using T4 DNA ligase (5 units/µl; Fermentas). Part of the ligations were electroporated into *E. coli* BL21(DE3) cells (F^- *omp*T *hsd*S_B(r_B^- m_B^-) *gal dcm* (DE3)) using a 1 mm cuvette and an ECM 630 set (BTX) using the parameters 1700 V, 200 Ω and 25 µF. The cells were plated on tryptose blood agar base (TBAB) plates supplemented with 50 µg/ml kanamycin and incubated over night at 37 °C. Positive clones were first verified on agarose gel of bacterial PCR products and finally with DNA sequence analysis.

Clones of pAY442 containing a successfully sub-cloned ABD variant encode a construct that is schematically described in Figure 8, i.e. essentially a His_6 tagged ABD variant.

Expression and purification of His_6 tagged ABD variants

ABDwt and the twelve ABD variants, all sub-cloned in pAY442 as described above, were expressed in *E. coli* BL21(DE3) as fusions to an N-terminal His_6-tag and purified by IMAC. A colony of each ABD variant was used to inoculate 5 ml TSB medium supplemented with 50 µg/ml kanamycin. The cultures were grown over night at 37 °C. The following day, 50 µl of each culture were inoculated separately to 100 ml TSB+YE medium supplemented with 50 µg/ml kanamycin in a 1 liter flask. The cultures were grown at 100 rpm at 37 °C to an OD_{600} of 0.7-1, after which IPTG was added to a final concentration of 0.5 mM and cells were incubated at room temperature over night at 100 rpm. Cultures were harvested by centrifugation at 8000 *g* for 5 minutes and pellets were stored in a freezer until protein preparation.

The His_6-tagged proteins were IMAC purified under denatured conditions using Ni-NTA Superflow columns and QIAsoft 4.1, protein/Ni-NTA Superflow 96 denaturing large scale 2 Vac4-24 samples, on a Biorobot 3000 (Qiagen). The buffer was exchanged to PBS using a dialysis cassette (Slide-A-Lyser, 3.5 kDa; Pierce cat. no. 66330) by dialyzing against 5 l PBS for 2 h followed by an additional dialysis over night.

Protein concentration was determined using A_{280} and the BCA Protein Assay Reagent Kit (Pierce) as recommended by the manufacturer. The purity of the proteins was analyzed by SDS-PAGE on 4-12 % Novex gels and

stained with Coomassie Blue R, and this analysis showed that only small amounts of impurities were present.

Biosensor analysis of ABD variants' affinity for HSA and MSA

Biosensor analysis on a Biacore2000 instrument (Biacore) was performed with MSA, HSA and RSA immobilized by amine coupling onto the carboxylated dextran layer on the surfaces of CM-5 chips (research grade; Biacore) according to the manufacturer's recommendations. Surface 1 on the chip was activated and deactivated and used as reference cell during injections, whereas surface 2 comprised MSA immobilized with 350 RU (resonance units), surface 3 comprised HSA immobilized with 360 RU and surface 4 comprised RSA immobilized with 340 RU. The ABD variants and ABDwt expressed and purified as described above were diluted in HBS-EP (Biacore) to 25 nM and injected at a constant flow-rate of 25 µl/min for 10 minutes, followed by injection of HBS-EP for 30 minutes. The surfaces were regenerated with two injections of 20 µl 15 mM HCl followed by 0.05 % SDS and one more injection of 20 µl HCl.

The Biacore study was not carried out with a view to determine the exact parameters for the variants' affinity for human and mouse serum albumin, but the results provide a qualitative measure of the relative affinities of these molecules for albumin. Results for binding to MSA and HSA are presented in Table 6.

Table 6: Biosensor analysis of ABD variants' binding to serum albumin from mouse and human

	MSA	HSA
	K_D (M)	K_D (M)
ABDwt	4.9×10^{-9}	1.5×10^{-9}
ABD00025	2.2×10^{-9}	2.7×10^{-11}
ABD00049	7.9×10^{-10}	2.2×10^{-11}
ABD00245	6.5×10^{-10}	6.0×10^{-11}
ABD00003	3.3×10^{-9}	1.6×10^{-11}
ABD00009	1.9×10^{-9}	5.4×10^{-11}
ABD00053	5.9×10^{-9}	1.1×10^{-11}
ABD00054	1.3×10^{-9}	2.0×10^{-11}
ABD00015	3.2×10^{-9}	4.5×10^{-11}
ABD00027	1.5×10^{-9}	4.1×10^{-11}
ABD00046	8.9×10^{-9}	1.2×10^{-10}
ABD00055	1.1×10^{-9}	5.4×10^{-11}

As is evident from this table, all tested ABD variants had a substantially higher affinity for human serum albumin than the wild type ABD molecule, as evidenced by K_D values at least one order of magnitude lower, frequently approaching two orders of magnitude lower. Furthermore, comparable and/or higher affinities towards mouse serum albumin was also exhibited by all variants.

Example 3

Additional biosensor characterization of selected ABD variants

Summary

In this example, selected ABD variants ABD00003, ABD00053 and ABD00239 plus ABDwt were all sub-cloned in pAY442 as described in Example 2 above, and expressed in a larger scale and purified with His Gravitrap™ kit. The expressed molecules were characterized for affinity to HSA using a Biacore instrument.

Protein expression and purification of His_6 tagged ABD variants

ABD00003, ABD00053, ABD00239 and ABDwt were expressed in *E. coli* BL21(DE3) cells as fusions to an N-terminal His_6-tag using constructs as described in Example 2, and purified by IMAC. A colony of each ABD variant was used to inoculate 10 ml TSB medium supplemented with 50 µg/ml kanamycin. The cultures were grown over night at 37 °C. The following day, 500 µl of each culture were inoculated separately to 500 ml TSB+YE medium supplemented with 50 µg/ml kanamycin in a 5 liter flask. The cultures were grown at 100 rpm and 37 °C to an OD_{600} of 0.7-1, which was followed by addition of IPTG to a final concentration of 0.5 mM and incubated at room temperature over night. Cultures were harvested by centrifugation at 8000 *g* for 5 minutes and pellets were stored at –20 °C until protein preparation.

The His_6-tagged proteins were IMAC purified under denaturing conditions using His-Gravitrap™ kit (GE Healthcare). The pellets were re-suspended (vortexed) in 20 ml of the denaturation buffer B-7M (100 mM NaH_2PO_4, 10 mM Tris-Cl, 7 M urea, pH 8) and 8 µl benzonase was added. The solutions were incubated for 30 minutes at room temperature and 200 rpm. An additional 20 ml of buffer B-7M was added and the solutions were

transferred to 50 ml Falcon tubes and sonicated on ice as follows: 3 s on/off during 3 min and with 40 % amplitude. Cell debris was removed by centrifugation at 25000 *g* for 40 min. The Gravitrap™ columns were equilibrated with buffer B-7M and the samples were applied. The columns were then washed with 10 ml buffer B-7M, 20 ml binding buffer (20 mM $NaPO_4$, 500 mM NaCl, 20 mM imidazole) and finally with 10 ml wash buffer (20 mN $NaPO_4$, 500 mM NaCl, 60 mM imidazole). The ABD molecules were eluted with 3 ml elution buffer (20 mN $NaPO_4$, 500 mM NaCl, 500 mM imidazole).

A buffer exchange to PBS pH 7.2 using a Slide-A-Lyser dialysis cassette (3.5 kDa; Pierce, cat. no. 66330) was made by dialyzing against 5 l PBS pH 7.2 for 2 hours followed by an additional dialysis over night and finally a buffer exchange to PBS pH 5 using PD 10 columns (GE Healthcare) was performed according to manufacturers' recommendations. Protein concentration was determined using Abs_{280}. The purity of the proteins were analyzed by SDS-PAGE on 4-12 % Novex gels and stained with Coomassie Blue R.

The proteins were successfully expressed and purified in an acceptable yield. The analysis with gel electrophoresis showed that no impurities were present (not shown).

Biosensor analysis for binding kinetics to human serum albumin

Biosensor analysis on a Biacore2000 instrument (Biacore) was performed with HSA (SIGMA, cat. no. A3782-5G) immobilized by amine coupling onto the carboxylated dextran layer on surfaces of a CM-5 chip (research grade; Biacore), according to the manufacturer's recommendations. The immobilization of HSA resulted in a signal of 450 resonance units. One cell surface on the chip was activated and deactivated and used as reference cell during injections. The purified His_6-ABD samples were diluted in HBS-EP (Biacore) to 4, 10, 40, 100 and 400 nM for ABDwt and to 0.2, 0.8, 2, 5 and 20 nM for the selected ABD variants. The samples were injected at a constant flow-rate of 25 µl/min for 10 min, followed by injection of HBS-EP for 3 hours. The surfaces were regenerated with two injections of 20 µl of 5 and 10 mM HCl. The K_D, k_a and k_d values were estimated and are given in Table 7, confirming the result of Example 2 that molecules exhibiting very high affinities to HSA had been obtained.

Table 7: Kinetic parameters (k_a, k_d and K_D) to HSA of purified ABD molecules

	k_a (Ms^{-1})	k_d (s^{-1})	K_D (M)
ABDwt	5.5×10^5	6.5×10^{-4}	1.2×10^{-9}
ABD00003	8.0×10^6	3.0×10^{-5}	3.8×10^{-12}
ABD00053	3.0×10^6	1.5×10^{-5}	5.0×10^{-12}
ABD00239	3.0×10^7	1.5×10^{-5}	5.0×10^{-13}

Example 4

Primate immunogenicity and pharmacokinetics of a Z variant polypeptide fused to a first ABD variant

Summary

Previous studies in mouse and rat have shown that various Z variant molecules fused to ABDwt generate a lower antibody response compared to the Z variant alone. The aim of this study was 1) to confirm these results in primates and expand it to a mutated variant of ABD exhibiting a 10^3-fold higher binding affinity for albumin compared to ABDwt, and 2) to compare the serum half-lives of ABD-fused and naked Z variants. A Z variant with affinity for the HER2 receptor was administered to primates with or without the ABD variant as fusion partner. Repeated immunization and bleeding proceeded over a 45-day period. The specific antibody responses against, and the serum half-lives of, the Z variant molecules were analyzed by ELISA assays.

Molecules studied

Z00342: a variant of protein Z, in turn derived from the B domain of staphylococcal protein A, with an affinity for the HER2 receptor. This variant was produced by recombinant DNA technology. Purification was performed using anion exchange and reverse phase chromatography methods followed by endotoxin removal on a Detoxi-Gel™ AffinityPak™ Pre-packed Column (Pierce, cat no 2034) according to the manufacturer's instructions. A detailed description of the Z00342 molecule is given in Orlova *et al*, Cancer Res 66:8, 4339-48 (2006), where it is denoted $Z_{Her2:342}$.

Z00342-ABD00003: a fusion protein between the Z variant Z00342 and the variant ABD molecule ABD00003 selected in Example 2. This fusion protein was produced by recombinant DNA technology. Purification was

performed using affinity capture on HSA-sepharose and reverse phase chromatography followed by endotoxin removal as above.

Methods

Administration and sampling schemes: The animal study was performed at SMI (Smittskyddsinsitutet) in Solna, Sweden, with permission from the local ethical animal committee (N196/06). The primates were sedated before administration of the test molecules and blood sampling, by an intramuscular administration of ketamine (Ketalar®). 10 individual cynomolgus primates, *Macaca fascicularis*, divided into two groups were intravenously injected with the test molecules according to the scheme in Table 8.

Table 8: Administration of test molecules

Group	Animal number	Molecule	Route of adm	mg/kg/injection	ml/animal/injection
1	9023, 9039, 10025, 10105, 11019	Z00342	i.v.	0.5	1
2	12031, 12041, 12047, 12061, 12065	Z00342-ABD00003	i.v.	0.5	1

Time points for administration and bleeding are summarized in Table 9. PK refers to samples taken for the pharmacokinetic study. Blood was stored at 4 °C over night and sera were subsequently kept at –20 °C.

Table 9: Time points for administration of test molecule and blood sampling

Day	Action
0	Bleeding at 0, 30, 60 minutes, 4 hours (PK) & Injection 1
1	Bleeding (PK)
2	Bleeding (PK)
3	Bleeding (PK)
7	Bleeding (PK) & Injection 2
14	Bleeding & Injection 3
21	Bleeding & Injection 4
28	Bleeding & Injection 5
35	Bleeding & Injection 6
45	Bleeding

General ELISA method: In general, a volume of 50 µl per well was used for all incubation steps except for blocking where a volume of 100 µl was used. Plates were coated over night at 4 °C in coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6) and washed with tap water. Blocking and dilutions were done in PBS with 0.5 % casein. Incubation times at room temperature were 1-2 hours for blocking and serum, 1 hour for secondary antibody and 10 min for substrate solution (ImmunoPure® TMB, Pierce, cat no 34021). Washing with 4x250 µl PBS-T (PBS with 0.05 % Tween 20) per well was carried out between all steps, using an automated ELISA SkanWasher 300 (Skatron). The color reaction was stopped by addition of 50 µl 2 M H_2SO_4 and plates were read at 450 nm using an Ultra384 plate reader (Tecan) equipped with the Magellan software v3.11 (Tecan).

Anti-Z00342 IgG specific ELISA: Plates were coated with 0.3 µg/ml of Z00342 diluted in coating buffer and incubated over night at 4 °C. After washing, plates were blocked as described above. Sera from primates were added in two-fold dilution series starting from 1/100. Purified serum from hyperimmunized primate was used as positive control and added in a two-fold dilution series starting from 8 µg/ml. Following incubation, plates were washed and a secondary, HRP conjugated anti-human IgG antibody (Southern Biotech cat. no. 2040-05) (diluted 1/10000) was added. After the final incubation, plates were washed and developed as described above.

Serum-Z specific ELISA for PK analysis: Plates were coated with 2 µg/ml affinity purified goat anti-Z Ig (produced in-house and specific for an epitope common to all Z variants) and incubated over night at 4 °C. After washing, plates were blocked as described above. Sera from primates injected with Z00342 or Z00342-ABD00003 were added in two-fold dilution series starting from 1/40 (for Z00342) or 1/80 (for Z00342-ABD00003). Standards of each molecule were added in a two-fold dilution series starting from 20 ng/ml. After incubation, plates were washed and the second step antibody was added (2 µg/ml of a rabbit IgG against Z (produced in-house) for Z00342; 1/5000 of a rabbit IgG against Z-ABD (produced in-house) for Z00342-ABD00003). Following incubation, plates were washed and HRP conjugated anti-rabbit Ig (Dako cat. no. 0448) diluted 1:5000 was added. After the final incubation, plates were washed and developed as described above.

Results

IgG specific for Z in primates injected with Z00342: The serum from each bleeding was analyzed by ELISA for the presence of IgG specific for Z variants (Figures 9A-9C). Low levels of IgG were detected at day 0 except for one primate (9039) that had moderate levels of pre-formed antibodies. After day 14, the antibody titer increased steadily and reached a maximum at day 28-35 in three of the animals (9039, 10025, 11019), whereas two showed a low antibody response (9023 and 10105) throughout the 45 day period.

IgG specific for Z in primates injected with Z00342-ABD00003: The serum from each bleeding was analyzed by ELISA for the presence of IgG specific for the Z-ABD00003 molecule (Figures 10A-10C). No antibody response was observed in two of the primates (12047 and 12065), whereas two primates (12041 and 12061) showed a high response. The fifth primate (12031) had high pre-serum levels of antibodies that were barely altered during the 45-day period.

Concentration of antibodies specific for Z variants: The concentration of IgG specific for Z variants in the sera was calculated by linear regression using the positive control as standard (Figure 11). Individual variations were seen within each group of primates. At day 45, the median concentration of specific IgG in groups 1 and 2 was 2 and 0.1 units/ml respectively, indicating that the fusion to ABD00003 decreases the antibody response against Z00342.

Pharmacokinetics of Z00342 in serum: The circulation times of Z00342 and Z00342-ABD00003 were compared in a pharmacokinetic analysis. The concentrations of the molecules over time were calculated from standard curves generated from dilution series of known amounts of Z00342 and Z00342-ABD00003, respectively. The results show that ABD-fused Z00342 fused to ABD00003 remains longer in the blood circulation compared to Z00342 alone (Figure 12). Z00342 disappeared from the circulation within 4 hours whereas the ABD00003-fused molecules were still detectable after 7 days.

Summary

The results of this study indicate that the ABD-fused Z variant molecule generates a lower immune response as well as exhibiting an extended elimination half-life in comparison with the Z variant molecule without an albumin binding fusion partner.

Example 5

Primate immunogenicity of a Z variant polypeptide fused to a second ABD variant

Molecules studied

In this extension of Example 4, a second variant of ABD with even higher affinity for albumin (K_D= 10^{-13} M), was fused to a dimeric Z variant and used for an immunogenicity study in primates.

$(Z01154)_2$: A dimeric variant of protein Z, in turn derived from the B domain of staphylococcal protein A. This dimer variant was produced by recombinant DNA technology. Purification was performed using anion exchange, reverse phase chromatography and cation exchange methods before endotoxin removal on a Detoxi-Gel™ AffinityPak™ Pre-packed Column (Pierce cat no 2034). A detailed description of the monomeric Z01154 molecule is given in Gunneriusson E *et al*, Protein Eng 12:10, 873-878 (1999), where it is denoted $Z_{Taq4:1}$.

$(Z01154)_2$-ABD00239: a fusion protein between the $(Z01154)_2$ dimer and the variant ABD molecule ABD00239 selected in Example 2. This fusion protein was produced by recombinant DNA technology. Purification was performed using affinity capture on HSA-sepharose and reverse phase chromatography followed by endotoxin removal as above.

Methods

Administration and sampling schemes: The animal study was performed at SMI (Smittskyddsinsitutet) in Solna, Sweden, with permission from the local ethical animal committee (N196/06). The primates were sedated before administration of test molecule and blood sampling, by an intramuscular administration of ketamine (Ketalar®). 7 individual cynomolgus primates, *Macaca fascicularis*, divided into two groups of three and four respectively, were intravenously injected with the test molecules as outlined in Table 10.

Table 10: Administration of test molecules

Group	Animal number	Molecule	Route of adm	mg/kg/injection	ml/animal/injection
1	E74, E78, E89	$(Z01154)_2$	i.v.	0.5	1
2	E75, E87, E88, E91	$(Z01154)_2$-ABD00239	i.v.	0.5	1

Time points for administration and blood sampling are summarized in Table 11.

Table 11: Time points for administration of test molecule and blood sampling

Day	Action
0	Bleeding 1 & Injection 1
7	Bleeding 2
14	Bleeding 3 & Injection 2
21	Bleeding 4
28	Bleeding 5 & Injection 3
35	Bleeding 6
45	Bleeding 7

Analysis of plasma samples by specific ELISA: Plates were coated with $(Z01154)_2$ or $(Z01154)_2$-ABD00239 diluted in coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6) to a final concentration of 2 µg/ml. 50 µl solution was used per well and plates were incubated at 4 °C over night. Blocking with PBS + 0.5 % casein was performed at room temperature for 1-2 h. Serum was added in two-fold dilution series starting from a 1/100 dilution in blocking buffer. Purified serum from hyperimmunized primate was used as a positive control. Incubation was performed at room temperature for 1-2 hours and washing with 4x175 µl PBS-T (PBS with 0.05 % Tween 20) per well was carried out using an automated ELISA SkanWasher 300 (Skatron). Incubation with secondary HRP-conjugated goat anti-human IgG F(ab)2 (Jackson cat. no. 109-035-097), diluted 1:5000 in blocking buffer, was performed at room temperature for 1-2 h. Automated washing was carried out as above. Detection was enabled using ImmunoPure® TMB (Pierce cat. no. 34021) where the reaction was quenched after 12 min by addition of 2 M H_2SO_4. Plates were read at 450 nm using an Ultra384 plate reader (Tecan) equipped with the Magellan software v3.11 (Tecan).

Results

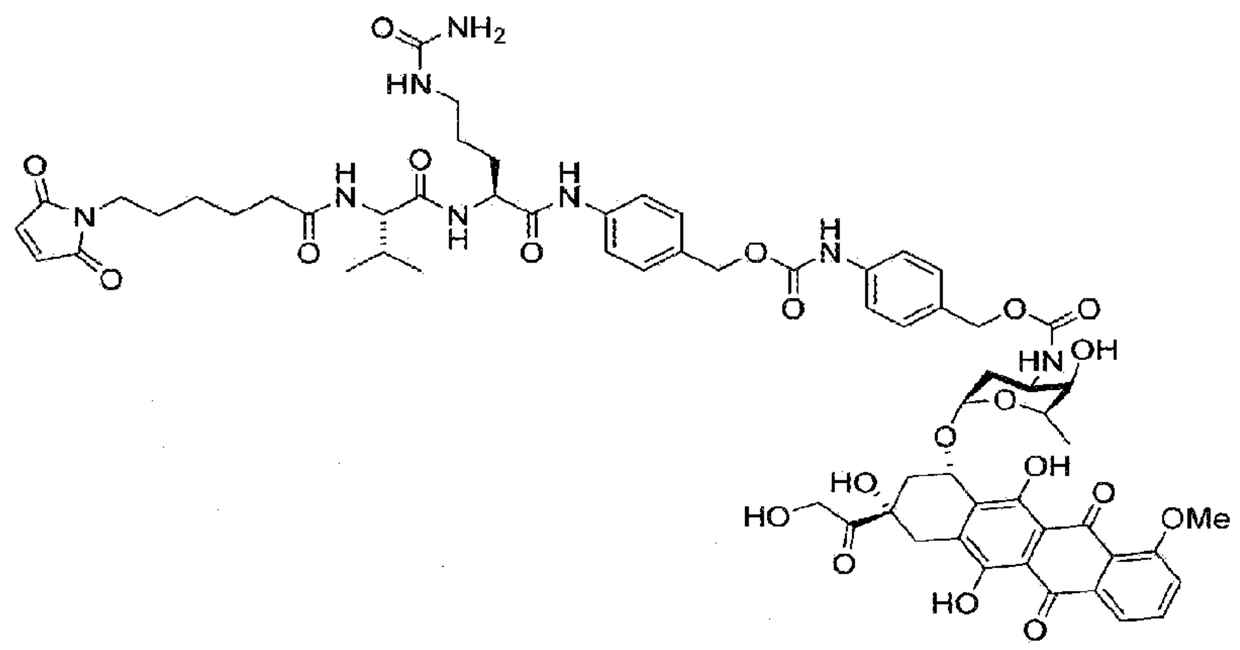
Immunization with $(Z01154)_2$ and $(Z01154)_2$-ABD00239: Sera from primates immunized with naked or ABD00239-fused $(Z01154)_2$ were titrated on $(Z01154)_2$ and $(Z01154)_2$-ABD00239 coated plates respectively, and the titration curves are shown in Figures 13A-13B and Figures 14A-14B. Serum from hyperimmunized monkey was included in the titration as positive control. The absorbance of the positive control at 1600 x dilution was set to 100 % and used for normalization. Figure 15 shows normalized values of the individual responses. The results show that all three animals responded against $(Z01154)_2$, although the response in one primate (E89) was of a lower magnitude. In contrast, only one out of four animals responded against $(Z01154)_2$-ABD00239.

Example 6

Increased solubility of doxorubicin conjugate after complexation with albumin

Summary

Conjugates were produced between the fusion protein ABDwt-$(Z00342)_2$-Cys (a recombinantly produced fusion protein comprising the wild-type ABD domain and a dimer of a Z variant, Z00342, with affinity for the HER2 receptor) and the non-polar molecule Maleimide-Spacelinker-Doxorubicin, the structural formula of which is given below.



Both the free linker and the conjugate have low solubility in aqueous solvents. For example, 30 % organic solvent is needed to keep the conjugate in solution. However, the addition of human serum albumin to an aqueous solution greatly improves solubility.

Conjugation

Maleimide-Spacelinker-Doxorubicin (Syntarga B.V., Netherlands) was dissolved in N,N-dimethyl formamide (Sigma, cat. no. D-4551) to a final concentration of 4 µmol/ml, and stored at –80 °C before use. 4 ml of the fusion protein ABDwt-$(Z00342)_2$-Cys, 1.9 mg/ml in PBS, was reduced with 20 mM DTT (Acros Organics, cat. no. 165680250) at 40 °C for 30 minutes. Excess DTT was removed by buffer exchange on PD-10 columns to PBS (2.68 mM KCl, 1.47 mM KH_2PO_4, 137 mM NaCl, 8.1 mM Na_2HPO_4, pH 7.4). The protein sample was adjusted to 30 % (v/v) organic solvent by addition of 3 ml acetonitrile (AcN, Merck, cat. no. 1.14291.2500). 198 µl, or a two-fold molar excess, of Maleimide-Spacelinker-Doxorubicin was added to the protein solution. After mixture for 30 minutes, the solution was incubated at 4 °C overnight. The reaction mixture was finally purified on a HiPrep 26/10 desalting column (GE, cat. no. 17-5087-01) equilibrated with deionized water/AcN (70:30, v/v). The protein concentration was determined to be 0.44 mg/ml by measuring the UV absorption at 280 nm.

