



(51) International Patent Classification:

C07K 14/00 (2006.01) *G01N 33/68* (2006.01)
C07K 14/195 (2006.01)

(21) International Application Number:

PCT/EP2013/072359

(22) International Filing Date:

25 October 2013 (25.10.2013)

(25) Filing Language:

English

(26) Publication Language:

English

(30) Priority Data:

12189932.2 25 October 2012 (25.10.2012) EP
61/718,238 25 October 2012 (25.10.2012) US

(71) Applicant: AFFIBODY AB [SE/SE]; Gunnar Asplunds
Allé 24, S-171 63 Solna (SE).

(72) Inventors: JONASSON, Per; Maria Prästgårdsgata 15A,
S-118 52 Stockholm (SE). EKLUND, Pär; Pålundsgatan
4, S-117 31 Stockholm (SE).

(74) Agent: MATTSSON, Niklas; Awapatent Ab, P.O. Box
45086, S-104 30 Stockholm (SE).

(81) Designated States (unless otherwise indicated, for every
kind of national protection available): AE, AG, AL, AM,
AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY,