Protein aliquots of 1.1 mg were lyophilized in an Alpha 2-4 LSC freeze dryer (Martin Christ GmbH, Germany). The vials were filled with nitrogen after finished lyophilization, capsulated and stored at 4 °C.

Solubility study

Three solutions were used for the solubility test:

1. DMEM, Dulbecco´s modified Eagle's medium (Cambrex Bio Science, cat. no. BE12-917F),
2. DMEM as in 1, but supplemented with human serum albumin (HSA), 6 mg/ml (Sigma, cat. no. A1887-5G), and
3. DMEM as in 1, but supplemented with 10 % fetal calf serum (FCS).

Solutions 1 and 2 were filtered through a 0.22 µ Millex-GV sterile filter (Millipore, cat. no. SLGV033RB).

Lyophilized conjugate was re-dissolved in vials containing 0.5 ml of each solution respectively. After 30 min incubation at 37 °C, samples were evaluated by visual inspection. A large fraction of undissolved material was

seen in the vials with solutions 1 and 3, while no visible precipitates were observed in the vial with solution 2.

LC-MS analysis of reconstituted conjugate

30 µl from each vial (solutions 1-3) was centrifuged at 13000 rpm for 10 min in an eppendorf centrifuge. 20 µl of the resulting supernatant was analyzed by liquid chromatography with online mass-spectrometric detection (Agilent 1100, LC-MS). The column, Zorbax 300SB-C18 (4.6x150 mm, 3.5 u), was equilibrated with 65 % solvent A (0.1 % TFA in deionized water) and 35 % solvent B (0.1 % TFA in AcN) at a flow rate of 0.5 ml/min. The UV absorption at 220, 280, 254 and 495 nm were recorded. Sample components were eluted with a shallow linear gradient from 50-60 % solvent B over 35 min. The peak area, corresponding to the amount of conjugate molecule in solution, was compared between the samples.

The results are shown in Table 12. Conjugate dissolved in DMEM supplemented with HSA (solution 2) showed a ten-fold larger area compared to the sample dissolved in DMEM only (solution 1), and a four-fold larger area compared to the sample dissolved in DMEM supplemented with 10 % FCS (solution 3).

Table 12: LC-MS analysis of reconstituted conjugate

Solvent	Peak area (mAU*s)	Ratio vs DMEM
DMEM	2347.4	1.00
DMEM+HSA	22659.6	9.65
DMEM+FCS	5872.0	2.50

SEQUENCE LISTING IN ELECTRONIC FORM

In accordance with Section 111(1) of the Patent Rules, this description contains a sequence listing in electronic form in ASCII text format (file: 22819-631 Seq 13-FEB-14 v1.txt).

A copy of the sequence listing in electronic form is available from the Canadian Intellectual Property Office.

CLAIMS:

1. Albumin binding polypeptide comprising an albumin binding motif, which motif consists of the amino acid residues according to the sequence:

$$GVSDX_5YKX_8X_9I \quad X_{11}X_{12}AX_{14}TVEGVX_{20} \quad ALX_{23}X_{24}X_{25}I$$

wherein, independently of each other,

X_5 is selected from Y and F;

X_8 is selected from N, R and S;

X_9 is selected from V, I, L, M, F and Y;

X_{11} is selected from N, S, E and D;

X_{12} is selected from R, K and N;

X_{14} is selected from K and R;

X_{20} is selected from D, N, Q, E, H, S, R and K;

X_{23} is selected from K, I and T;

X_{24} is selected from A, S, T, G, H, L and D; and

X_{25} is selected from H, E and D;

with the proviso that the amino acid sequence is not

GVSDYYKNLI NNAKTVEGVK ALIDEI;

the albumin binding polypeptide binding to albumin such that the KD value of the interaction is at most 1×10^{-9} M.

2. Albumin binding polypeptide according to claim 1, wherein X_5 is Y.

3. Albumin binding polypeptide according to any one of claims 1 and 2, wherein X_8 is selected from N and R.

4. Albumin binding polypeptide according to claim 3, wherein X_8 is R.

5. Albumin binding polypeptide according to any one of claims 1 to 4, wherein X_9 is L.

6. Albumin binding polypeptide according to any one of claims 1 to 5, wherein X_{11} is selected from N and S.

7. Albumin binding polypeptide according to claim 6, wherein X_{11} is N.

8. Albumin binding polypeptide according to any one of claims 1 to 7, wherein X_{12} is selected from R and K.

9. Albumin binding polypeptide according to claim 8, wherein X_{12} is R.

10. Albumin binding polypeptide according to claim 8, wherein X_{12} is K.

11. Albumin binding polypeptide according to any one of claims 1 to 10, wherein X_{14} is K.

12. Albumin binding polypeptide according to any one of claims 1 to 11, wherein X_{20} is selected from D, N, Q, E, H, R and S.

13. Albumin binding polypeptide according to claim 12, wherein X_{20} is E.

14. Albumin binding polypeptide according to any one of claims 1 to 13, wherein X_{23} is selected from K and I.

15. Albumin binding polypeptide according to claim 14, wherein X_{23} is K.

16. Albumin binding polypeptide according to any one of claims 1 to 15, wherein X_{24} is selected from A, S, T, G, H and L.

17. Albumin binding polypeptide according to claim 16, wherein X_{24} is L.

18. Albumin binding polypeptide according to claim 17, wherein $X_{23}X_{24}$ is KL.

19. Albumin binding polypeptide according to claim 17 when claim 16 is dependent on any one of claims 1-13, wherein $X_{23}X_{24}$ is TL.

20. Albumin binding polypeptide according to claim 16, wherein X_{24} is selected from A, S, T, G and H.

21. Albumin binding polypeptide according to claim 20 when claim 16 is dependent on any one of claims 1-14, wherein X_{23} is I.

22. Albumin binding polypeptide according to any one of claims 1 to 21, wherein X_{25} is H.

23. Albumin binding polypeptide according to any one of claims 1 to 22, in which said albumin binding motif consists of an amino acid sequence selected from SEQ ID NO:1-257.

24. Albumin binding polypeptide according to claim 23, in which said albumin binding motif consists of amino acid residues according to a sequence selected from SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:9, SEQ ID NO:15, SEQ ID NO:25, SEQ ID NO:27, SEQ ID NO:46, SEQ ID NO:49, SEQ ID NO:53, SEQ ID NO:54, SEQ ID NO:55, SEQ ID NO:155, SEQ ID NO:239, SEQ ID NO:240, SEQ ID NO:241, SEQ ID NO:242, SEQ ID NO:243, SEQ ID NO:244 and SEQ ID NO:245.

25. Albumin binding polypeptide according to claim 24, in which said albumin binding motif consists of amino acid residues according to a sequence selected from SEQ ID NO:3, SEQ ID NO:53 and SEQ ID NO:239.

26. Albumin binding polypeptide according to any one of claims 1 to 25, in which said albumin binding motif forms part of a three-helix bundle protein domain.

27. Albumin binding polypeptide according to claim 26, in which said three-helix bundle protein domain is selected from the group consisting of three-helix domains of bacterial receptor proteins.

28. Albumin binding polypeptide according to claim 27, in which said bacterial receptor protein is selected from the group consisting of albumin binding receptor proteins from species of *Streptococcus*, *Peptostreptococcus* and *Finegoldia*.

29. Albumin binding polypeptide according to claim 28, in which said albumin binding receptor protein is selected from the group consisting of G; MAG; ZAG; PPL; and PAB.

30. Albumin binding polypeptide according to claim 29, in which said albumin binding receptor protein is protein G.

31. Albumin binding polypeptide according to claim 30, in which said albumin binding receptor protein is protein G from *Streptococcus* strain G148.

32. Albumin binding polypeptide according to claim 31, in which said three-helix bundle protein domain is selected from the group consisting of domain GA1, domain GA2 and domain GA3 of protein G from *Streptococcus* strain G148.

33. Albumin binding polypeptide according to claim 32, in which said three-helix bundle protein domain is domain GA3 of protein G from *Streptococcus* strain G148.

34. Albumin binding polypeptide according to claim 27, in which said bacterial receptor protein is protein A from *Staphylococcus aureus*.

35. Albumin binding polypeptide according to claim 34, in which said three-helix bundle protein domain is selected from the group consisting of protein A domains A, B, C, D and E.

36. Albumin binding polypeptide according to claim 34, in which said three-helix bundle protein domain is protein Z derived from domain B of protein A from *Staphylococcus aureus*.

37. Albumin binding polypeptide according to claim 26, which comprises the amino acid sequence:

$$LAEAKX_aX_bAX_cX_d\ ELX_eKY\text{-}[ABM]\text{-}LAALP$$

wherein

[*ABM*] is an albumin binding motif as defined in any one of claims 1-25,

and, independently of each other,

X_a is selected from V and E;

X_b is selected from L, E and D;

X_c is selected from N, L and I;

X_d is selected from R and K; and

X_e is selected from D and K.

38. Albumin binding polypeptide according to claim 37, wherein X_a is V.

39. Albumin binding polypeptide according to any one of claims 37-38, wherein X_b is L.

40. Albumin binding polypeptide according to any one of claims 37-39, wherein X_c is N.

41. Albumin binding polypeptide according to any one of claims 37-40, wherein X_d is R.

42. Albumin binding polypeptide according to any one of claims 37-41, wherein X_e is D.

43. Albumin binding polypeptide, whose amino acid sequence comprises a sequence selected from SEQ ID NOs:258-513 and 514.

44. Albumin binding polypeptide according to claim 43, whose amino acid sequence comprises a sequence selected from SEQ ID NO:259, SEQ ID NO:260, SEQ ID NO:266, SEQ ID NO:272, SEQ ID NO:282, SEQ ID NO:284, SEQ ID NO:303, SEQ ID NO:306, SEQ ID NO:310, SEQ ID NO:311, SEQ ID NO:312, SEQ ID NO:412, SEQ ID NO:496, SEQ ID NO:497, SEQ ID NO:498, SEQ ID NO:499, SEQ ID NO:500, SEQ ID NO:501 and SEQ ID NO:502.

45. Albumin binding polypeptide according to claim 44, whose amino acid sequence comprises a sequence selected from SEQ ID NO:260, SEQ ID NO:310 and SEQ ID NO:496.

46. Albumin binding polypeptide according to any one of claims 1 to 45, which binds to albumin such that the K_D value of the interaction is at most 1×10^{-10} M.

47. Albumin binding polypeptide according to claim 46, which binds to albumin such that the K_D value of the interaction is at most 1×10^{-11} M.

48. Albumin binding polypeptide according to claim 47, which binds to albumin such that the K_D value of the interaction is at most 1×10^{-12} M.

49. Albumin binding polypeptide according to any one of claims 1 to 48, which binds to human serum albumin.

50. Albumin binding polypeptide according to any one of claims 1 to 49, further comprising one or more additional amino acid(s) positioned on one or both sides of the albumin binding motif.

51. Albumin binding polypeptide according to claim 50, in which said additional amino acid(s) enhance binding of albumin by the polypeptide.

52. Albumin binding polypeptide according to claim 50, in which said additional amino acid(s) improves a characteristic selected from production, purification, stabilization *in vivo* or *in vitro*, coupling and detection of the polypeptide, and any combination thereof.

53. Multimer of albumin binding polypeptides as defined in any one of claims 1 to 52, comprising at least two albumin binding polypeptides as monomer units.

54. Multimer according to claim 53, in which the amino acid sequences of the monomer units are the same.

55. Multimer according to claim 53, in which the amino acid sequences of the monomer units are different.

56. Fusion protein or conjugate comprising

i) a first moiety consisting of an albumin binding polypeptide according to any one of claims 1-52 or multimer according to any one of claims 53 to 55; and

ii) a second moiety consisting of a second polypeptide having a desired biological activity.

57. Fusion protein or conjugate according to claim 56, in which said desired biological activity is a therapeutic activity.

58. Fusion protein or conjugate according to claim 56, in which said desired biological activity is a binding activity.

59. Fusion protein or conjugate according to claim 56, in which said desired biological activity is an enzymatic activity.

60. Fusion protein or conjugate according to claim 56, in which said second moiety is selected from the group consisting of GLP-1; HGH; G-CSF; IL-1 receptor agonist; TNF-α; and blood clotting factors VII, VIII, IX and X.

61. Fusion protein or conjugate according to claim 56, in which said second moiety is a binding moiety capable of selective interaction with a target molecule, which binding moiety is selected from the group consisting of

antibodies and fragments and domains thereof substantially retaining antibody binding activity;

microbodies, maxybodies, avimers and other small disulfide-bonded proteins; and

binding proteins derived from a scaffold selected from the group consisting of staphylococcal protein A and domains thereof, lipocalins, ankyrin repeat domains, cellulose binding domains, γ crystallines, green fluorescent protein, human cytotoxic T lymphocyte-associated antigen 4, protease inhibitors, PDZ domains, peptide aptamers, staphylococcal nuclease, tendamistats, fibronectin type III domain, zinc fingers, conotoxins, and Kunitz domains.

62. Fusion protein or conjugate according to claim 61, in which said target molecule is selected from the group consisting of Aß peptide; other disease-associated amyloid peptides; toxins; blood clotting factors; interleukins; myostatin; pro-inflammatory factors; complement factors; hypersensitivity mediators, and tumor-related antigens.

63. Fusion protein or conjugate according to claim 62, in which said target molecule is a toxin selected from bacterial toxin and snake venoms.

64. Fusion protein or conjugate according to claim 62, in which said blood clotting factor is von Willebrand factor.

65. Fusion protein or conjugate according to claim 62, in which said interleukin is IL13.

66. Fusion protein or conjugate according to claim 62, in which said target molecule is a pro-inflammatory factor selected from TNF-α, TNF-α receptor and IL-8.

67. Fusion protein or conjugate according to claim 62, in which said target molecule is a complement factor selected from C3a and C5a.

68. Fusion protein or conjugate according to claim 62, in which said target molecule is a hypersensitivity mediator selected from histamine and IgE.

69. Fusion protein or conjugate according to claim 62, in which said target molecule is a tumor-related antigen selected from CD19, CD20, CD22, CD30, CD33, CD40, CD52, CD70, cMet, HER1, HER2, HER3, HER4, CA9, CEA, IL-2 receptor, MUC1, PSMA and TAG-72.

70. Polypeptide according to any one of claims 1 to 52, multimer according to any one of claims 53 to 55, or fusion protein or conjugate according to any one of claims 56 to 69, further comprising a label.

71. Polypeptide, multimer, fusion protein or conjugate according to claim 70, in which said label is selected from the group consisting of fluorescent dyes and metals, chromophoric dyes, chemiluminescent compounds and bioluminescent proteins, enzymes, radionuclides and particles.

72. Polynucleotide encoding a polypeptide according to any one of claims 1-52, a multimer according to any one of claims 53 to 55, or a fusion protein according to any one of claims 56 to 69.

73. Method of producing a polypeptide according to any one of claims 1- 52, a multimer according to any one of claims 53 to 55, or a fusion protein

according to any one of claims 56 to 69, comprising expressing a polynucleotide according to claim 72.

74. Expression vector comprising a polynucleotide according to claim 72.

75. Host cell comprising an expression vector according to claim 74.

76. Method of producing a polypeptide according to any one of claims 1-52, a multimer according to any one of claims 53 to 55, or a fusion protein according to any one of claims 56 to 69, comprising

culturing a host cell according to claim 75 under conditions permitting expression of said polypeptide from said expression vector, and

isolating the polypeptide.

77. Use of a fusion protein or conjugate according to any one of claims 56-69 for the preparation of a medicament which exhibits a half-life *in vivo* which is longer than the half-life *in vivo* of the second polypeptide having a desired biological activity *per se*.

78. Use of a fusion protein or conjugate according to any one of claims 56-69 for the preparation of a medicament which elicits no or a reduced immune response upon administration to a mammal, as compared to the immune response elicited upon administration to the mammal of the second polypeptide having a desired biological activity *per se*.

Polypeptide	Amino acid sequence	SEQ ID NO:
ABM00001	GVSDFYKNLI NRAKTVEGVH ALIGHI	1
ABM00002	GVSDFYKNVI NRAKTVEGVH ALIDHI	2
ABM00003	GVSDYYKNII NRAKTVEGVR ALKLHI	3
ABM00004	GVSDFYKNVI NRAKTVEGVS ALIHEI	4
ABM00005	GVSDFYKRLI NRAKTVEGVN TLIADI	5
ABM00006	GVSDFYKNLI NRAKTVEGVN TLIADI	6
ABM00007	GVSDYYKNLI NRAKTVEGVN SLISHI	7
ABM00008	GVSDFYKRLI NRAKTVGGVQ SLISEI	8
ABM00009	GVSDFYKNLI NRAKTVEGVS SLKGHI	9
ABM00010	GVSDFYKNVI NRAKTVEGVD SLIAEI	10
ABM00011	GVSDFYKNLI NRARTVEGVQ TLISDI	11
ABM00012	GVSDFYKKFI NKAKTVEGVE TLISEI	12
ABM00013	GVSDFYKSLI NRAKTVEGVH SLTDEI	13
ABM00014	GVSDYYKNVI NKAKTVEGVS SLTAEI	14
ABM00015	GVSDFYKSLI NRAKTVEGVD ALTSHI	15
ABM00016	GVSDFYKNLI NKAKTVEGVS TLIHDI	16
ABM00017	GVSDFYKNLI NRAKTVEGVS TLIHDI	17
ABM00018	GVSDFYKNLI NRAKTVEGVQ ALISEI	18
ABM00019	GVSDYYKSLI NKAKTVEGVD SLIVHI	19
ABM00020	GVSDFYKNLI NRAKTVEGVQ SLITEI	20
ABM00021	GVSDYYKNLI NRAKTVEGVD ALITHI	21
ABM00022	GVSDFYKSMI NRAKTVEGVD SLITHI	22
ABM00023	GVSDFYKNLI NRAKTVEGVT TLTTDI	23
ABM00024	GVSDFYKNLI NRAKTVEGVE SLIDHI	24
ABM00025	GVSDFYKSYI NRAKTVEGVH TLIGHI	25
ABM00026	GVSDFYKNLI NRAKTVEGVQ TLISDI	26
ABM00027	GVSDFYKNLI NRAKTVEGVN SLTSHI	27
ABM00028	GVSDFYKNLI NRAKTVEGVN TLIHDI	28
ABM00029	GVSDFYKNLI NRAKTVEGVE SLIGEI	29
ABM00030	GVSDFYKNLI NKAKTVEGVH TLIHDI	30
ABM00031	GVSDYYKNLI NKAKTVEGVS ALKMHI	31

FIGURE 1

Polypeptide	Amino acid sequence	SEQ ID NO:
ABM00032	GVSDFYKNLI NKAKTVEGVD ALIVHI	32
ABM00033	GVSDYYKRLI NRAKTVEGVH ALIAEI	33
ABM00034	GVSDYYKNLI NRARTVEGVD TLIHDI	34
ABM00035	GVSDFYKKVI NRARTVEGVQ ALIADI	35
ABM00036	GVSDFYKNLI NRAKTVEGVE SLIADI	36
ABM00037	GVSDYYKNLI NKAKTVEGVD ALIAHI	37
ABM00038	GVSDYYKNLI NRAKTVEGVE SLITHI	38
ABM00039	GVSDFYKNLI NRARTVEGVD SLIVEI	39
ABM00040	GVSDFYKNVI NRAKTVEGVS ALIREI	40
ABM00041	GVSDFYKNLI NRAKTVEGVN ALISDI	41
ABM00042	GVSDFYKNLI NRAKTVEGVS ALIQEI	42
ABM00043	GVSDFYKNLI NRAKTVEGVQ SLIDHI	43
ABM00044	GVSDFYKNLI NRAKTVEGVD ALICHI	44
ABM00045	GVSDYYKRLI NKAKTVEGVN ALITHI	45
ABM00046	GVSDFYKNVI NKAKTVEGVE ALIADI	46
ABM00047	GVSDFYKNLI NRAKTVEGVE TLIRDI	47
ABM00048	GVSDFYKNLI NRARTVEGVQ TLITDI	48
ABM00049	GVSDFYKRLI NKAKTVEGVN ALTHHI	49
ABM00050	GVSDYYKNLI NRAKTVEGVQ ALIAHI	50
ABM00051	GVSDFYKNVI NRAKTVEGVN SLINHI	51
ABM00052	GVSDFYKSLI NRARTVEGVD SLIRHI	52
ABM00053	GVSDYYKNLI NKAKTVEGVE ALTLHI	53
ABM00054	GVSDFYKNLI NRAKTVEGVD ALIAHI	54
ABM00055	GVSDYYKNLI NKAKTVEGVQ ALIAHI	55
ABM00056	GVSDFYKRLI NRAKTVEGVH ALIGHI	56
ABM00057	GVSDFYKRVI NRAKTVEGVH ALIDHI	57
ABM00058	GVSDYYKRII NRAKTVEGVR ALKLHI	58
ABM00059	GVSDFYKRVI NRAKTVEGVS ALIHEI	59
ABM00060	GVSDFYKRLI NRAKTVEGVN TLIADI	60
ABM00061	GVSDYYKRLI NRAKTVEGVN SLISHI	61
ABM00062	GVSDFYKRLI NRAKTVEGVS SLKGHI	62

FIGURE 1

Polypeptide	Amino acid sequence	SEQ ID NO:
ABM00063	GVSDFYKRVI NRAKTVEGVD SLIAEI	63
ABM00064	GVSDFYKRLI NRARTVEGVQ TLISDI	64
ABM00065	GVSDFYKRFI NKAKTVEGVE TLISEI	65
ABM00066	GVSDFYKRLI NRAKTVEGVH SLTDEI	66
ABM00067	GVSDYYKRVI NKAKTVEGVS SLTAEI	67
ABM00068	GVSDFYKRLI NRAKTVEGVD ALTSHI	68
ABM00069	GVSDFYKRLI NKAKTVEGVS TLIHDI	69
ABM00070	GVSDFYKRLI NRAKTVEGVS TLIHDI	70
ABM00071	GVSDFYKRLI NRAKTVEGVQ ALISEI	71
ABM00072	GVSDYYKRLI NKAKTVEGVD SLIVHI	72
ABM00073	GVSDFYKRLI NRAKTVEGVQ SLITEI	73
ABM00074	GVSDYYKRLI NRAKTVEGVD ALITHI	74
ABM00075	GVSDFYKRMI NRAKTVEGVD SLITHI	75
ABM00076	GVSDFYKRLI NRAKTVEGVT TLTTDI	76
ABM00077	GVSDFYKRLI NRAKTVEGVE SLIDHI	77
ABM00078	GVSDFYKRYI NRAKTVEGVH TLIGHI	78
ABM00079	GVSDFYKRLI NRAKTVEGVQ TLISDI	79
ABM00080	GVSDFYKRLI NRAKTVEGVN SLTSHI	80
ABM00081	GVSDFYKRLI NRAKTVEGVN TLIHDI	81
ABM00082	GVSDFYKRLI NRAKTVEGVE SLIGEI	82
ABM00083	GVSDFYKRLI NKAKTVEGVH TLIHDI	83
ABM00084	GVSDYYKRLI NKAKTVEGVS ALKMHI	84
ABM00085	GVSDFYKRLI NKAKTVEGVD ALIVHI	85
ABM00086	GVSDYYKRLI NRARTVEGVD TLIHDI	86
ABM00087	GVSDFYKRVI NRARTVEGVQ ALIADI	87
ABM00088	GVSDFYKRLI NRAKTVEGVE SLIADI	88
ABM00089	GVSDYYKRLI NKAKTVEGVD ALIAHI	89
ABM00090	GVSDYYKRLI NRAKTVEGVE SLITHI	90
ABM00091	GVSDFYKRLI NRARTVEGVD SLIVEI	91
ABM00092	GVSDFYKRVI NRAKTVEGVS ALIREI	92
ABM00093	GVSDFYKRLI NRAKTVEGVN ALISDI	93

FIGURE 1

Polypeptide	Amino acid sequence	SEQ ID NO:
ABM00094	GVSDFYKRLI NRAKTVEGVS ALIQEI	94
ABM00095	GVSDFYKRLI NRAKTVEGVQ SLIDHI	95
ABM00096	GVSDFYKRLI NRAKTVEGVD ALICHI	96
ABM00097	GVSDFYKRVI NKAKTVEGVE ALIADI	97
ABM00098	GVSDFYKRLI NRAKTVEGVE TLIRDI	98
ABM00099	GVSDFYKRLI NRARTVEGVQ TLITDI	99
ABM00100	GVSDYYKRLI NRAKTVEGVQ ALIAHI	100
ABM00101	GVSDFYKRVI NRAKTVEGVN SLINHI	101
ABM00102	GVSDFYKRLI NRARTVEGVD SLIRHI	102
ABM00103	GVSDYYKRLI NKAKTVEGVE ALTLHI	103
ABM00104	GVSDFYKRLI NRAKTVEGVD ALIAHI	104
ABM00105	GVSDYYKRLI NKAKTVEGVQ ALIAHI	105
ABM00106	GVSDFYKNLI NRAKTVEGVH ALKGHI	106
ABM00107	GVSDFYKNVI NRAKTVEGVH ALKDHI	107
ABM00108	GVSDFYKNVI NRAKTVEGVS ALKHEI	108
ABM00109	GVSDFYKRLI NRAKTVEGVN TLKADI	109
ABM00110	GVSDFYKNLI NRAKTVEGVN TLKADI	110
ABM00111	GVSDYYKNLI NRAKTVEGVN SLKSHI	111
ABM00112	GVSDFYKRLI NRAKTVGGVQ SLKSEI	112
ABM00113	GVSDFYKNVI NRAKTVEGVD SLKAEI	113
ABM00114	GVSDFYKNLI NRARTVEGVQ TLKSDI	114
ABM00115	GVSDFYKKFI NKAKTVEGVE TLKSEI	115
ABM00116	GVSDFYKSLI NRAKTVEGVH SLKDEI	116
ABM00117	GVSDYYKNVI NKAKTVEGVS SLKAEI	117
ABM00118	GVSDFYKSLI NRAKTVEGVD ALKSHI	118
ABM00119	GVSDFYKNLI NKAKTVEGVS TLKHDI	119
ABM00120	GVSDFYKNLI NRAKTVEGVS TLKHDI	120
ABM00121	GVSDFYKNLI NRAKTVEGVQ ALKSEI	121
ABM00122	GVSDYYKSLI NKAKTVEGVD SLKVHI	122
ABM00123	GVSDFYKNLI NRAKTVEGVQ SLKTEI	123
ABM00124	GVSDYYKNLI NRAKTVEGVD ALKTHI	124

FIGURE 1

Polypeptide	Amino acid sequence	SEQ ID NO:
ABM00125	GVSDFYKSMI NRAKTVEGVD SLKTHI	125
ABM00126	GVSDFYKNLI NRAKTVEGVT TLKTDI	126
ABM00127	GVSDFYKNLI NRAKTVEGVE SLKDHI	127
ABM00128	GVSDFYKSYI NRAKTVEGVH TLKGHI	128
ABM00129	GVSDFYKNLI NRAKTVEGVQ TLKSDI	129
ABM00130	GVSDFYKNLI NRAKTVEGVN SLKSHI	130
ABM00131	GVSDFYKNLI NRAKTVEGVN TLKHDI	131
ABM00132	GVSDFYKNLI NRAKTVEGVE SLKGEI	132
ABM00133	GVSDFYKNLI NKAKTVEGVH TLKHDI	133
ABM00134	GVSDFYKNLI NKAKTVEGVD ALKVHI	134
ABM00135	GVSDYYKRLI NRAKTVEGVH ALKAEI	135
ABM00136	GVSDYYKNLI NRARTVEGVD TLKHDI	136
ABM00137	GVSDFYKKVI NRARTVEGVQ ALKADI	137
ABM00138	GVSDFYKNLI NRAKTVEGVE SLKADI	138
ABM00139	GVSDYYKNLI NKAKTVEGVD ALKAHI	139
ABM00140	GVSDYYKNLI NRAKTVEGVE SLKTHI	140
ABM00141	GVSDFYKNLI NRARTVEGVD SLKVEI	141
ABM00142	GVSDFYKNVI NRAKTVEGVS ALKREI	142
ABM00143	GVSDFYKNLI NRAKTVEGVN ALKSDI	143
ABM00144	GVSDFYKNLI NRAKTVEGVS ALKQEI	144
ABM00145	GVSDFYKNLI NRAKTVEGVQ SLKDHI	145
ABM00146	GVSDFYKNLI NRAKTVEGVD ALKCHI	146
ABM00147	GVSDYYKRLI NKAKTVEGVN ALKTHI	147
ABM00148	GVSDFYKNVI NKAKTVEGVE ALKADI	148
ABM00149	GVSDFYKNLI NRAKTVEGVE TLKRDI	149
ABM00150	GVSDFYKNLI NRARTVEGVQ TLKTDI	150
ABM00151	GVSDFYKRLI NKAKTVEGVN ALKHHI	151
ABM00152	GVSDYYKNLI NRAKTVEGVQ ALKAHI	152
ABM00153	GVSDFYKNVI NRAKTVEGVN SLKNHI	153
ABM00154	GVSDFYKSLI NRARTVEGVD SLKRHI	154
ABM00155	GVSDYYKNLI NKAKTVEGVE ALKLHI	155

FIGURE 1

Polypeptide	Amino acid sequence	SEQ ID NO:
ABM00156	GVSDFYKNLI NRAKTVEGVD ALKAHI	156
ABM00157	GVSDYYKNLI NKAKTVEGVQ ALKAHI	157
ABM00158	GVSDFYKRLI NRAKTVEGVH ALKGHI	158
ABM00159	GVSDFYKRVI NRAKTVEGVH ALKDHI	159
ABM00160	GVSDYYKRII NRAKTVEGVR ALKLHI	160
ABM00161	GVSDFYKRVI NRAKTVEGVS ALKHEI	161
ABM00162	GVSDYYKRLI NRAKTVEGVN SLKSHI	162
ABM00163	GVSDFYKRLI NRAKTVGGVQ SLKSEI	163
ABM00164	GVSDFYKRLI NRAKTVEGVS SLKGHI	164
ABM00165	GVSDFYKRVI NRAKTVEGVD SLKAEI	165
ABM00166	GVSDFYKRLI NRARTVEGVQ TLKSDI	166
ABM00167	GVSDFYKRFI NKAKTVEGVE TLKSEI	167
ABM00168	GVSDFYKRLI NRAKTVEGVH SLKDEI	168
ABM00169	GVSDYYKRVI NKAKTVEGVS SLKAEI	169
ABM00170	GVSDFYKRLI NRAKTVEGVD ALKSHI	170
ABM00171	GVSDFYKRLI NKAKTVEGVS TLKHDI	171
ABM00172	GVSDFYKRLI NRAKTVEGVS TLKHDI	172
ABM00173	GVSDFYKRLI NRAKTVEGVQ ALKSEI	173
ABM00174	GVSDYYKRLI NKAKTVEGVD SLKVHI	174
ABM00175	GVSDFYKRLI NRAKTVEGVQ SLKTEI	175
ABM00176	GVSDYYKRLI NRAKTVEGVD ALKTHI	176
ABM00177	GVSDFYKRMI NRAKTVEGVD SLKTHI	177
ABM00178	GVSDFYKRLI NRAKTVEGVT TLKTDI	178
ABM00179	GVSDFYKRLI NRAKTVEGVE SLKDHI	179
ABM00180	GVSDFYKRYI NRAKTVEGVH TLKGHI	180
ABM00181	GVSDFYKRLI NRAKTVEGVQ TLKSDI	181
ABM00182	GVSDFYKRLI NRAKTVEGVN SLKSHI	182
ABM00183	GVSDFYKRLI NRAKTVEGVN TLKHDI	183
ABM00184	GVSDFYKRLI NRAKTVEGVE SLKGEI	184
ABM00185	GVSDFYKRLI NKAKTVEGVH TLKHDI	185
ABM00186	GVSDYYKRLI NKAKTVEGVS ALKMHI	186

FIGURE 1

Polypeptide	Amino acid sequence	SEQ ID NO:
ABM00187	GVSDFYKRLI NKAKTVEGVD ALKVHI	187
ABM00188	GVSDYYKRLI NRAKTVEGVH ALKAEI	188
ABM00189	GVSDYYKRLI NRARTVEGVD TLKHDI	189
ABM00190	GVSDFYKRVI NRARTVEGVQ ALKADI	190
ABM00191	GVSDFYKRLI NRAKTVEGVE SLKADI	191
ABM00192	GVSDYYKRLI NKAKTVEGVD ALKAHI	192
ABM00193	GVSDYYKRLI NRAKTVEGVE SLKTHI	193
ABM00194	GVSDFYKRLI NRARTVEGVD SLKVEI	194
ABM00195	GVSDFYKRVI NRAKTVEGVS ALKREI	195
ABM00196	GVSDFYKRLI NRAKTVEGVN ALKSDI	196
ABM00197	GVSDFYKRLI NRAKTVEGVS ALKQEI	197
ABM00198	GVSDFYKRLI NRAKTVEGVQ SLKDHI	198
ABM00199	GVSDFYKRLI NRAKTVEGVD ALKCHI	199
ABM00200	GVSDYYKRLI NKAKTVEGVN ALKTHI	200
ABM00201	GVSDFYKRVI NKAKTVEGVE ALKADI	201
ABM00202	GVSDFYKRLI NRAKTVEGVE TLKRDI	202
ABM00203	GVSDFYKRLI NRARTVEGVQ TLKTDI	203
ABM00204	GVSDFYKRLI NKAKTVEGVN ALKHHI	204
ABM00205	GVSDYYKRLI NRAKTVEGVQ ALKAHI	205
ABM00206	GVSDFYKRVI NRAKTVEGVN SLKNHI	206
ABM00207	GVSDFYKRLI NRARTVEGVD SLKRHI	207
ABM00208	GVSDYYKRLI NKAKTVEGVE ALKLHI	208
ABM00209	GVSDFYKRLI NRAKTVEGVD ALKAHI	209
ABM00210	GVSDYYKRLI NKAKTVEGVQ ALKAHI	210
ABM00211	GVSDFYKRVI NRAKTVEGVS ALKHHI	211
ABM00212	GVSDFYKRLI NRAKTVEGVN TLKAHI	212
ABM00213	GVSDFYKRLI NRAKTVGGVQ SLKSHI	213
ABM00214	GVSDFYKRVI NRAKTVEGVD SLKAHI	214
ABM00215	GVSDFYKRLI NRARTVEGVQ TLKSHI	215
ABM00216	GVSDFYKRFI NKAKTVEGVE TLKSHI	216
ABM00217	GVSDFYKRLI NRAKTVEGVH SLKDHI	217

FIGURE 1

Polypeptide	Amino acid sequence	SEQ ID NO:
ABM00218	GVSDYYKRVI NKAKTVEGVS SLKAHI	218
ABM00219	GVSDFYKRLI NKAKTVEGVS TLKHHI	219
ABM00220	GVSDFYKRLI NRAKTVEGVS TLKHHI	220
ABM00221	GVSDFYKRLI NRAKTVEGVQ ALKSHI	221
ABM00222	GVSDFYKRLI NRAKTVEGVQ SLKTHI	222
ABM00223	GVSDFYKRLI NRAKTVEGVT TLKTHI	223
ABM00224	GVSDFYKRLI NRAKTVEGVQ TLKSHI	224
ABM00225	GVSDFYKRLI NRAKTVEGVN TLKHHI	225
ABM00226	GVSDFYKRLI NRAKTVEGVE SLKGHI	226
ABM00227	GVSDFYKRLI NKAKTVEGVH TLKHHI	227
ABM00228	GVSDYYKRLI NRAKTVEGVH ALKAHI	228
ABM00229	GVSDYYKRLI NRARTVEGVD TLKHHI	229
ABM00230	GVSDFYKRVI NRARTVEGVQ ALKAHI	230
ABM00231	GVSDFYKRLI NRAKTVEGVE SLKAHI	231
ABM00232	GVSDFYKRLI NRARTVEGVD SLKVHI	232
ABM00233	GVSDFYKRVI NRAKTVEGVS ALKRHI	233
ABM00234	GVSDFYKRLI NRAKTVEGVN ALKSHI	234
ABM00235	GVSDFYKRLI NRAKTVEGVS ALKQHI	235
ABM00236	GVSDFYKRVI NKAKTVEGVE ALKAHI	236
ABM00237	GVSDFYKRLI NRAKTVEGVE TLKRHI	237
ABM00238	GVSDFYKRLI NRARTVEGVQ TLKTHI	238
ABM00239	GVSDFYKRLI NKAKTVEGVE ALKLHI	239
ABM00240	GVSDYYKNLI NRARTVEGVE ALKLHI	240
ABM00241	GVSDYYKNII NRAKTVEGVE ALKLHI	241
ABM00242	GVSDFYKNLI NRAKTVEGVE ALKLHI	242
ABM00243	GVSDFYKNVI NKAKTVEGVE ALKLHI	243
ABM00244	GVSDYYKNLI NRAKTVEGVE ALKLHI	244
ABM00245	GVSDYYKNLI NRARTVEGVH ALIDHI	245
ABM00246	GVSDFYKRLI NKAKTVEGVE ALKLHI	246
ABM00247	GVSDYYKRLI SKAKTVEGVK ALISEI	247
ABM00248	GVSDFYKRLI NKAKTVEGVE ALKLHI	248

FIGURE 1

Polypeptide	Amino acid sequence	SEQ ID NO:
ABM00249	GVSDYYKRLI SKAKTVEGVK ALISEI	249
ABM00250	GVSDYYKRLI SKAKTVEGVK ALISEI	250
ABM00251	GVSDYYKRLI SKAKTVEGVK ALISEI	251
ABM00252	GVSDYYKRLI SKAKTVEGVK ALISEI	252
ABM00253	GVSDYYKRLI SKAKTVEGVK ALISEI	253
ABM00254	GVSDYYKRLI SKAKTVEGVK ALISEI	254
ABM00255	GVSDYYKRLI SKAKTVEGVK ALISEI	255
ABM00256	GVSDYYKRLI SKAKTVEGVK ALISEI	256
ABM00257	GVSDYYKRLI SKAKTVEGVK ALISEI	257
ABD00001	LAEAKVLANR ELDKYGVSDF YKNLINRAKT VEGVHALIGH ILAALP	258
ABD00002	LAEAKVLANR ELDKYGVSDF YKNVINRAKT VEGVHALIDH ILAALP	259
ABD00003	LAEAKVLANR ELDKYGVSDY YKNIINRAKT VEGVRALKLH ILAALP	260
ABD00004	LAEAKVLANR ELDKYGVSDF YKNVINRAKT VEGVSALIHE ILAALP	261
ABD00005	LAEAKVLANR ELDKYGVSDF YKRLINRAKT VEGVNTLIAD ILAALP	262
ABD00006	LAEAKVLANR ELDKYGVSDF YKNLINRAKT VEGVNTLIAD ILAALP	263
ABD00007	LAEAKVLANR ELDKYGVSDY YKNLINRAKT VEGVNSLISH ILAALP	264
ABD00008	LAEAKVLANR ELDKYGVSDF YKRLINRAKT VGGVQSLISE ILAALP	265
ABD00009	LAEAKVLANR ELDKYGVSDF YKNLINRAKT VEGVSSLKGH ILAALP	266
ABD00010	LAEAKVLANR ELDKYGVSDF YKNVINRAKT VEGVDSLIAE ILAALP	267
ABD00011	LAEAKVLANR ELDKYGVSDF YKNLINRART VEGVQTLISD ILAALP	268
ABD00012	LAEAKVLANR ELDKYGVSDF YKKFINKAKT VEGVETLISE ILAALP	269
ABD00013	LAEAKVLANR ELDKYGVSDF YKSLINRAKT VEGVHSLTDE ILAALP	270
ABD00014	LAEAKVLANR ELDKYGVSDY YKNVINKAKT VEGVSSLTAE ILAALP	271
ABD00015	LAEAKVLANR ELDKYGVSDF YKSLINRAKT VEGVDALTSH ILAALP	272
ABD00016	LAEAKVLANR ELDKYGVSDF YKNLINKAKT VEGVSTLIHD ILAALP	273
ABD00017	LAEAKVLANR ELDKYGVSDF YKNLINRAKT VEGVSTLIHD ILAALP	274
ABD00018	LAEAKVLANR ELDKYGVSDF YKNLINRAKT VEGVQALISE ILAALP	275
ABD00019	LAEAKVLANR ELDKYGVSDY YKSLINKAKT VEGVDSLIVH ILAALP	276
ABD00020	LAEAKVLANR ELDKYGVSDF YKNLINRAKT VEGVQSLITE ILAALP	277
ABD00021	LAEAKVLANR ELDKYGVSDY YKNLINRAKT VEGVDALITH ILAALP	278
ABD00022	LAEAKVLANR ELDKYGVSDF YKSMINRAKT VEGVDSLITH ILAALP	279

FIGURE 1

Polypeptide	Amino acid sequence	SEQ ID NO:
ABD00023	LAEAKVLANR ELDKYGVSDF YKNLINRAKT VEGVTTLTTD ILAALP	280
ABD00024	LAEAKVLANR ELDKYGVSDF YKNLINRAKT VEGVESLIDH ILAALP	281
ABD00025	LAEAKVLANR ELDKYGVSDF YKSYINRAKT VEGVHTLIGH ILAALP	282
ABD00026	LAEAKVLANR ELDKYGVSDF YKNLINRAKT VEGVQTLISD ILAALP	283
ABD00027	LAEAKVLANR ELDKYGVSDF YKNLINRAKT VEGVNSLTSH ILAALP	284
ABD00028	LAEAKVLANR ELDKYGVSDF YKNLINRAKT VEGVNTLIHD ILAALP	285
ABD00029	LAEAKVLANR ELDKYGVSDF YKNLINRAKT VEGVESLIGE ILAALP	286
ABD00030	LAEAKVLANR ELDKYGVSDF YKNLINKAKT VEGVHTLIHD ILAALP	287
ABD00031	LAEAKVLANR ELDKYGVSDY YKNLINKAKT VEGVSALKMH ILAALP	288
ABD00032	LAEAKVLANR ELDKYGVSDF YKNLINKAKT VEGVDALIVH ILAALP	289
ABD00033	LAEAKVLANR ELDKYGVSDY YKRLINRAKT VEGVHALIAE ILAALP	290
ABD00034	LAEAKVLANR ELDKYGVSDY YKNLINRART VEGVDTLIHD ILAALP	291
ABD00035	LAEAKVLANR ELDKYGVSDF YKKVINRART VEGVQALIAD ILAALP	292
ABD00036	LAEAKVLANR ELDKYGVSDF YKNLINRAKT VEGVESLIAD ILAALP	293
ABD00037	LAEAKVLANR ELDKYGVSDY YKNLINKAKT VEGVDALIAH ILAALP	294
ABD00038	LAEAKVLANR ELDKYGVSDY YKNLINRAKT VEGVESLITH ILAALP	295
ABD00039	LAEAKVLANR ELDKYGVSDF YKNLINRART VEGVDSLIVE ILAALP	296
ABD00040	LAEAKVLANR ELDKYGVSDF YKNVINRAKT VEGVSALIRE ILAALP	297
ABD00041	LAEAKVLANR ELDKYGVSDF YKNLINRAKT VEGVNALISD ILAALP	298
ABD00042	LAEAKVLANR ELDKYGVSDF YKNLINRAKT VEGVSALIQE ILAALP	299
ABD00043	LAEAKVLANR ELDKYGVSDF YKNLINRAKT VEGVQSLIDH ILAALP	300
ABD00044	LAEAKVLANR ELDKYGVSDF YKNLINRAKT VEGVDALICH ILAALP	301
ABD00045	LAEAKVLANR ELDKYGVSDY YKRLINKAKT VEGVNALITH ILAALP	302
ABD00046	LAEAKVLANR ELDKYGVSDF YKNVINKAKT VEGVEALIAD ILAALP	303
ABD00047	LAEAKVLANR ELDKYGVSDF YKNLINRAKT VEGVETLIRD ILAALP	304
ABD00048	LAEAKVLANR ELDKYGVSDF YKNLINRART VEGVQTLITD ILAALP	305
ABD00049	LAEAKVLANR ELDKYGVSDF YKRLINKAKT VEGVNALTHH ILAALP	306
ABD00050	LAEAKVLANR ELDKYGVSDY YKNLINRAKT VEGVQALIAH ILAALP	307
ABD00051	LAEAKVLANR ELDKYGVSDF YKNVINRAKT VEGVNSLINH ILAALP	308
ABD00052	LAEAKVLANR ELDKYGVSDF YKSLINRART VEGVDSLIRH ILAALP	309
ABD00053	LAEAKVLANR ELDKYGVSDY YKNLINKAKT VEGVEALTLH ILAALP	310

FIGURE 1

Polypeptide	Amino acid sequence	SEQ ID NO:
ABD00054	LAEAKVLANR ELDKYGVSDF YKNLINRAKT VEGVDALIAH ILAALP	311
ABD00055	LAEAKVLANR ELDKYGVSDY YKNLINKAKT VEGVQALIAH ILAALP	312
ABD00056	LAEAKVLANR ELDKYGVSDF YKRLINRAKT VEGVHALIGH ILAALP	313
ABD00057	LAEAKVLANR ELDKYGVSDF YKRVINRAKT VEGVHALIDH ILAALP	314
ABD00058	LAEAKVLANR ELDKYGVSDY YKRIINRAKT VEGVRALKLH ILAALP	315
ABD00059	LAEAKVLANR ELDKYGVSDF YKRVINRAKT VEGVSALIHE ILAALP	316
ABD00060	LAEAKVLANR ELDKYGVSDF YKRLINRAKT VEGVNTLIAD ILAALP	317
ABD00061	LAEAKVLANR ELDKYGVSDY YKRLINRAKT VEGVNSLISH ILAALP	318
ABD00062	LAEAKVLANR ELDKYGVSDF YKRLINRAKT VEGVSSLKGH ILAALP	319
ABD00063	LAEAKVLANR ELDKYGVSDF YKRVINRAKT VEGVDSLIAE ILAALP	320
ABD00064	LAEAKVLANR ELDKYGVSDF YKRLINRART VEGVQTLISD ILAALP	321
ABD00065	LAEAKVLANR ELDKYGVSDF YKRFINKAKT VEGVETLISE ILAALP	322
ABD00066	LAEAKVLANR ELDKYGVSDF YKRLINRAKT VEGVHSLTDE ILAALP	323
ABD00067	LAEAKVLANR ELDKYGVSDY YKRVINKAKT VEGVSSLTAE ILAALP	324
ABD00068	LAEAKVLANR ELDKYGVSDF YKRLINRAKT VEGVDALTSH ILAALP	325
ABD00069	LAEAKVLANR ELDKYGVSDF YKRLINKAKT VEGVSTLIHD ILAALP	326
ABD00070	LAEAKVLANR ELDKYGVSDF YKRLINRAKT VEGVSTLIHD ILAALP	327
ABD00071	LAEAKVLANR ELDKYGVSDF YKRLINRAKT VEGVQALISE ILAALP	328
ABD00072	LAEAKVLANR ELDKYGVSDY YKRLINKAKT VEGVDSLIVH ILAALP	329
ABD00073	LAEAKVLANR ELDKYGVSDF YKRLINRAKT VEGVQSLITE ILAALP	330
ABD00074	LAEAKVLANR ELDKYGVSDY YKRLINRAKT VEGVDALITH ILAALP	331
ABD00075	LAEAKVLANR ELDKYGVSDF YKRMINRAKT VEGVDSLITH ILAALP	332
ABD00076	LAEAKVLANR ELDKYGVSDF YKRLINRAKT VEGVTTLTTD ILAALP	333
ABD00077	LAEAKVLANR ELDKYGVSDF YKRLINRAKT VEGVESLIDH ILAALP	334
ABD00078	LAEAKVLANR ELDKYGVSDF YKRYINRAKT VEGVHTLIGH ILAALP	335
ABD00079	LAEAKVLANR ELDKYGVSDF YKRLINRAKT VEGVQTLISD ILAALP	336
ABD00080	LAEAKVLANR ELDKYGVSDF YKRLINRAKT VEGVNSLTSH ILAALP	337
ABD00081	LAEAKVLANR ELDKYGVSDF YKRLINRAKT VEGVNTLIHD ILAALP	338
ABD00082	LAEAKVLANR ELDKYGVSDF YKRLINRAKT VEGVESLIGE ILAALP	339
ABD00083	LAEAKVLANR ELDKYGVSDF YKRLINKAKT VEGVHTLIHD ILAALP	340
ABD00084	LAEAKVLANR ELDKYGVSDY YKRLINKAKT VEGVSALKMH ILAALP	341

FIGURE 1

Polypeptide	Amino acid sequence	SEQ ID NO:
ABD00085	LAEAKVLANR ELDKYGVSDF YKRLINKAKT VEGVDALIVH ILAALP	342
ABD00086	LAEAKVLANR ELDKYGVSDY YKRLINRART VEGVDTLIHD ILAALP	343
ABD00087	LAEAKVLANR ELDKYGVSDF YKRVINRART VEGVQALIAD ILAALP	344
ABD00088	LAEAKVLANR ELDKYGVSDF YKRLINRAKT VEGVESLIAD ILAALP	345
ABD00089	LAEAKVLANR ELDKYGVSDY YKRLINKAKT VEGVDALIAH ILAALP	346
ABD00090	LAEAKVLANR ELDKYGVSDY YKRLINRAKT VEGVESLITH ILAALP	347
ABD00091	LAEAKVLANR ELDKYGVSDF YKRLINRART VEGVDSLIVE ILAALP	348
ABD00092	LAEAKVLANR ELDKYGVSDF YKRVINRAKT VEGVSALIRE ILAALP	349
ABD00093	LAEAKVLANR ELDKYGVSDF YKRLINRAKT VEGVNALISD ILAALP	350
ABD00094	LAEAKVLANR ELDKYGVSDF YKRLINRAKT VEGVSALIQE ILAALP	351
ABD00095	LAEAKVLANR ELDKYGVSDF YKRLINRAKT VEGVQSLIDH ILAALP	352
ABD00096	LAEAKVLANR ELDKYGVSDF YKRLINRAKT VEGVDALICH ILAALP	353
ABD00097	LAEAKVLANR ELDKYGVSDF YKRVINKAKT VEGVEALIAD ILAALP	354
ABD00098	LAEAKVLANR ELDKYGVSDF YKRLINRAKT VEGVETLIRD ILAALP	355
ABD00099	LAEAKVLANR ELDKYGVSDF YKRLINRART VEGVQTLITD ILAALP	356
ABD00100	LAEAKVLANR ELDKYGVSDY YKRLINRAKT VEGVQALIAH ILAALP	357
ABD00101	LAEAKVLANR ELDKYGVSDF YKRVINRAKT VEGVNSLINH ILAALP	358
ABD00102	LAEAKVLANR ELDKYGVSDF YKRLINRART VEGVDSLIRH ILAALP	359
ABD00103	LAEAKVLANR ELDKYGVSDY YKRLINKAKT VEGVEALTLH ILAALP	360
ABD00104	LAEAKVLANR ELDKYGVSDF YKRLINRAKT VEGVDALIAH ILAALP	361
ABD00105	LAEAKVLANR ELDKYGVSDY YKRLINKAKT VEGVQALIAH ILAALP	362
ABD00106	LAEAKVLANR ELDKYGVSDF YKNLINRAKT VEGVHALKGH ILAALP	363
ABD00107	LAEAKVLANR ELDKYGVSDF YKNVINRAKT VEGVHALKDH ILAALP	364
ABD00108	LAEAKVLANR ELDKYGVSDF YKNVINRAKT VEGVSALKHE ILAALP	365
ABD00109	LAEAKVLANR ELDKYGVSDF YKRLINRAKT VEGVNTLKAD ILAALP	366
ABD00110	LAEAKVLANR ELDKYGVSDF YKNLINRAKT VEGVNTLKAD ILAALP	367
ABD00111	LAEAKVLANR ELDKYGVSDY YKNLINRAKT VEGVNSLKSH ILAALP	368
ABD00112	LAEAKVLANR ELDKYGVSDF YKRLINRAKT VGGVQSLKSE ILAALP	369
ABD00113	LAEAKVLANR ELDKYGVSDF YKNVINRAKT VEGVDSLKAE ILAALP	370
ABD00114	LAEAKVLANR ELDKYGVSDF YKNLINRART VEGVQTLKSD ILAALP	371
ABD00115	LAEAKVLANR ELDKYGVSDF YKKFINKAKT VEGVETLKSE ILAALP	372

FIGURE 1

Polypeptide	Amino acid sequence	SEQ ID NO:
ABD00116	LAEAKVLANR ELDKYGVSDF YKSLINRAKT VEGVHSLKDE ILAALP	373
ABD00117	LAEAKVLANR ELDKYGVSDY YKNVINKAKT VEGVSSLKAE ILAALP	374
ABD00118	LAEAKVLANR ELDKYGVSDF YKSLINRAKT VEGVDALKSH ILAALP	375
ABD00119	LAEAKVLANR ELDKYGVSDF YKNLINKAKT VEGVSTLKHD ILAALP	376
ABD00120	LAEAKVLANR ELDKYGVSDF YKNLINRAKT VEGVSTLKHD ILAALP	377
ABD00121	LAEAKVLANR ELDKYGVSDF YKNLINRAKT VEGVQALKSE ILAALP	378
ABD00122	LAEAKVLANR ELDKYGVSDY YKSLINKAKT VEGVDSLKVH ILAALP	379
ABD00123	LAEAKVLANR ELDKYGVSDF YKNLINRAKT VEGVQSLKTE ILAALP	380
ABD00124	LAEAKVLANR ELDKYGVSDY YKNLINRAKT VEGVDALKTH ILAALP	381
ABD00125	LAEAKVLANR ELDKYGVSDF YKSMINRAKT VEGVDSLKTH ILAALP	382
ABD00126	LAEAKVLANR ELDKYGVSDF YKNLINRAKT VEGVTTLKTD ILAALP	383
ABD00127	LAEAKVLANR ELDKYGVSDF YKNLINRAKT VEGVESLKDH ILAALP	384
ABD00128	LAEAKVLANR ELDKYGVSDF YKSYINRAKT VEGVHTLKGH ILAALP	385
ABD00129	LAEAKVLANR ELDKYGVSDF YKNLINRAKT VEGVQTLKSD ILAALP	386
ABD00130	LAEAKVLANR ELDKYGVSDF YKNLINRAKT VEGVNSLKSH ILAALP	387
ABD00131	LAEAKVLANR ELDKYGVSDF YKNLINRAKT VEGVNTLKHD ILAALP	388
ABD00132	LAEAKVLANR ELDKYGVSDF YKNLINRAKT VEGVESLKGE ILAALP	389
ABD00133	LAEAKVLANR ELDKYGVSDF YKNLINKAKT VEGVHTLKHD ILAALP	390
ABD00134	LAEAKVLANR ELDKYGVSDF YKNLINKAKT VEGVDALKVH ILAALP	391
ABD00135	LAEAKVLANR ELDKYGVSDY YKRLINRAKT VEGVHALKAE ILAALP	392
ABD00136	LAEAKVLANR ELDKYGVSDY YKNLINRART VEGVDTLKHD ILAALP	393
ABD00137	LAEAKVLANR ELDKYGVSDF YKKVINRART VEGVQALKAD ILAALP	394
ABD00138	LAEAKVLANR ELDKYGVSDF YKNLINRAKT VEGVESLKAD ILAALP	395
ABD00139	LAEAKVLANR ELDKYGVSDY YKNLINKAKT VEGVDALKAH ILAALP	396
ABD00140	LAEAKVLANR ELDKYGVSDY YKNLINRAKT VEGVESLKTH ILAALP	397
ABD00141	LAEAKVLANR ELDKYGVSDF YKNLINRART VEGVDSLKVE ILAALP	398
ABD00142	LAEAKVLANR ELDKYGVSDF YKNVINRAKT VEGVSALKRE ILAALP	399
ABD00143	LAEAKVLANR ELDKYGVSDF YKNLINRAKT VEGVNALKSD ILAALP	400
ABD00144	LAEAKVLANR ELDKYGVSDF YKNLINRAKT VEGVSALKQE ILAALP	401
ABD00145	LAEAKVLANR ELDKYGVSDF YKNLINRAKT VEGVQSLKDH ILAALP	402
ABD00146	LAEAKVLANR ELDKYGVSDF YKNLINRAKT VEGVDALKCH ILAALP	403

FIGURE 1

Polypeptide	Amino acid sequence	SEQ ID NO:
ABD00147	LAEAKVLANR ELDKYGVSDY YKRLINKAKT VEGVNALKTH ILAALP	404
ABD00148	LAEAKVLANR ELDKYGVSDF YKNVINKAKT VEGVEALKAD ILAALP	405
ABD00149	LAEAKVLANR ELDKYGVSDF YKNLINRAKT VEGVETLKRD ILAALP	406
ABD00150	LAEAKVLANR ELDKYGVSDF YKNLINRART VEGVQTLKTD ILAALP	407
ABD00151	LAEAKVLANR ELDKYGVSDF YKRLINKAKT VEGVNALKHH ILAALP	408
ABD00152	LAEAKVLANR ELDKYGVSDY YKNLINRAKT VEGVQALKAH ILAALP	409
ABD00153	LAEAKVLANR ELDKYGVSDF YKNVINRAKT VEGVNSLKNH ILAALP	410
ABD00154	LAEAKVLANR ELDKYGVSDF YKSLINRART VEGVDSLKRH ILAALP	411
ABD00155	LAEAKVLANR ELDKYGVSDY YKNLINKAKT VEGVEALKLH ILAALP	412
ABD00156	LAEAKVLANR ELDKYGVSDF YKNLINRAKT VEGVDALKAH ILAALP	413
ABD00157	LAEAKVLANR ELDKYGVSDY YKNLINKAKT VEGVQALKAH ILAALP	414
ABD00158	LAEAKVLANR ELDKYGVSDF YKRLINRAKT VEGVHALKGH ILAALP	415
ABD00159	LAEAKVLANR ELDKYGVSDF YKRVINRAKT VEGVHALKDH ILAALP	416
ABD00160	LAEAKVLANR ELDKYGVSDY YKRIINRAKT VEGVRALKLH ILAALP	417
ABD00161	LAEAKVLANR ELDKYGVSDF YKRVINRAKT VEGVSALKHE ILAALP	418
ABD00162	LAEAKVLANR ELDKYGVSDY YKRLINRAKT VEGVNSLKSH ILAALP	419
ABD00163	LAEAKVLANR ELDKYGVSDF YKRLINRAKT VGGVQSLKSE ILAALP	420
ABD00164	LAEAKVLANR ELDKYGVSDF YKRLINRAKT VEGVSSLKGH ILAALP	421
ABD00165	LAEAKVLANR ELDKYGVSDF YKRVINRAKT VEGVDSLKAE ILAALP	422
ABD00166	LAEAKVLANR ELDKYGVSDF YKRLINRART VEGVQTLKSD ILAALP	423
ABD00167	LAEAKVLANR ELDKYGVSDF YKRFINKAKT VEGVETLKSE ILAALP	424
ABD00168	LAEAKVLANR ELDKYGVSDF YKRLINRAKT VEGVHSLKDE ILAALP	425
ABD00169	LAEAKVLANR ELDKYGVSDY YKRVINKAKT VEGVSSLKAE ILAALP	426
ABD00170	LAEAKVLANR ELDKYGVSDF YKRLINRAKT VEGVDALKSH ILAALP	427
ABD00171	LAEAKVLANR ELDKYGVSDF YKRLINKAKT VEGVSTLKHD ILAALP	428
ABD00172	LAEAKVLANR ELDKYGVSDF YKRLINRAKT VEGVSTLKHD ILAALP	429
ABD00173	LAEAKVLANR ELDKYGVSDF YKRLINRAKT VEGVQALKSE ILAALP	430
ABD00174	LAEAKVLANR ELDKYGVSDY YKRLINKAKT VEGVDSLKVH ILAALP	431
ABD00175	LAEAKVLANR ELDKYGVSDF YKRLINRAKT VEGVQSLKTE ILAALP	432
ABD00176	LAEAKVLANR ELDKYGVSDY YKRLINRAKT VEGVDALKTH ILAALP	433
ABD00177	LAEAKVLANR ELDKYGVSDF YKRMINRAKT VEGVDSLKTH ILAALP	434

FIGURE 1

Polypeptide	Amino acid sequence	SEQ ID NO:
ABD00178	LAEAKVLANR ELDKYGVSDF YKRLINRAKT VEGVTTLKTD ILAALP	435
ABD00179	LAEAKVLANR ELDKYGVSDF YKRLINRAKT VEGVESLKDH ILAALP	436
ABD00180	LAEAKVLANR ELDKYGVSDF YKRYINRAKT VEGVHTLKGH ILAALP	437
ABD00181	LAEAKVLANR ELDKYGVSDF YKRLINRAKT VEGVQTLKSD ILAALP	438
ABD00182	LAEAKVLANR ELDKYGVSDF YKRLINRAKT VEGVNSLKSH ILAALP	439
ABD00183	LAEAKVLANR ELDKYGVSDF YKRLINRAKT VEGVNTLKHD ILAALP	440
ABD00184	LAEAKVLANR ELDKYGVSDF YKRLINRAKT VEGVESLKGE ILAALP	441
ABD00185	LAEAKVLANR ELDKYGVSDF YKRLINKAKT VEGVHTLKHD ILAALP	442
ABD00186	LAEAKVLANR ELDKYGVSDY YKRLINKAKT VEGVSALKMH ILAALP	443
ABD00187	LAEAKVLANR ELDKYGVSDF YKRLINKAKT VEGVDALKVH ILAALP	444
ABD00188	LAEAKVLANR ELDKYGVSDY YKRLINRAKT VEGVHALKAE ILAALP	445
ABD00189	LAEAKVLANR ELDKYGVSDY YKRLINRART VEGVDTLKHD ILAALP	446
ABD00190	LAEAKVLANR ELDKYGVSDF YKRVINRART VEGVQALKAD ILAALP	447
ABD00191	LAEAKVLANR ELDKYGVSDF YKRLINRAKT VEGVESLKAD ILAALP	448
ABD00192	LAEAKVLANR ELDKYGVSDY YKRLINKAKT VEGVDALKAH ILAALP	449
ABD00193	LAEAKVLANR ELDKYGVSDY YKRLINRAKT VEGVESLKTH ILAALP	450
ABD00194	LAEAKVLANR ELDKYGVSDF YKRLINRART VEGVDSLKVE ILAALP	451
ABD00195	LAEAKVLANR ELDKYGVSDF YKRVINRAKT VEGVSALKRE ILAALP	452
ABD00196	LAEAKVLANR ELDKYGVSDF YKRLINRAKT VEGVNALKSD ILAALP	453
ABD00197	LAEAKVLANR ELDKYGVSDF YKRLINRAKT VEGVSALKQE ILAALP	454
ABD00198	LAEAKVLANR ELDKYGVSDF YKRLINRAKT VEGVQSLKDH ILAALP	455
ABD00199	LAEAKVLANR ELDKYGVSDF YKRLINRAKT VEGVDALKCH ILAALP	456
ABD00200	LAEAKVLANR ELDKYGVSDY YKRLINKAKT VEGVNALKTH ILAALP	457
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ABD00202	LAEAKVLANR ELDKYGVSDF YKRLINRAKT VEGVETLKRD ILAALP	459
ABD00203	LAEAKVLANR ELDKYGVSDF YKRLINRART VEGVQTLKTD ILAALP	460
ABD00204	LAEAKVLANR ELDKYGVSDF YKRLINKAKT VEGVNALKHH ILAALP	461
ABD00205	LAEAKVLANR ELDKYGVSDY YKRLINRAKT VEGVQALKAH ILAALP	462
ABD00206	LAEAKVLANR ELDKYGVSDF YKRVINRAKT VEGVNSLKNH ILAALP	463
ABD00207	LAEAKVLANR ELDKYGVSDF YKRLINRART VEGVDSLKRH ILAALP	464
ABD00208	LAEAKVLANR ELDKYGVSDY YKRLINKAKT VEGVEALKLH ILAALP	465

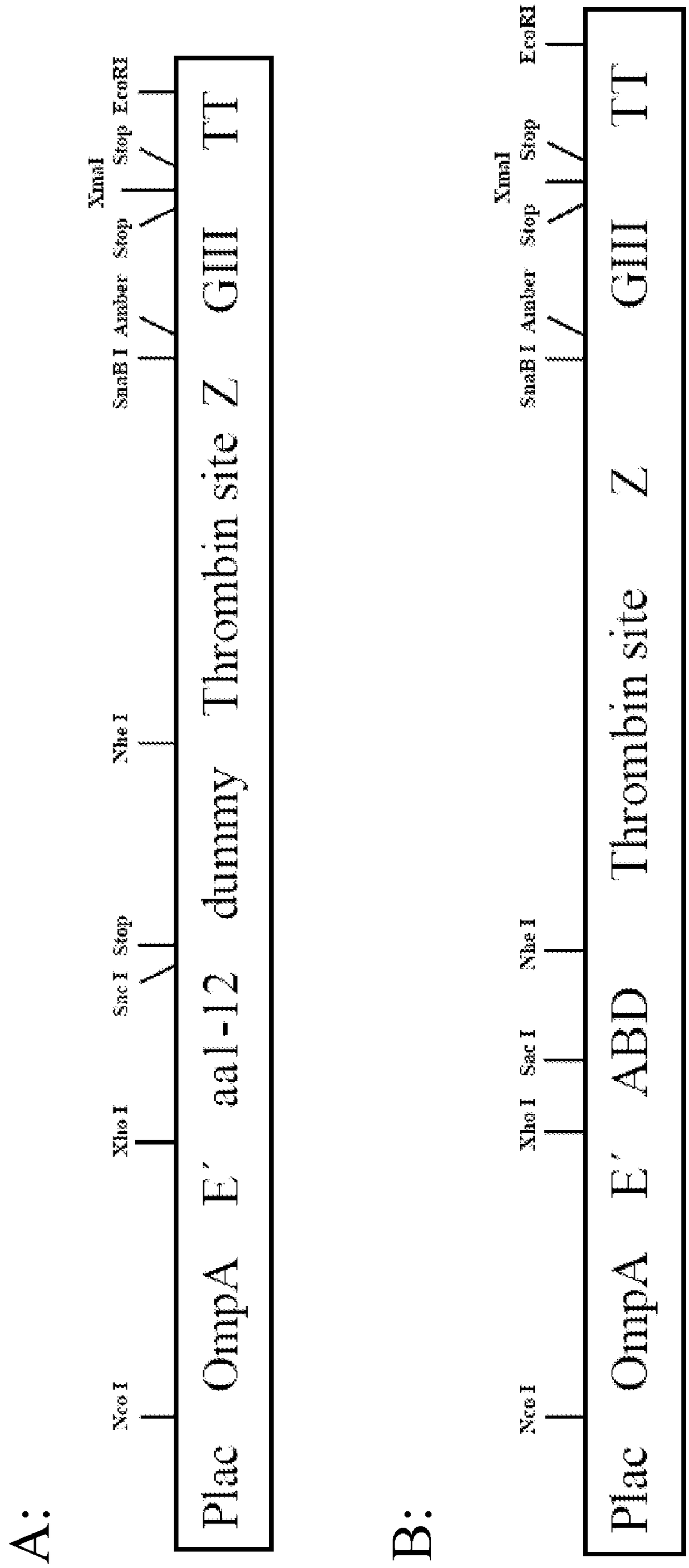
FIGURE 1

Polypeptide	Amino acid sequence	SEQ ID NO:
ABD00209	LAEAKVLANR ELDKYGVSDF YKRLINRAKT VEGVDALKAH ILAALP	466
ABD00210	LAEAKVLANR ELDKYGVSDY YKRLINKAKT VEGVQALKAH ILAALP	467
ABD00211	LAEAKVLANR ELDKYGVSDF YKRVINRAKT VEGVSALKHH ILAALP	468
ABD00212	LAEAKVLANR ELDKYGVSDF YKRLINRAKT VEGVNTLKAH ILAALP	469
ABD00213	LAEAKVLANR ELDKYGVSDF YKRLINRAKT VGGVQSLKSH ILAALP	470
ABD00214	LAEAKVLANR ELDKYGVSDF YKRVINRAKT VEGVDSLKAH ILAALP	471
ABD00215	LAEAKVLANR ELDKYGVSDF YKRLINRART VEGVQTLKSH ILAALP	472
ABD00216	LAEAKVLANR ELDKYGVSDF YKRFINKAKT VEGVETLKSH ILAALP	473
ABD00217	LAEAKVLANR ELDKYGVSDF YKRLINRAKT VEGVHSLKDH ILAALP	474
ABD00218	LAEAKVLANR ELDKYGVSDY YKRVINKAKT VEGVSSLKAH ILAALP	475
ABD00219	LAEAKVLANR ELDKYGVSDF YKRLINKAKT VEGVSTLKHH ILAALP	476
ABD00220	LAEAKVLANR ELDKYGVSDF YKRLINRAKT VEGVSTLKHH ILAALP	477
ABD00221	LAEAKVLANR ELDKYGVSDF YKRLINRAKT VEGVQALKSH ILAALP	478
ABD00222	LAEAKVLANR ELDKYGVSDF YKRLINRAKT VEGVQSLKTH ILAALP	479
ABD00223	LAEAKVLANR ELDKYGVSDF YKRLINRAKT VEGVTTLKTH ILAALP	480
ABD00224	LAEAKVLANR ELDKYGVSDF YKRLINRAKT VEGVQTLKSH ILAALP	481
ABD00225	LAEAKVLANR ELDKYGVSDF YKRLINRAKT VEGVNTLKHH ILAALP	482
ABD00226	LAEAKVLANR ELDKYGVSDF YKRLINRAKT VEGVESLKGH ILAALP	483
ABD00227	LAEAKVLANR ELDKYGVSDF YKRLINKAKT VEGVHTLKHH ILAALP	484
ABD00228	LAEAKVLANR ELDKYGVSDY YKRLINRAKT VEGVHALKAH ILAALP	485
ABD00229	LAEAKVLANR ELDKYGVSDY YKRLINRART VEGVDTLKHH ILAALP	486
ABD00230	LAEAKVLANR ELDKYGVSDF YKRVINRART VEGVQALKAH ILAALP	487
ABD00231	LAEAKVLANR ELDKYGVSDF YKRLINRAKT VEGVESLKAH ILAALP	488
ABD00232	LAEAKVLANR ELDKYGVSDF YKRLINRART VEGVDSLKVH ILAALP	489
ABD00233	LAEAKVLANR ELDKYGVSDF YKRVINRAKT VEGVSALKRH ILAALP	490
ABD00234	LAEAKVLANR ELDKYGVSDF YKRLINRAKT VEGVNALKSH ILAALP	491
ABD00235	LAEAKVLANR ELDKYGVSDF YKRLINRAKT VEGVSALKQH ILAALP	492
ABD00236	LAEAKVLANR ELDKYGVSDF YKRVINKAKT VEGVEALKAH ILAALP	493
ABD00237	LAEAKVLANR ELDKYGVSDF YKRLINRAKT VEGVETLKRH ILAALP	494
ABD00238	LAEAKVLANR ELDKYGVSDF YKRLINRART VEGVQTLKTH ILAALP	495
ABD00239	LAEAKVLANR ELDKYGVSDF YKRLINKAKT VEGVEALKLH ILAALP	496

FIGURE 1

Polypeptide	Amino acid sequence	SEQ ID NO:
ABD00240	LAEAKVLANR ELDKYGVSDY YKNLINRART VEGVEALKLH ILAALP	497
ABD00241	LAEAKVLANR ELDKYGVSDY YKNIINRAKT VEGVEALKLH ILAALP	498
ABD00242	LAEAKVLANR ELDKYGVSDF YKNLINRAKT VEGVEALKLH ILAALP	499
ABD00243	LAEAKVLANR ELDKYGVSDF YKNVINKAKT VEGVEALKLH ILAALP	500
ABD00244	LAEAKVLANR ELDKYGVSDY YKNLINRAKT VEGVEALKLH ILAALP	501
ABD00245	LAEAKVLANR ELDKYGVSDY YKNLINRART VEGVHALIDH ILAALP	502
ABD00246	LAEAKVLALR ELDKYGVSDF YKRLINKAKT VEGVEALKLH ILAALP	503
ABD00247	LAEAKVLALR ELDKYGVSDY YKRLISKAKT VEGVKALISE ILAALP	504
ABD00248	LAEAKVLAIR ELDKYGVSDF YKRLINKAKT VEGVEALKLH ILAALP	505
ABD00249	LAEAKVLAIR ELDKYGVSDY YKRLISKAKT VEGVKALISE ILAALP	506
ABD00250	LAEAKVLAIK ELDKYGVSDY YKRLISKAKT VEGVKALISE ILAALP	507
ABD00251	LAEAKELANR ELDKYGVSDY YKRLISKAKT VEGVKALISE ILAALP	508
ABD00252	LAEAKVDANR ELDKYGVSDY YKRLISKAKT VEGVKALISE ILAALP	509
ABD00253	LAEAKEDANR ELDKYGVSDY YKRLISKAKT VEGVKALISE ILAALP	510
ABD00254	LAEAKEDAIK ELDKYGVSDY YKRLISKAKT VEGVKALISE ILAALP	511
ABD00255	LAEAKVLALK ELDKYGVSDY YKRLISKAKT VEGVKALISE ILAALP	512
ABD00256	LAEAKELAIK ELDKYGVSDY YKRLISKAKT VEGVKALISE ILAALP	513
ABD00257	LAEAKVDAIK ELDKYGVSDY YKRLISKAKT VEGVKALISE ILAALP	514
ABDwt	LAEAKVLANR ELDKYGVSDY YKNLINNAKT VEGVKALIDE ILAALP	515

FIGURE 1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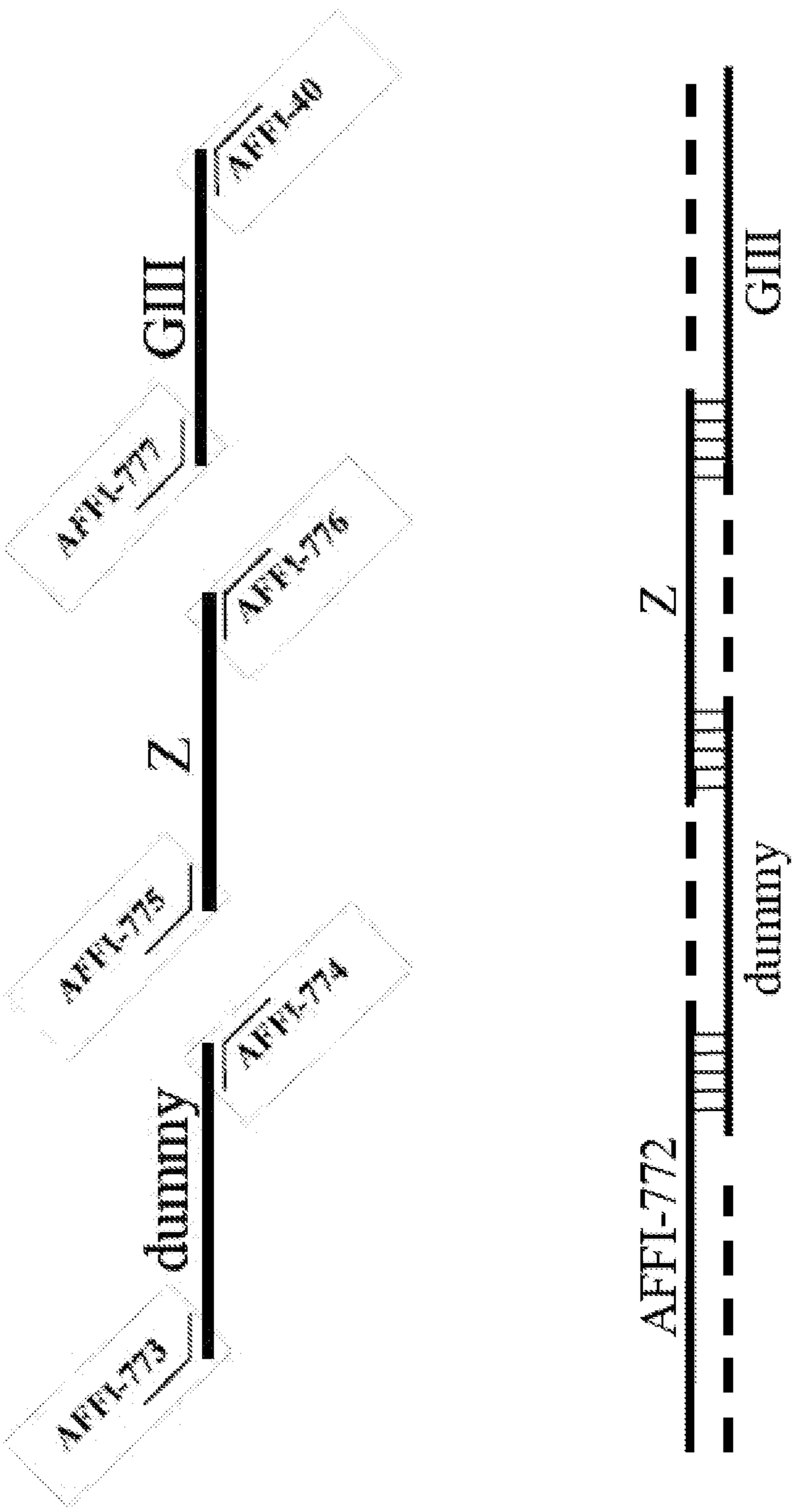
SnaB I Amber Stop
Xma I
Stop
EcoRI
Plac OmpA GIII TT
Xho I
Sac I Stop
E´ aal-12 dummy Thrombin site Z
Nco I
B:
Nhe I
SnaB I Amber Stop
Xma I
Stop
EcoRI
Plac OmpA GIII TT
Xho I
Sac I
Nhe I
E´ ABD Thrombin site Z
Nco I

FIGURE 2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AFFL-773
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GIII
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B:
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dummy
Z
GIII

FIGURE 3

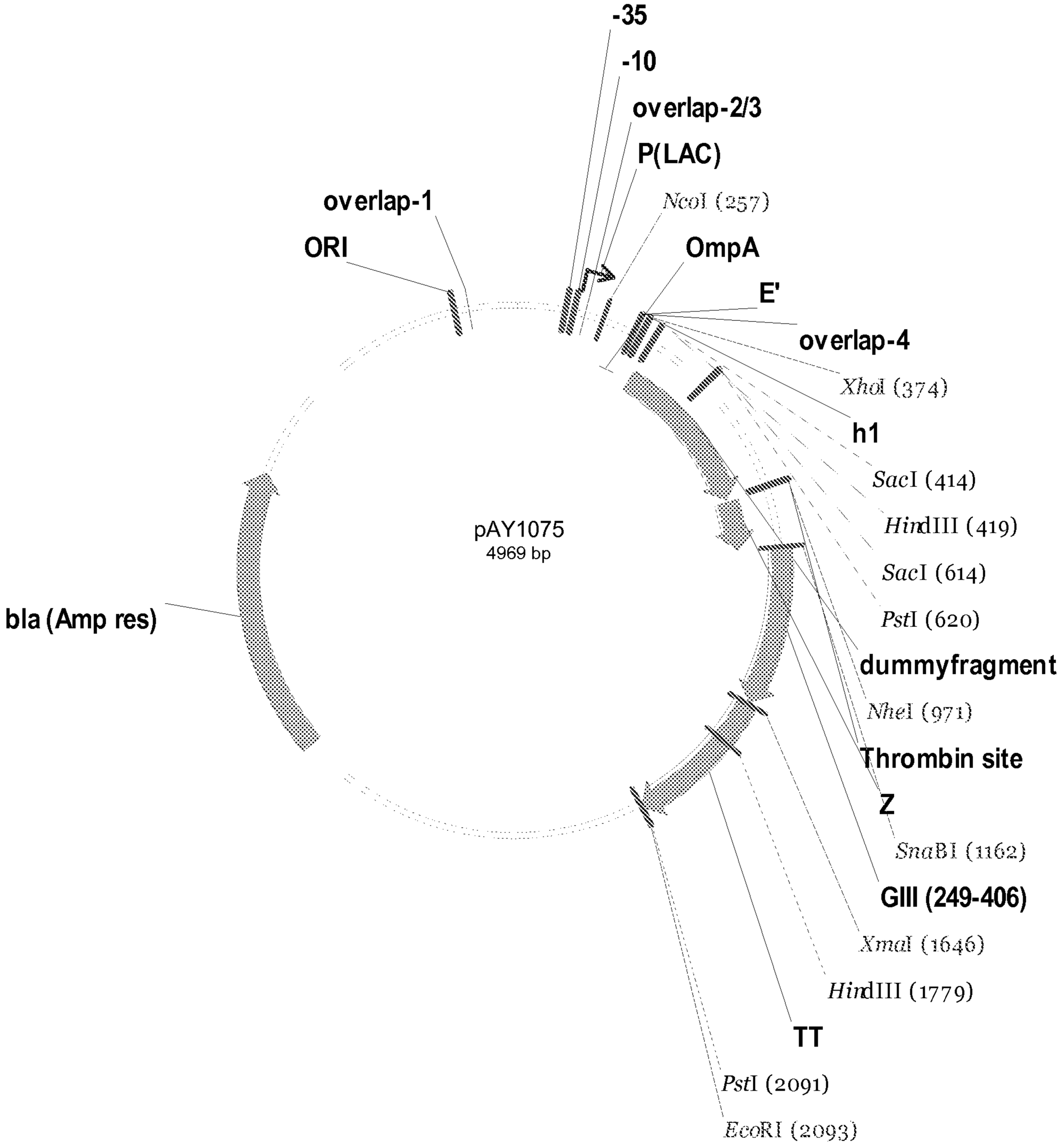

-35
-10
overlap-2/3
P(LAC)
overlap-1
NcoI (257)
ORI
OmpA
E'
overlap-4
XhoI (374)
h1
SacI (414)
HindIII (419)
SacI (614)
PstI (620)
dummyfragment
NheI (971)
Thrombin site
Z
SnaBI (1162)
GIII (249-406)
XmaI (1646)
HindIII (1779)
TT
PstI (2091)
EcoRI (2093)
pAY1075
4969 bp
bla (Amp res)

FIGURE 4

-35
-10
overlap-2/3
P(LAC)
overlap-1
ORI
NotI (239)
NcoI (257)
OmpA
E'
overlap-4
XhoI (374)
SacI (414)
ABD
pAY1075-ABD
4516 bp
NheI (518)
Thrombin site
Z
SnaBI (709)
GIII (249-406)
NdeI (1063)
XmaI (1193)
SmaI (1195)
HindIII (1326)
bla (Amp res)
TT
EcoRI (1640)

FIGURE 5

	Pos18	Pos18	Pos20	Pos20	Pos23	Pos23	Pos24	Pos24	Pos27	Pos27	Pos29	Pos29	Pos30	Pos30	Pos32	Pos32	Pos33	Pos33	Pos35	Pos35	Pos36	Pos36	Pos37	Pos37	Pos39	Pos39	Pos40	Pos40	Pos41	Pos41
Ala (A)															5,53	1,33	8,5	8	6,25	10,7	34	26,7			6,25	5,3				
Arg (R)					25	21			25	17	10	11			0,93	2,67	12,38	11	9,38	4					9,38	11				
Asn (N)	10	6,67			25	37			25	39			10	12	0,55	0	4,125	1,3	3,13	2,67					3,13	2,7				
Asp (D)															6,24	2,67	4,25	1,3	3,13	5,33					3,13	2,7	25	27		
Cys (C)															0,05	0			3,13	1,33					3,13	2,7				
Gln (Q)															5	8	4,125	1,3	3,13	0					3,13	0	25	23		
Glu (E)															56,17	52	4,24	2,7	3,13	2,67					3,13	0	25	16		
Gly (G)															5,53	5,33	8,5	6,7	6,25	2,67					6,25	2,7				
His (H)															0,55	4	4,125	4	3,13	1,33					3,13	1,3	25	35		
Ile (I)							5	8							0,05	0	4,125	8	3,13	2,67			5	9,3	3,13	1,3			79	77
Leu (L)							75	76							0,93	1,33	8,25	12	9,38	18,7			75	76	9,38	17			4,4	6,7
Lys (K)					25	20			25	15	90	89			5	9,33	4,125	0	3,13	0					3,13	0				
Met (M)							5	2,7							0,44	1,33	4,125	4	3,13	2,67			5	2,7	3,13	2,7			8,8	9,3
Phe (F)			50	54,7			5	0							0,05	0			3,13	6,67			5	4	3,13	2,7			3,6	0
Pro (P)															0,49	0	8,25	23	6,25	5,33					6,25	16				
Ser (S)	80	88			25	21			25	29			10	9,3	0,54	0	4,125	2,7	9,38	9,33	33	52			9,38	16				
Thr (T)	10	5,33											80	79	0,49	0	8,25	6,7	6,25	2,67	33	20			6,25	2,7				
Trp (w)															0,44	0			3,13	5,33					3,13	4				
Tyr (Y)			50	45,3											0,55	0			3,13	6,67					3,13	2,7				
Val (V)							10	13							5,53	4	8,5	8	6,25	5,33			10	11	6,25	6,7			4	6,7
Stop (.)															5	8			3,13	4					3,13	0				

FIGURE 6

	"Pos18"	"Pos18"	Pos18	Pos20	Pos23	Pos24	Pos27	Pos29	Pos30	Pos32	Pos33	Pos35	Pos36	Pos37	Pos39	Pos40	Pos40	Pos41	Pos41
Ala (A)	50	45	80	50	25	5	25	90	10	5,53	7,8	11,7	34		6,5				
Arg (R)	50	55	10	50	25	75	25	10	10	0,93	10	11,7			16				
Asn (N)			11,7		42		39		10	0,55	1,3	0			2,6				
Asp (D)										6,24	1,3	3,9			1,3	25			
Cys (C)										0,05		0			2,6		25		
Gln (Q)										5	1,3	3,9			2,6	25			
Glu (E)										56,17	2,6	1,3			1,3	25	17		
Gly (G)										5,53	6,5	1,3			3,9				
His (H)										0,55	3,9	2,6			1,3	25	44		
Ile (I)						9,1				0,05	7,8	0		5	3,13			79	
Leu (L)						78				0,93	12	23,4		75	9,1			4,4	
Lys (K)					16		7,8	86		5	0	1,3			0				
Met (M)						3,9				0,44	3,9	1,3		5	3,9			8,8	14
Phe (F)	50	5,2		58,4		5				0,05	2,6	6,49		5	2,6			3,6	2,6
Pro (P)										0,49	7,8	13			7,8				
Ser (S)	50	45	72,7		31		38		7,8	0,54	13	6,49	40,3	1,3	7,8				
Thr (T)	50	55	15,6						82	0,49	2,6	2,6	27,3	71	3,9				
Trp (w)										0,44	1,3	1,3			2,6				
Tyr (Y)				41,6						0,55	0	0			7,8				3,9
Val (V)						3,9				5,53	8,5	6,49		17	6,25				
Stop (.)										5		1,3			3,13				

FIGURE 7

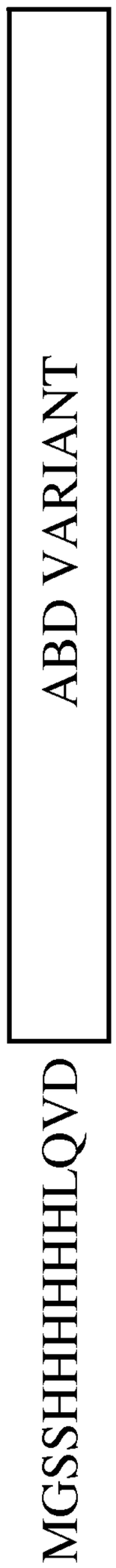
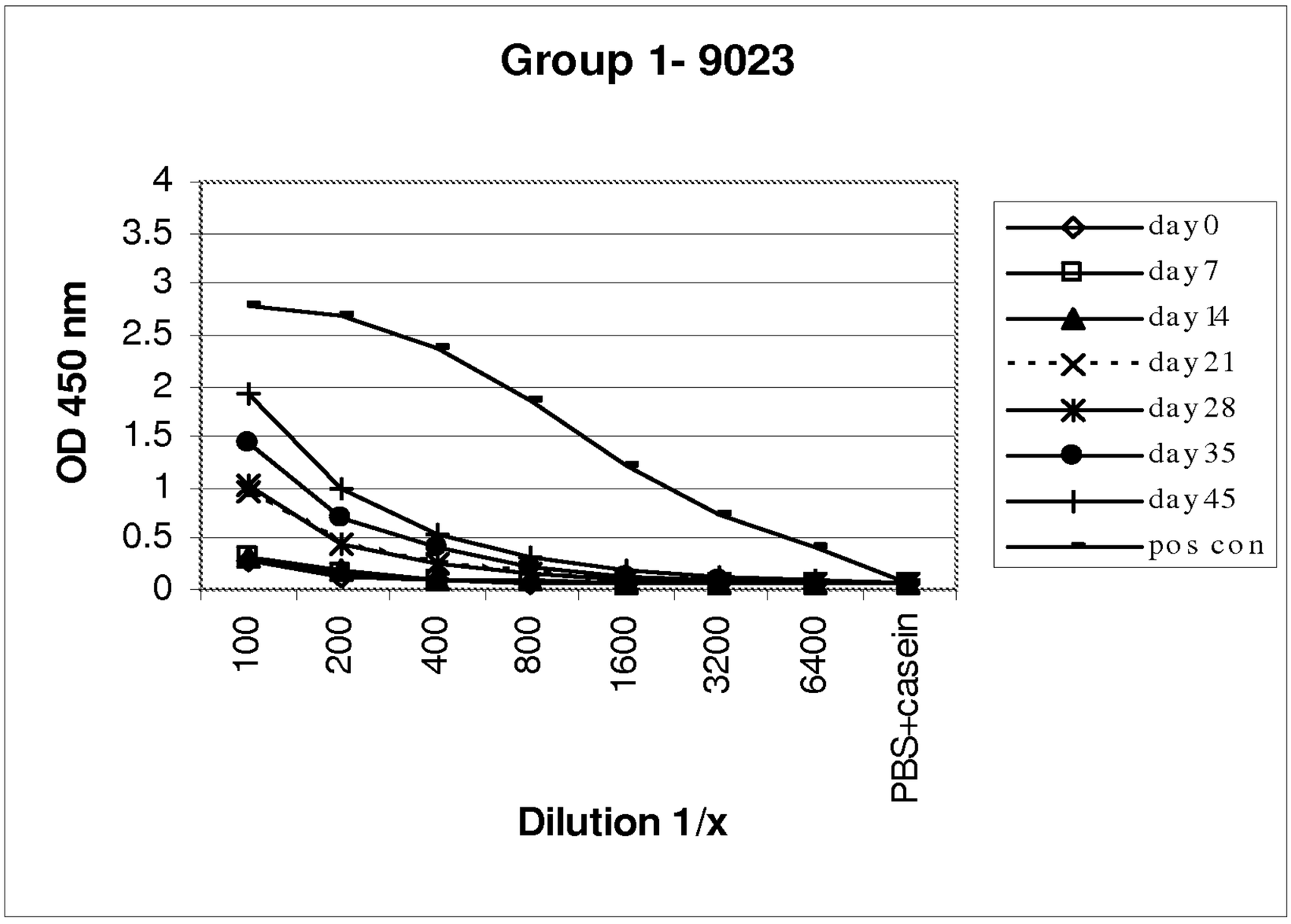


FIGURE 8


Group 1- 9023
OD 450 nm
4
3.5
3
2.5
2
1.5
1
0.5
0
100
200
400
800
1600
3200
6400
PBS+casein
Dilution 1/x
day 0
day 7
day 14
day 21
day 28
day 35
day 45
pos con

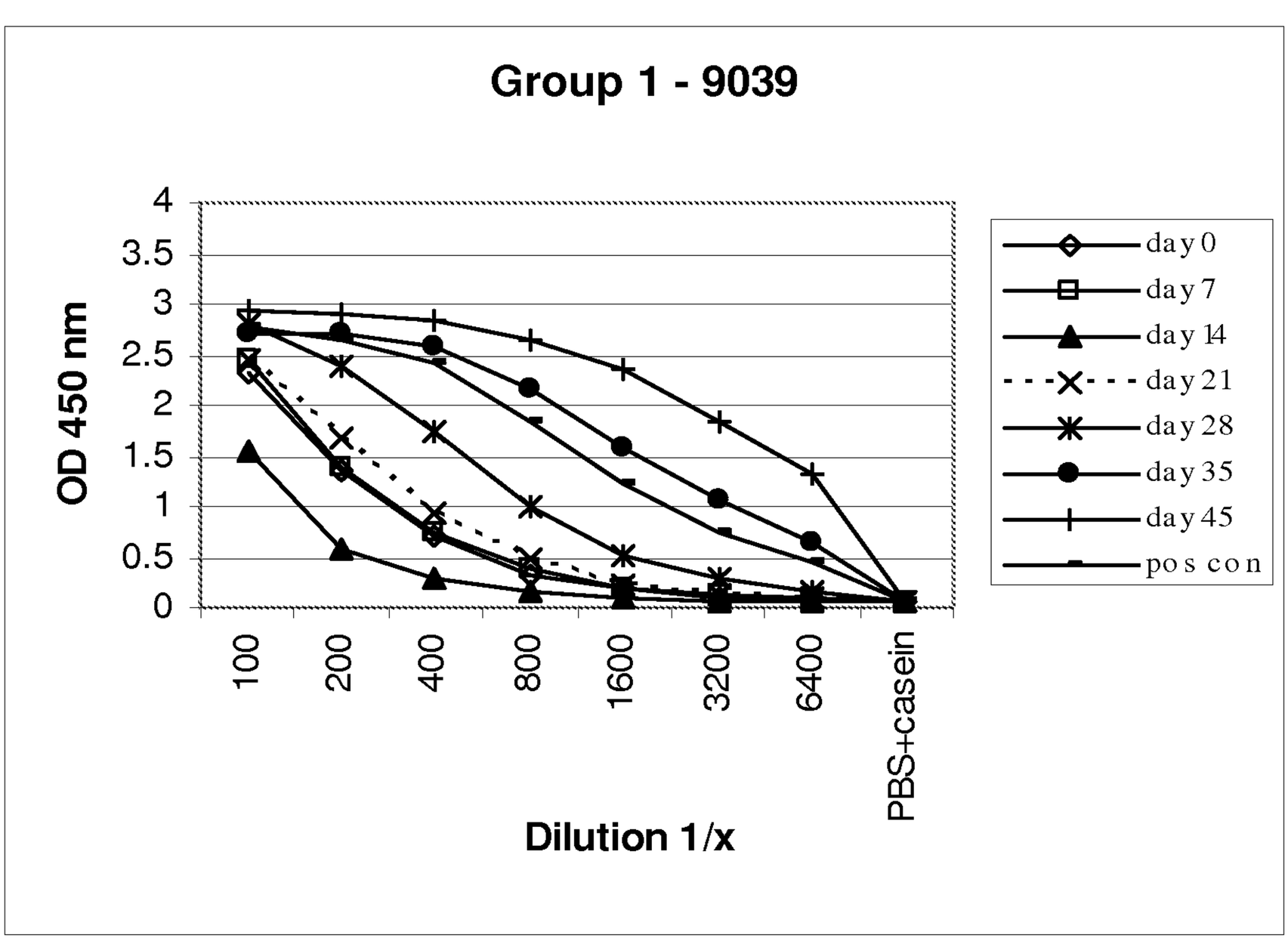

Group 1 - 9039
OD 450 nm
4
3.5
3
2.5
2
1.5
1
0.5
0
100
200
400
800
1600
3200
6400
PBS+casein
Dilution 1/x
day 0
day 7
day 14
day 21
day 28
day 35
day 45
pos con

FIGURE 9A

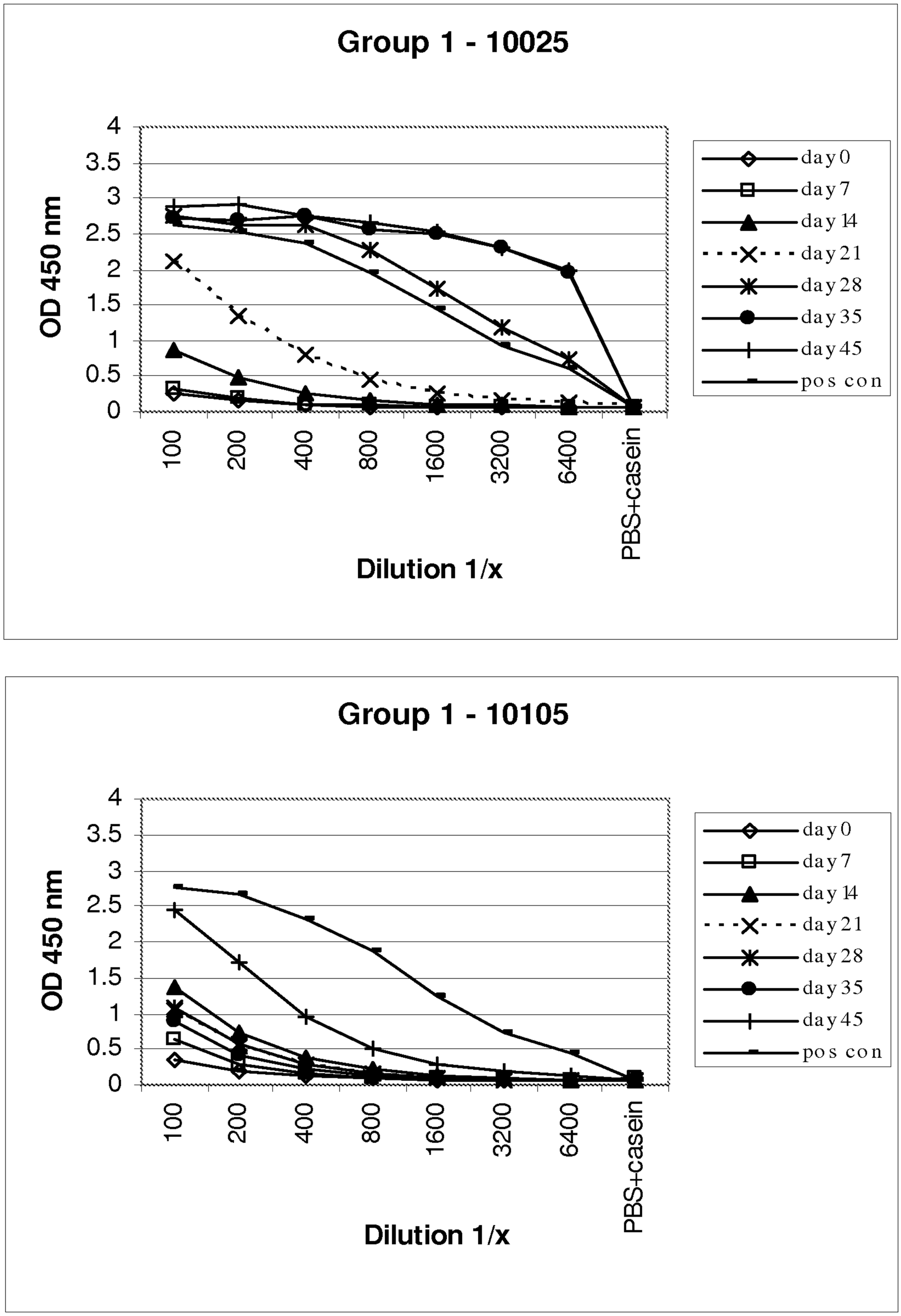

Group 1 - 10025
OD 450 nm
4
3.5
3
2.5
2
1.5
1
0.5
0
100
200
400
800
1600
3200
6400
PBS+casein
Dilution 1/x
day 0
day 7
day 14
day 21
day 28
day 35
day 45
pos con
Group 1 - 10105
OD 450 nm
4
3.5
3
2.5
2
1.5
1
0.5
0
100
200
400
800
1600
3200
6400
PBS+casein
Dilution 1/x
day 0
day 7
day 14
day 21
day 28
day 35
day 45
pos con

FIGURE 9B

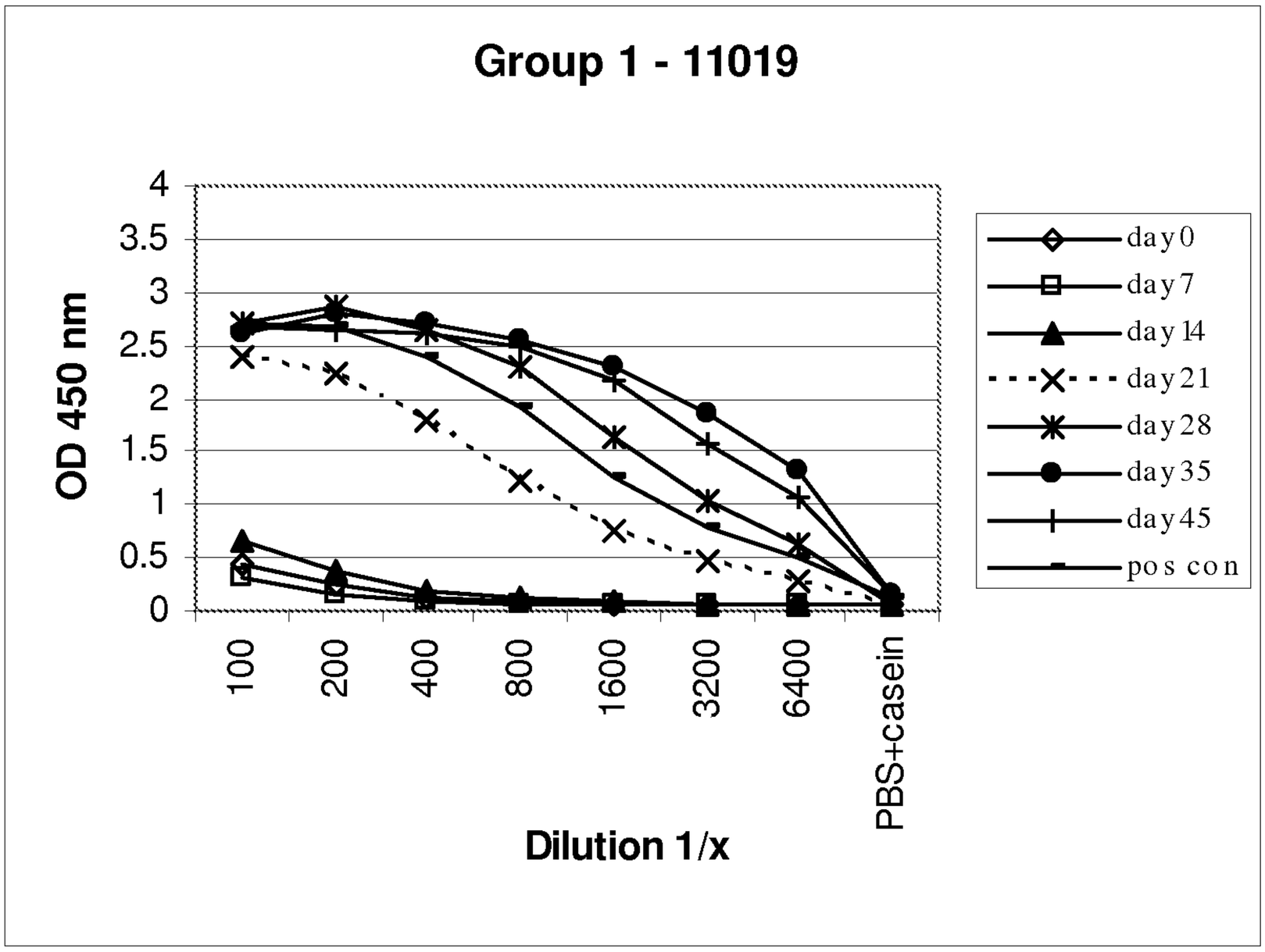
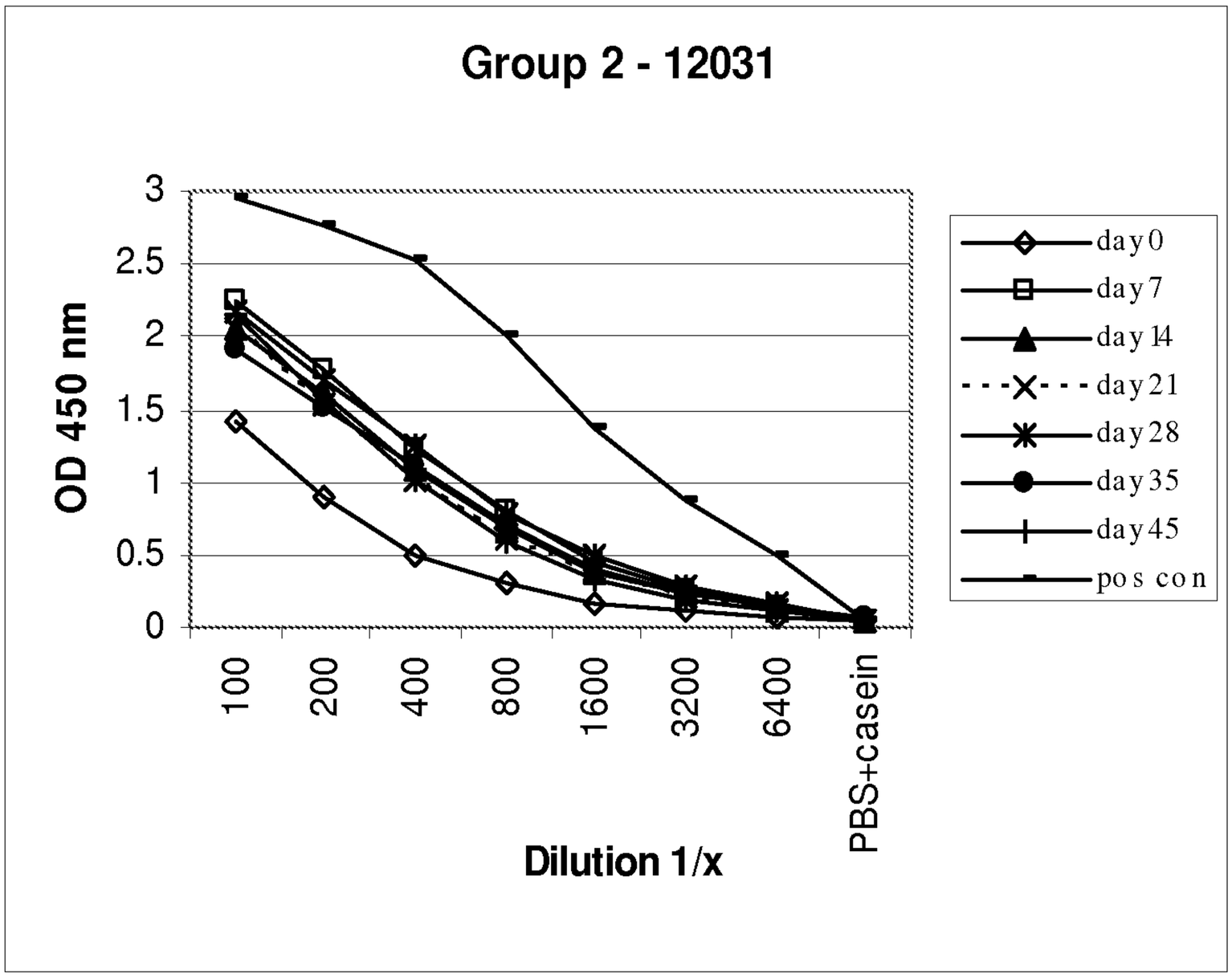

Group 1 - 11019
OD 450 nm
4
3.5
3
2.5
2
1.5
1
0.5
0
100
200
400
800
1600
3200
6400
PBS+casein
Dilution 1/x
day 0
day 7
day 14
day 21
day 28
day 35
day 45
pos con

FIGURE 9C


Group 2 - 12031
OD 450 nm
3
2.5
2
1.5
1
0.5
0
100
200
400
800
1600
3200
6400
PBS+casein
Dilution 1/x
day 0
day 7
day 14
day 21
day 28
day 35
day 45
pos con

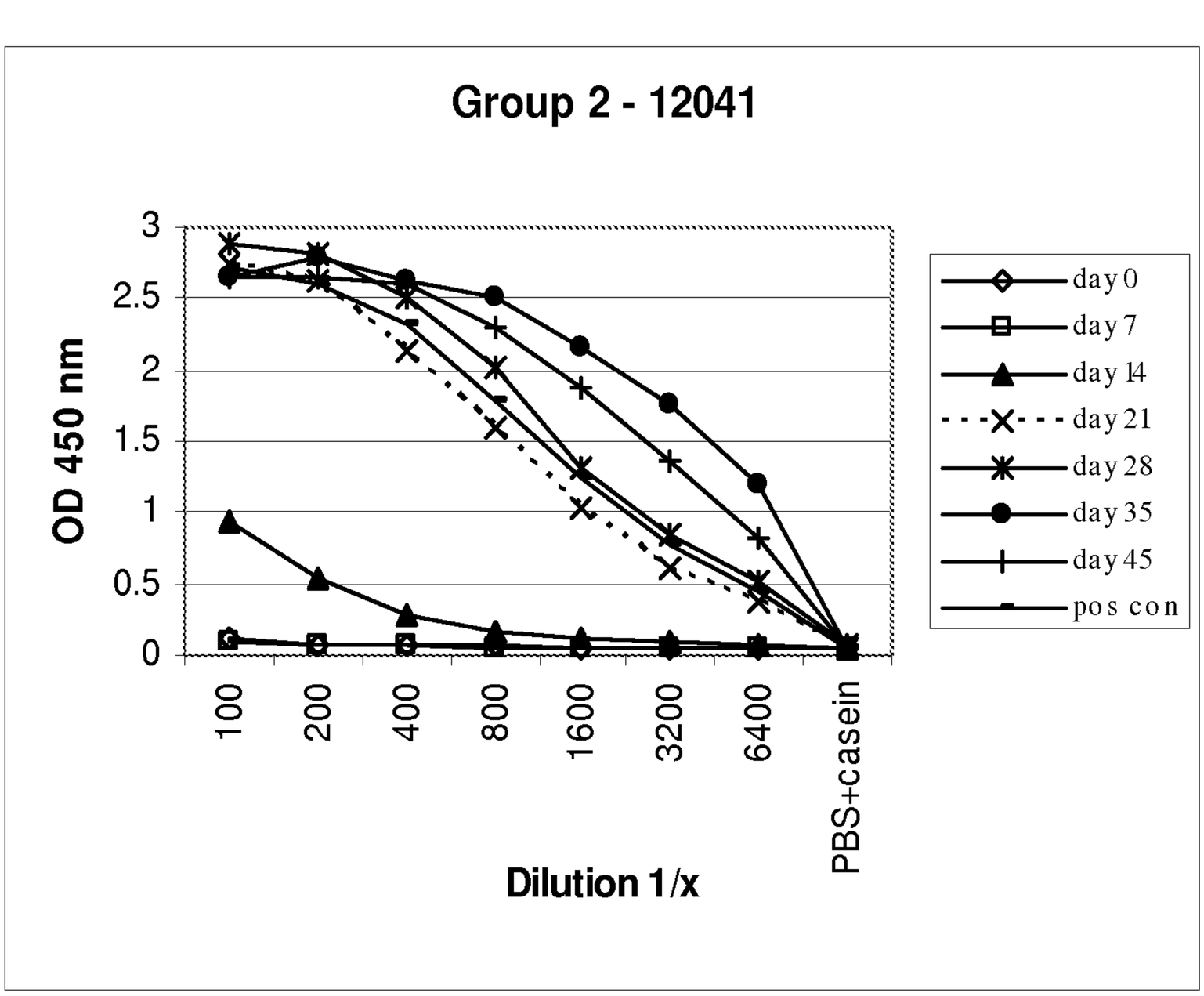
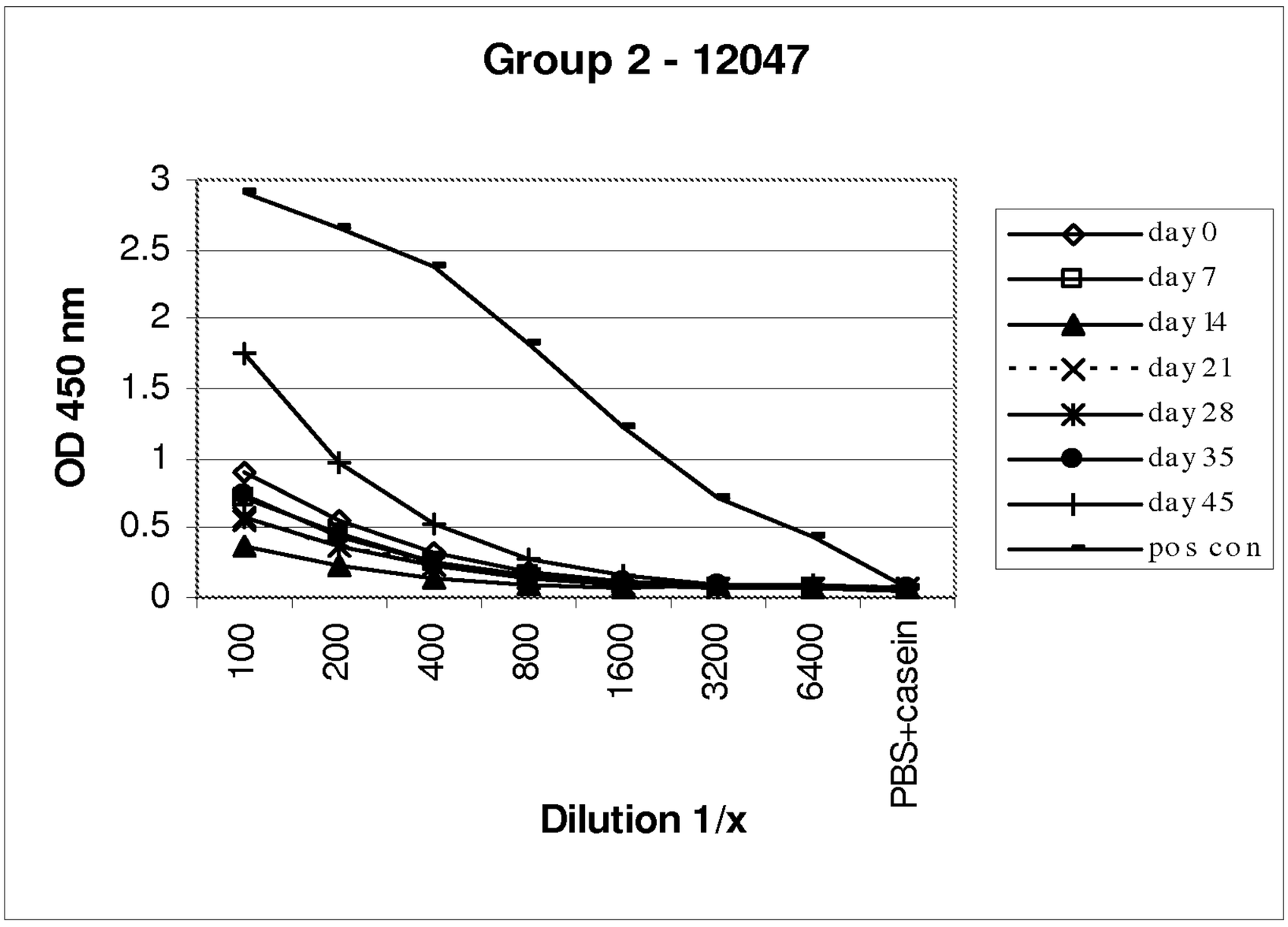

Group 2 - 12041
OD 450 nm
3
2.5
2
1.5
1
0.5
0
100
200
400
800
1600
3200
6400
PBS+casein
Dilution 1/x
day 0
day 7
day 14
day 21
day 28
day 35
day 45
pos con

FIGURE 10A


Group 2 - 12047
OD 450 nm
3
2.5
2
1.5
1
0.5
0
100
200
400
800
1600
3200
6400
PBS+casein
Dilution 1/x
day 0
day 7
day 14
day 21
day 28
day 35
day 45
pos con

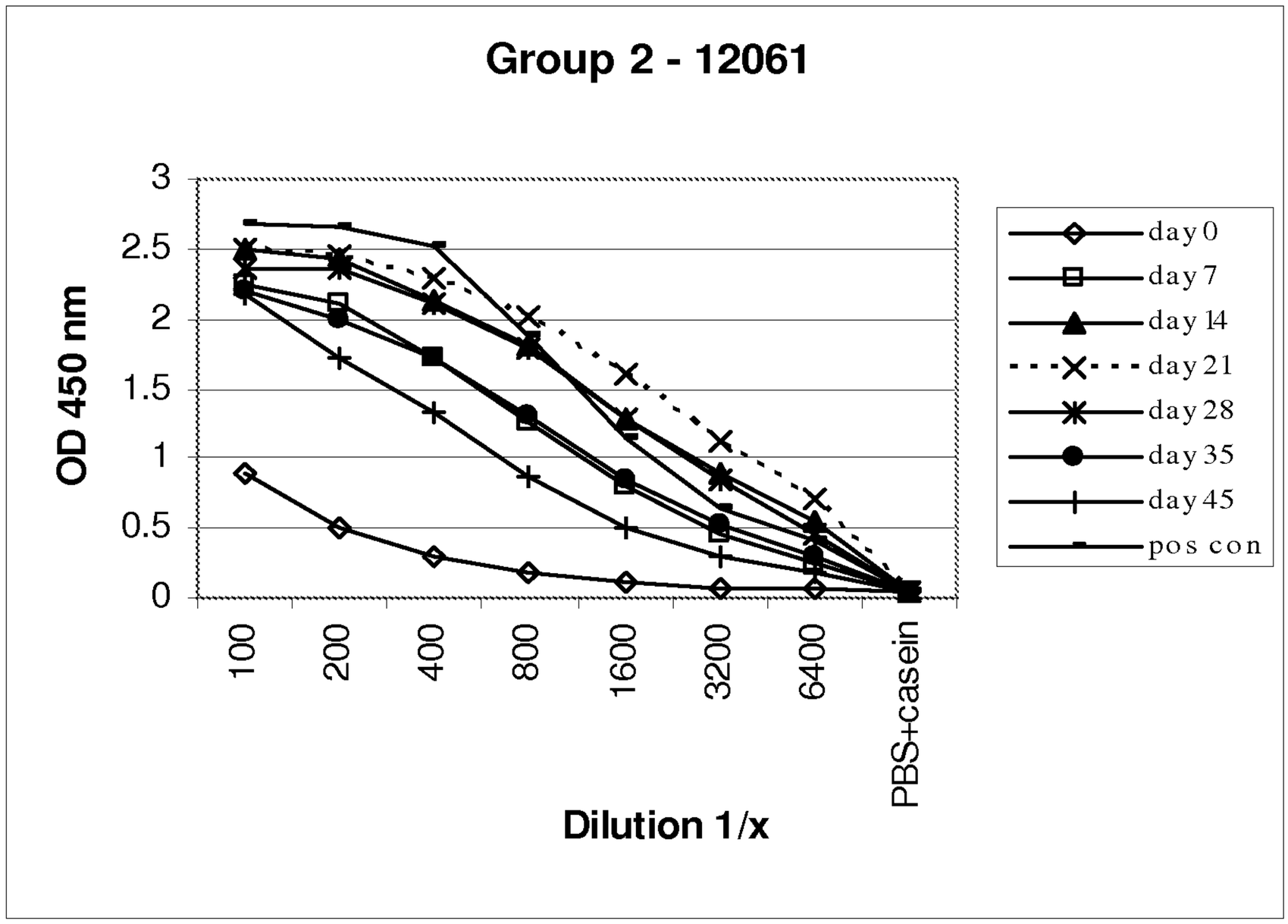

Group 2 - 12061
OD 450 nm
3
2.5
2
1.5
1
0.5
0
100
200
400
800
1600
3200
6400
PBS+casein
Dilution 1/x
day 0
day 7
day 14
day 21
day 28
day 35
day 45
pos con

FIGURE 10B

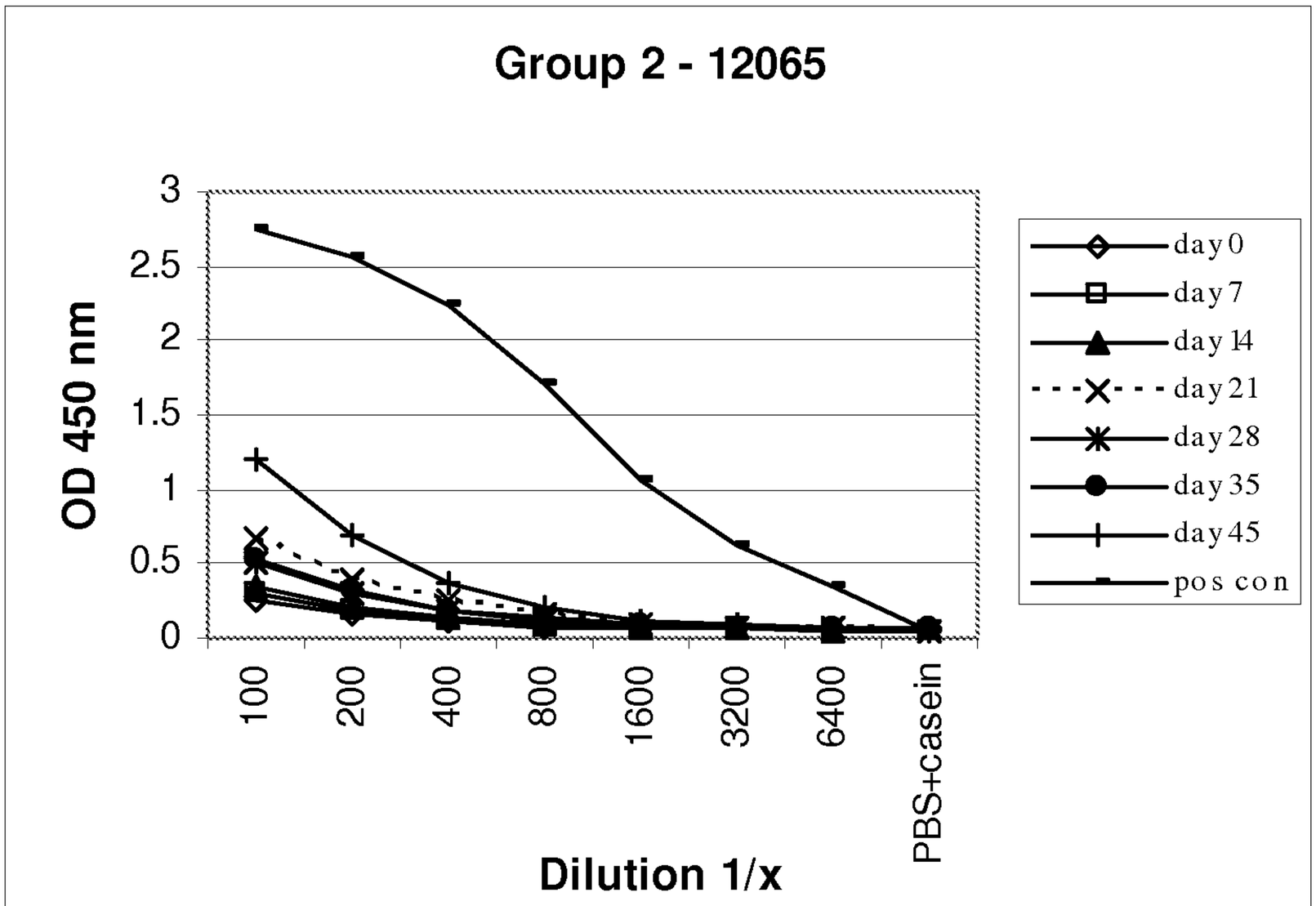

Group 2 - 12065
OD 450 nm
3
2.5
2
1.5
1
0.5
0
100
200
400
800
1600
3200
6400
PBS+casein
Dilution 1/x
day 0
day 7
day 14
day 21
day 28
day 35
day 45
pos con

FIGURE 10C

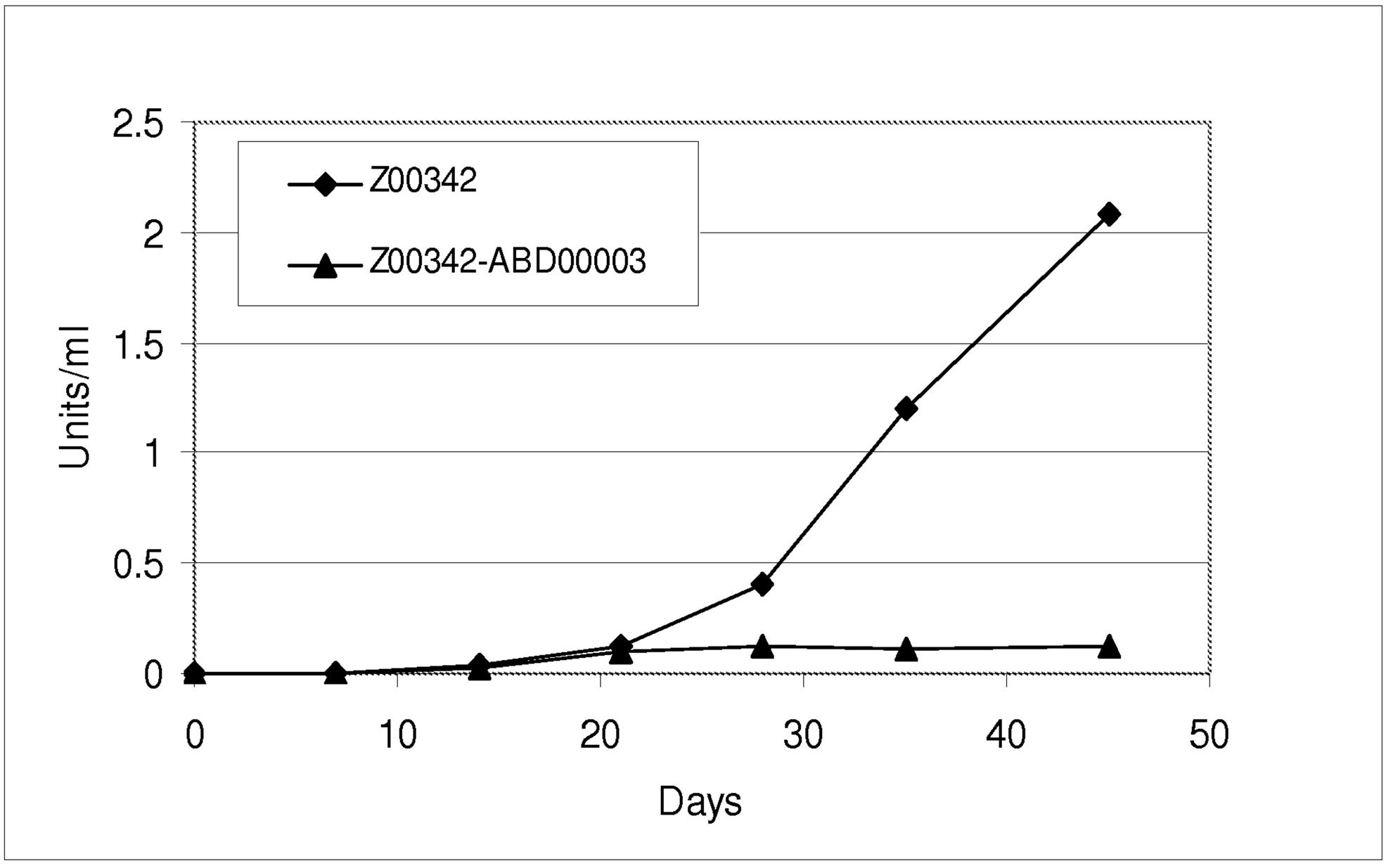
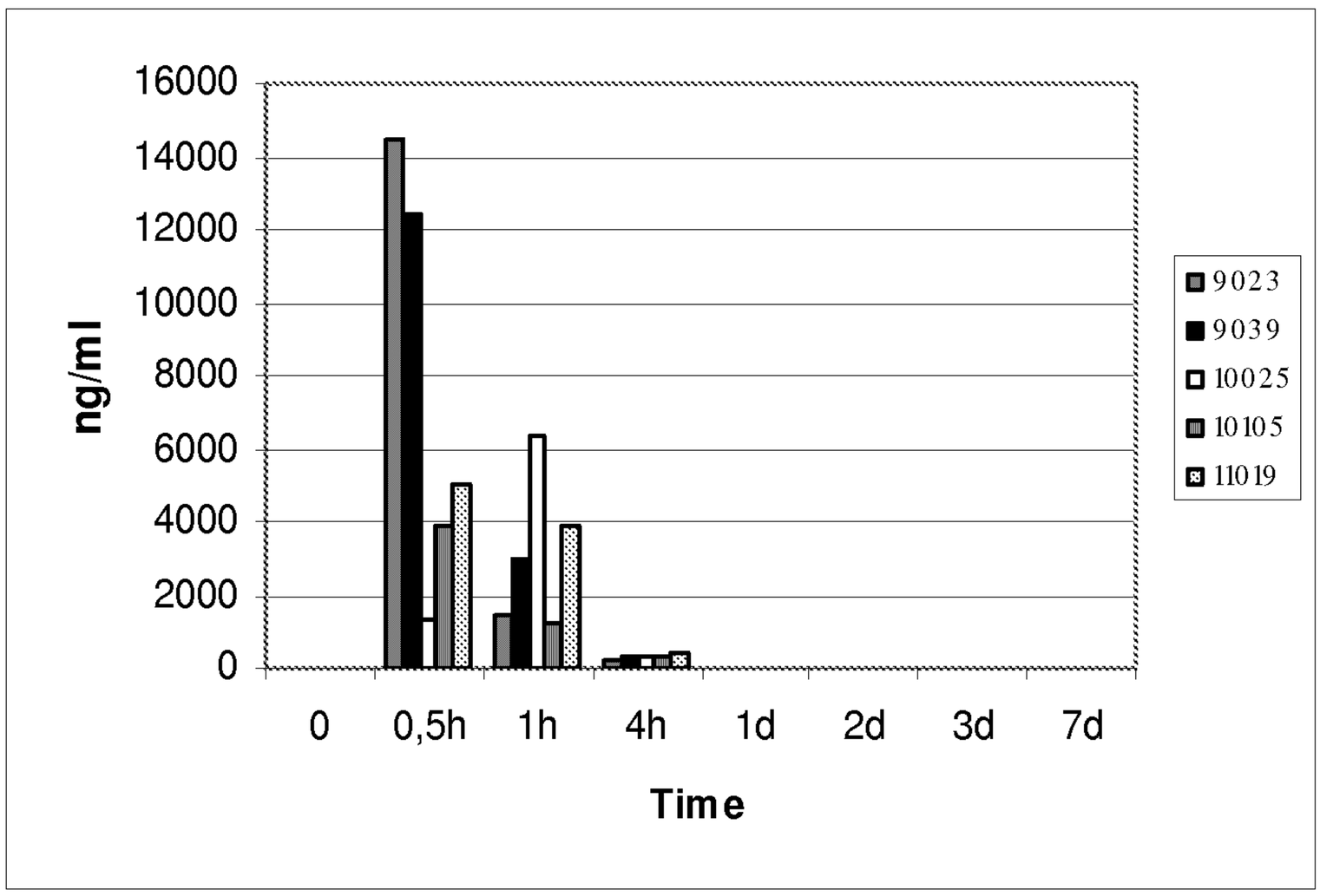

2.5
2
1.5
1
0.5
0
Units/ml
Z00342
Z00342-ABD00003
0
10
20
30
40
50
Days

FIGURE 11

A


16000
14000
12000
10000
8000
6000
4000
2000
0
ng/ml
0
0,5h
1h
4h
1d
2d
3d
7d
Time
9023
9039
10025
10105
11019

B

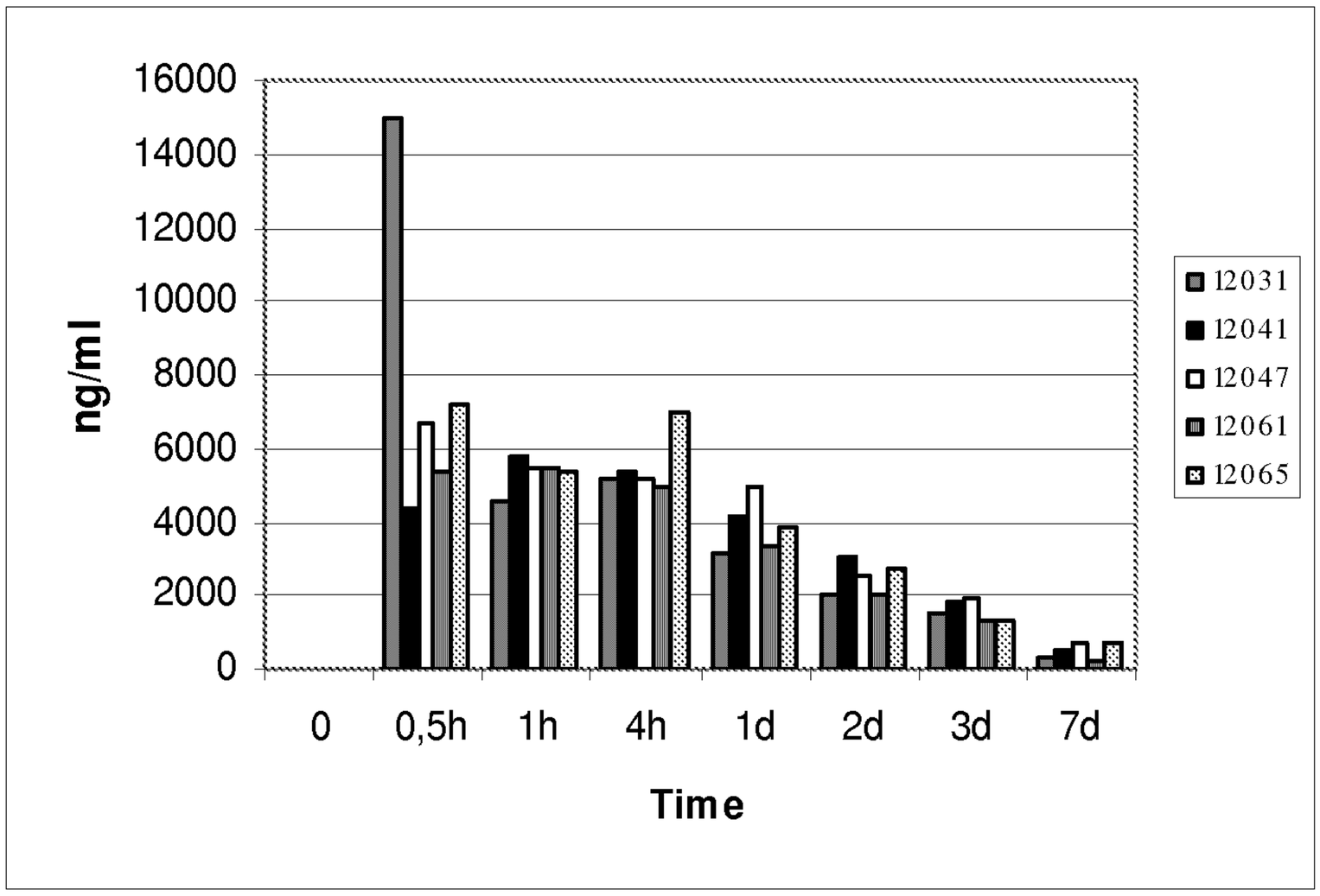
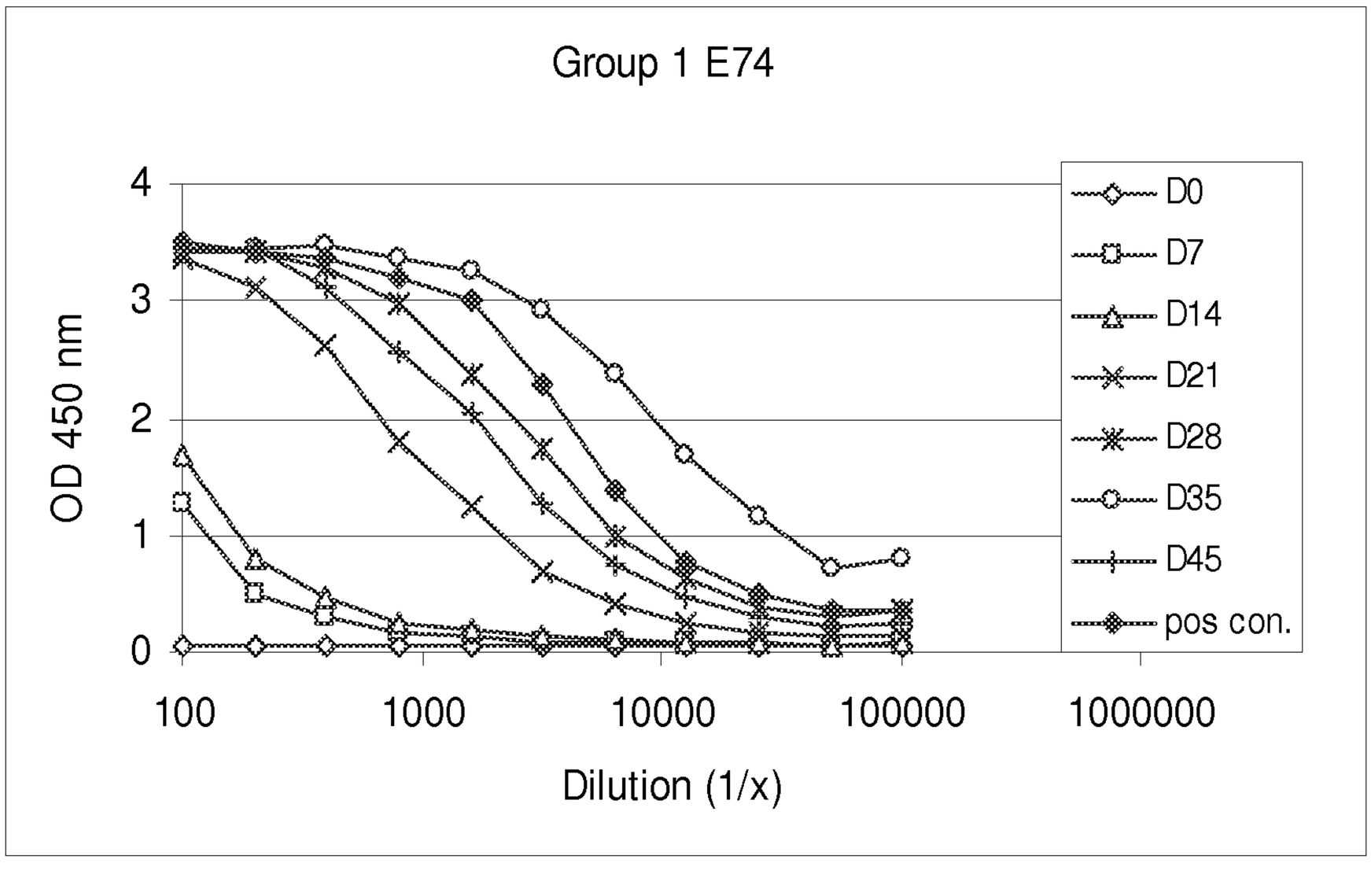

16000
14000
12000
10000
8000
6000
4000
2000
0
ng/ml
0
0,5h
1h
4h
1d
2d
3d
7d
Time
12031
12041
12047
12061
12065

FIGURE 12


Group 1 E74
OD 450 nm
0
1
2
3
4
100
1000
10000
100000
1000000
Dilution (1/x)
D0
D7
D14
D21
D28
D35
D45
pos con.

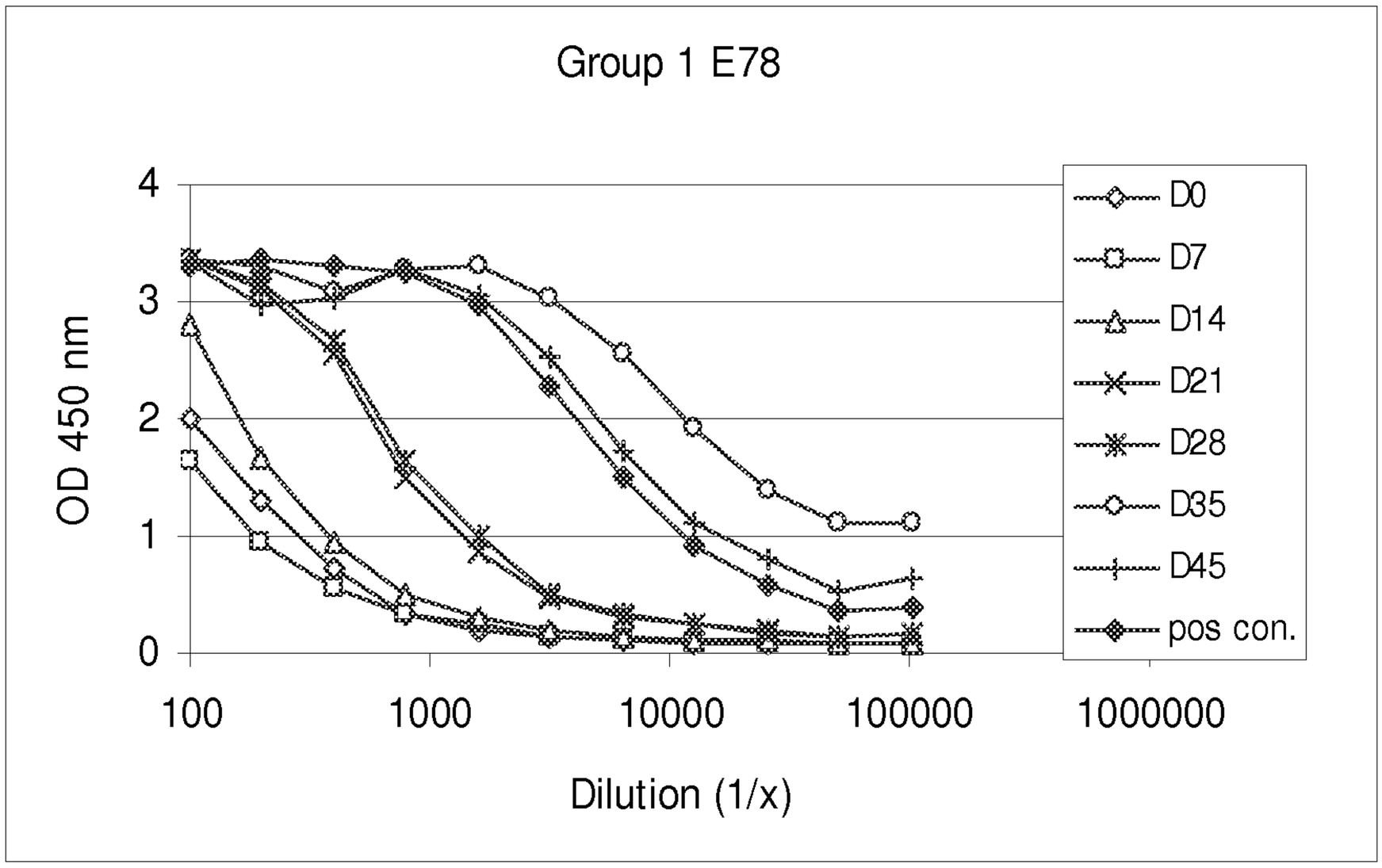

Group 1 E78
OD 450 nm
0
1
2
3
4
100
1000
10000
100000
1000000
Dilution (1/x)
D0
D7
D14
D21
D28
D35
D45
pos con.

FIGURE 13A

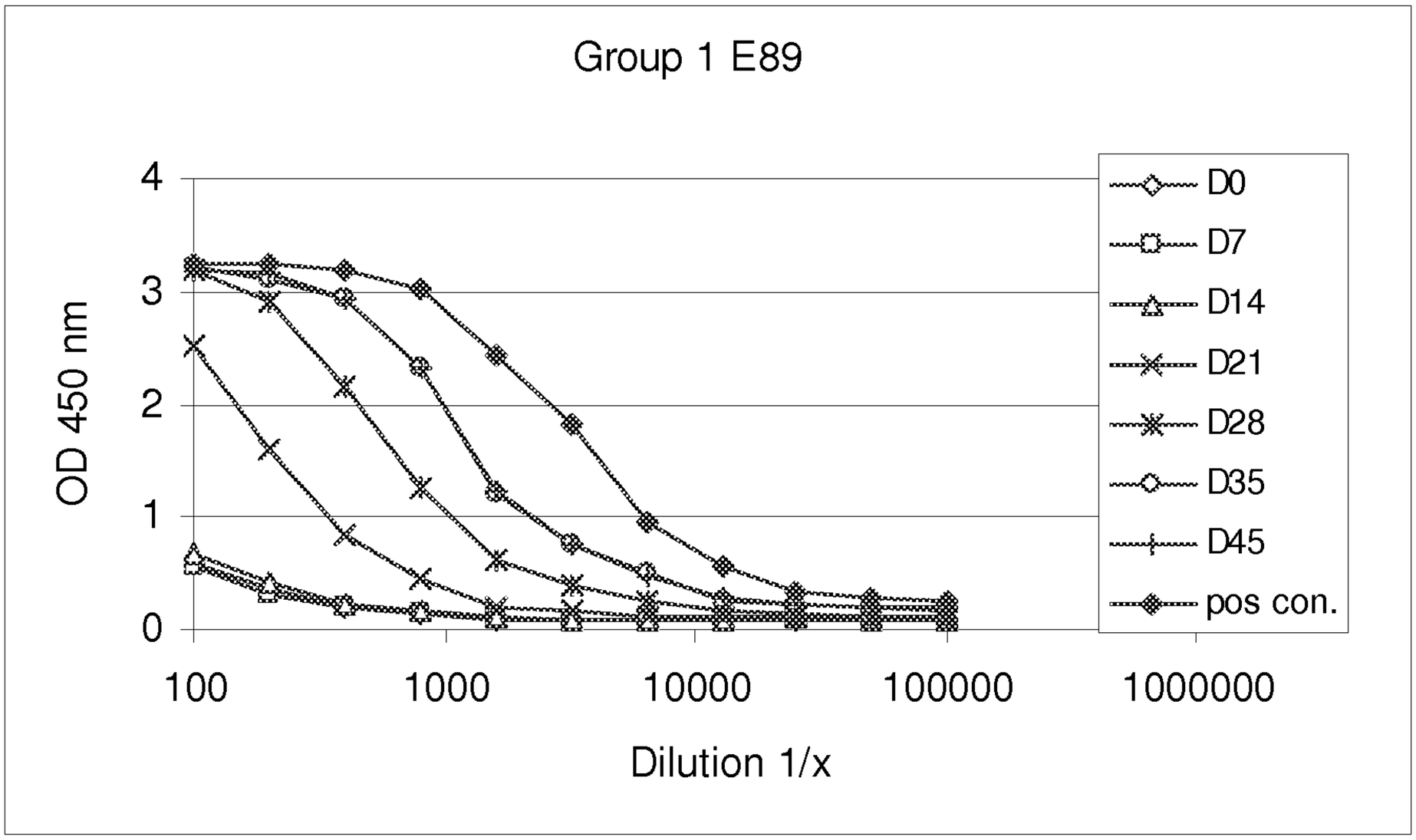
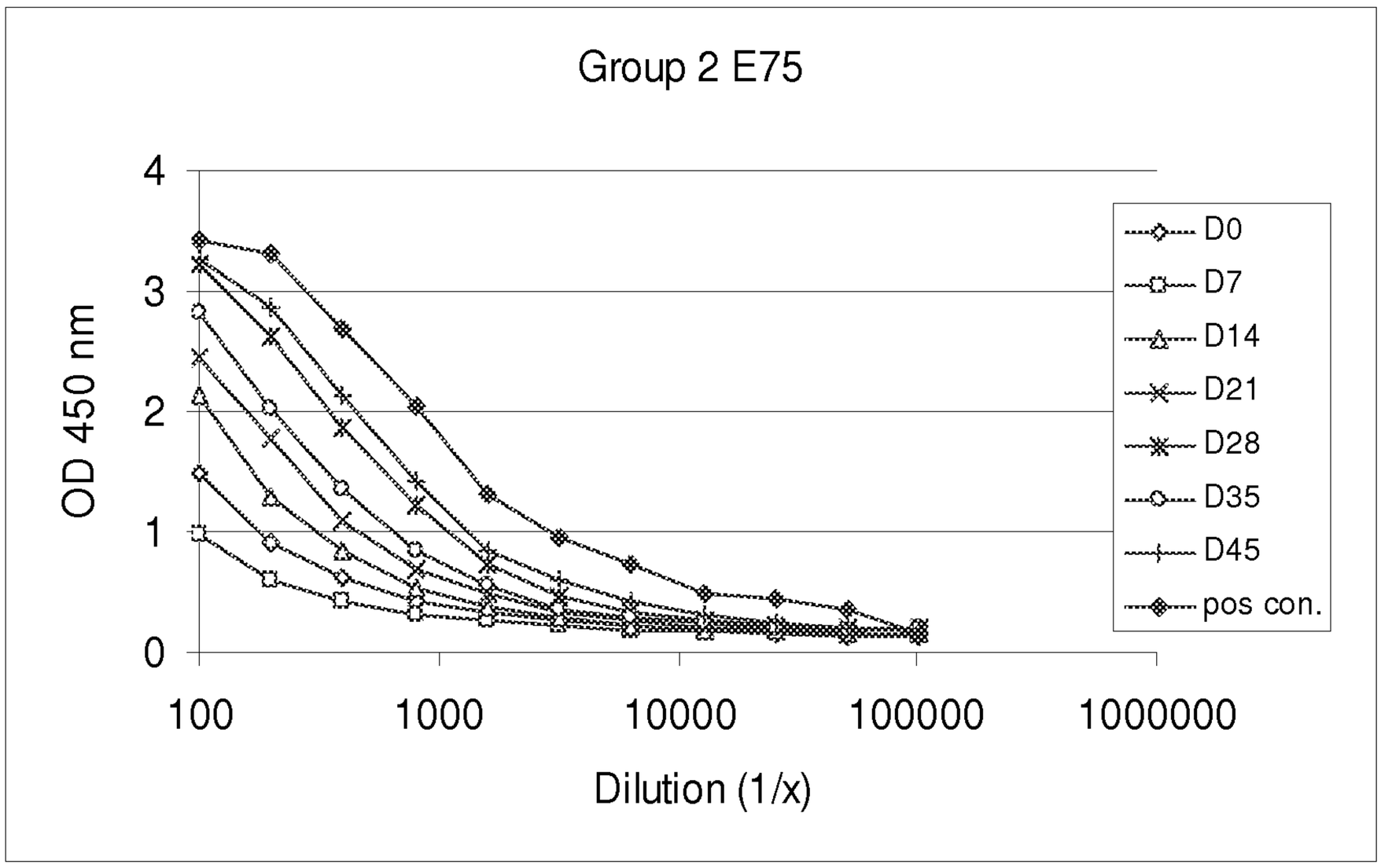

Group 1 E89
OD 450 nm
4
3
2
1
0
100
1000
10000
100000
1000000
Dilution 1/x
D0
D7
D14
D21
D28
D35
D45
pos con.

FIGURE 13B


Group 2 E75
OD 450 nm
4
3
2
1
0
100
1000
10000
100000
1000000
Dilution (1/x)
D0
D7
D14
D21
D28
D35
D45
pos con.

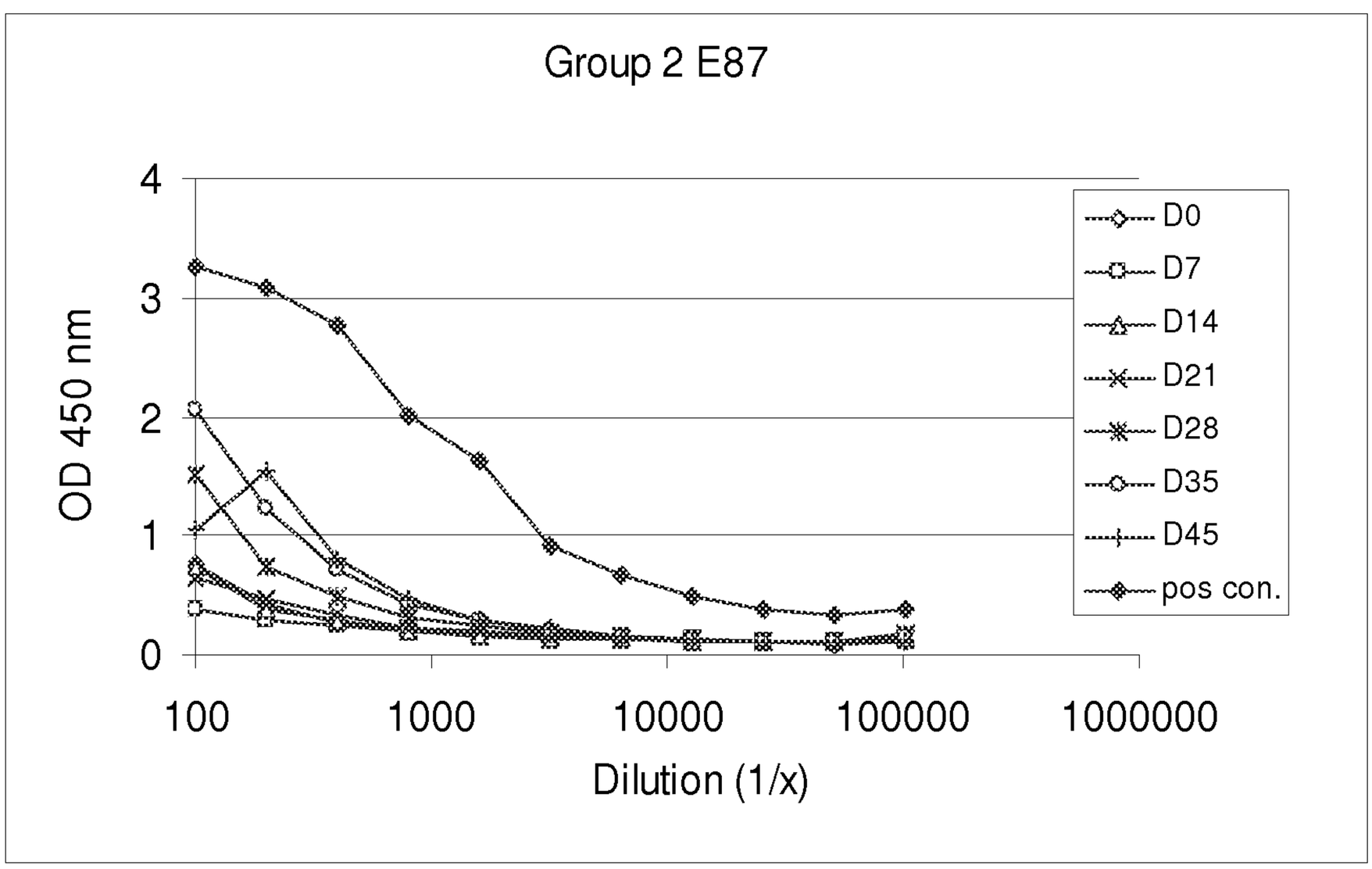
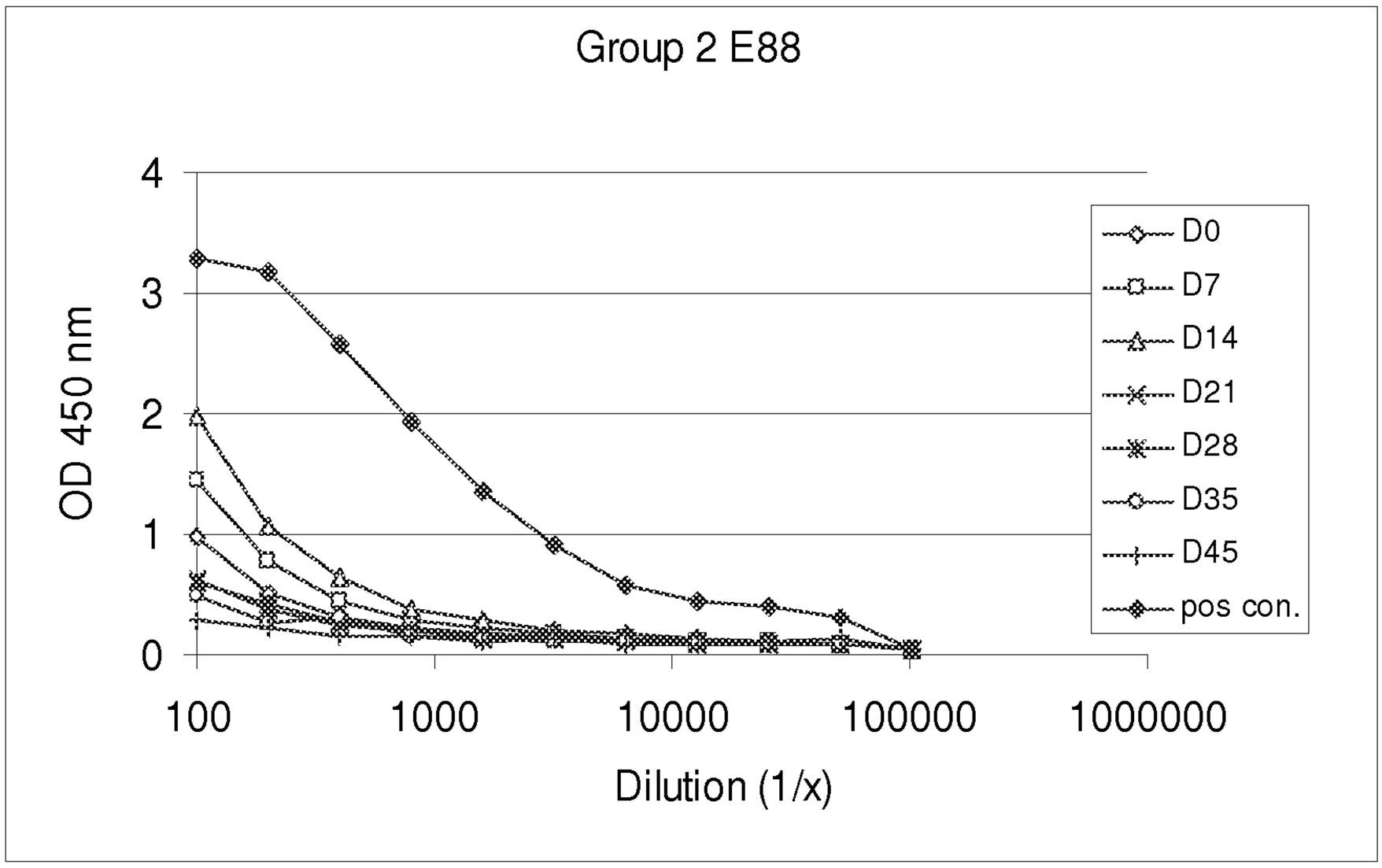

Group 2 E87
OD 450 nm
4
3
2
1
0
100
1000
10000
100000
1000000
Dilution (1/x)
D0
D7
D14
D21
D28
D35
D45
pos con.

FIGURE 14A


Group 2 E88
OD 450 nm
4
3
2
1
0
100
1000
10000
100000
1000000
Dilution (1/x)
D0
D7
D14
D21
D28
D35
D45
pos con.

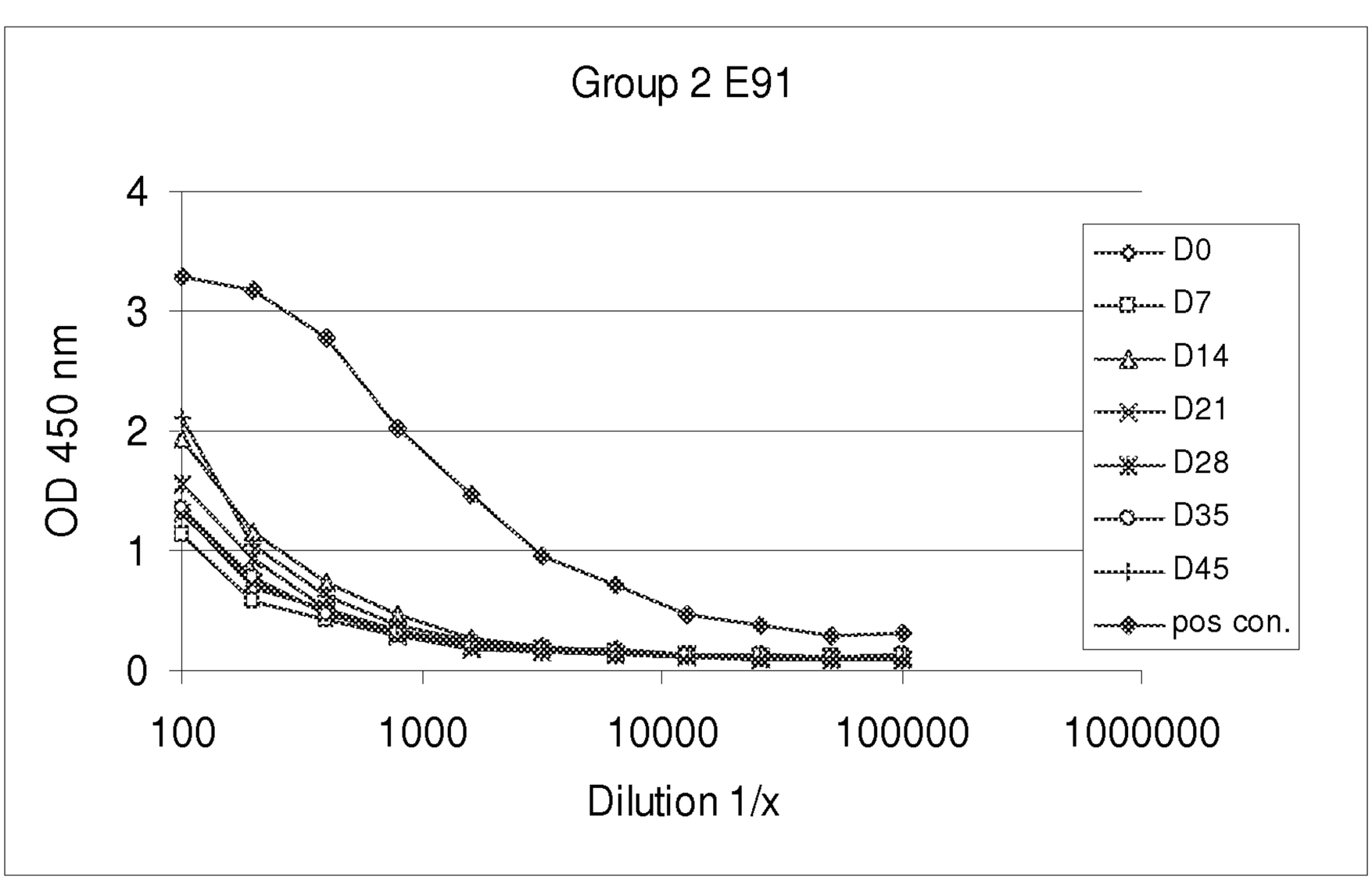
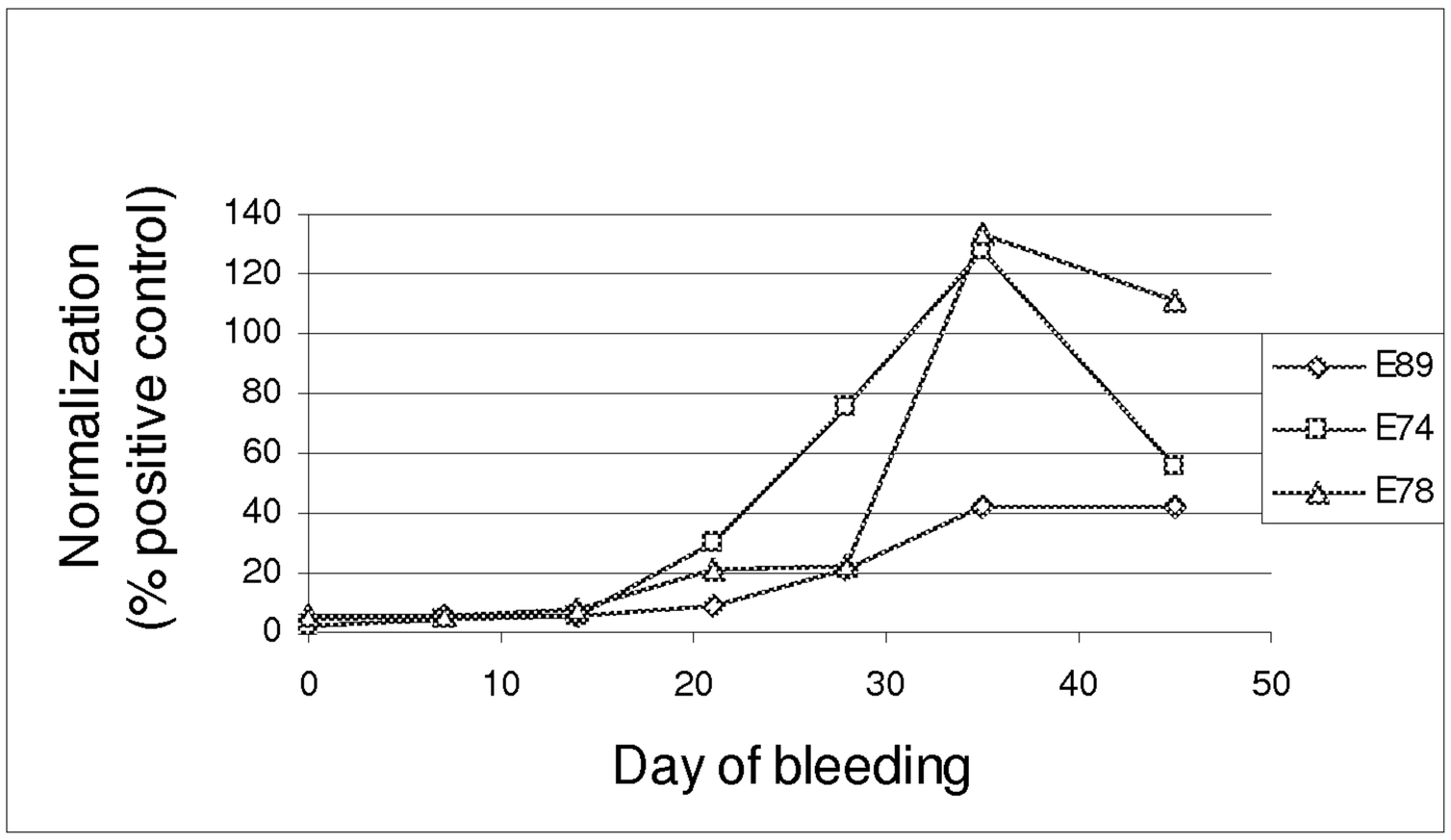

Group 2 E91
OD 450 nm
4
3
2
1
0
100
1000
10000
100000
1000000
Dilution 1/x
D0
D7
D14
D21
D28
D35
D45
pos con.

FIGURE 14B

A


Normalization
(% positive control)
140
120
100
80
60
40
20
0
0
10
20
30
40
50
Day of bleeding
E89
E74
E78

B

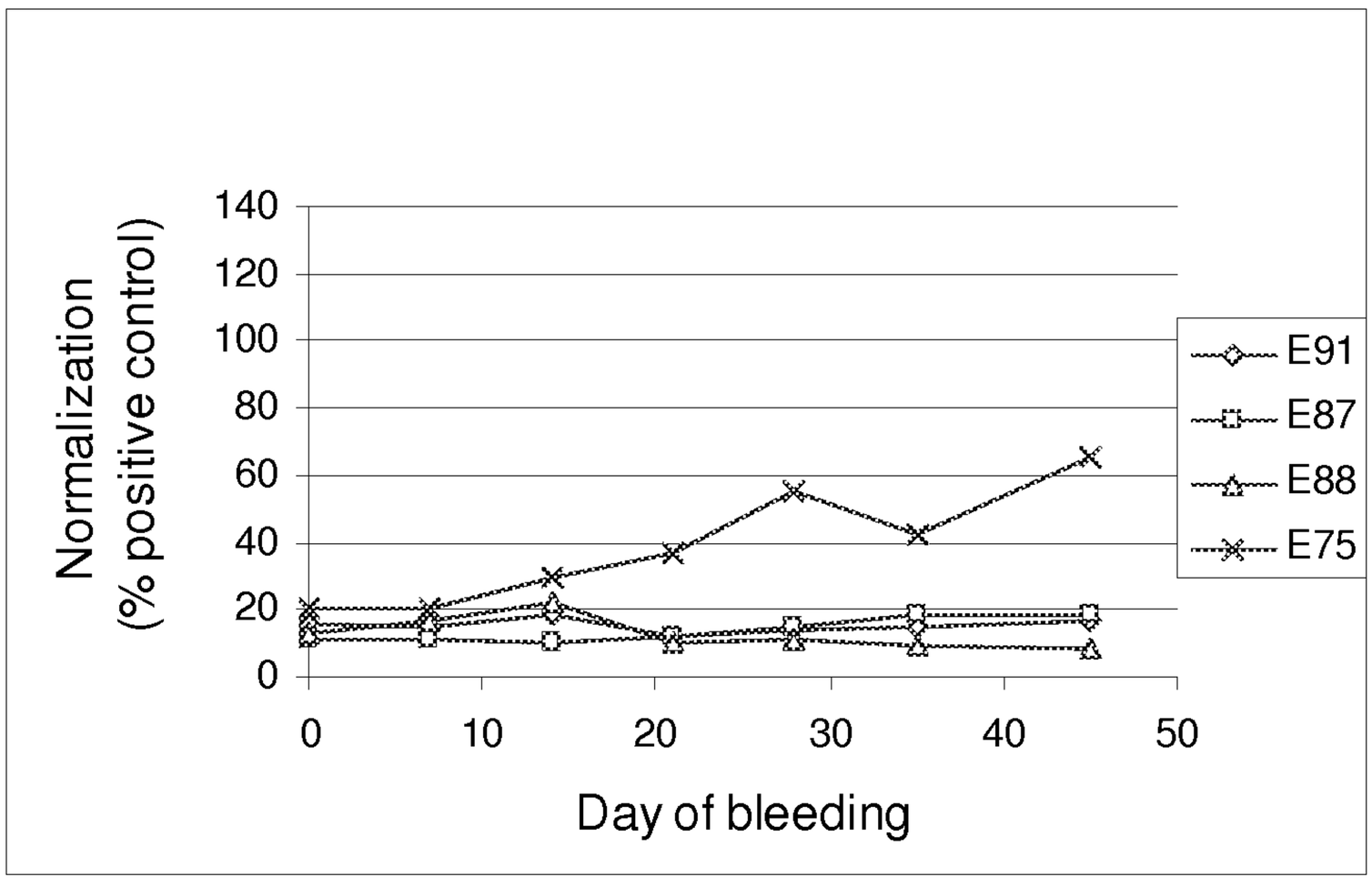

Normalization
(% positive control)
140
120
100
80
60
40
20
0
0
10
20
30
40
50
Day of bleeding
E91
E87
E88
E75

FIGURE 15

MGSSHHHHHHLQVD ABD VARIANT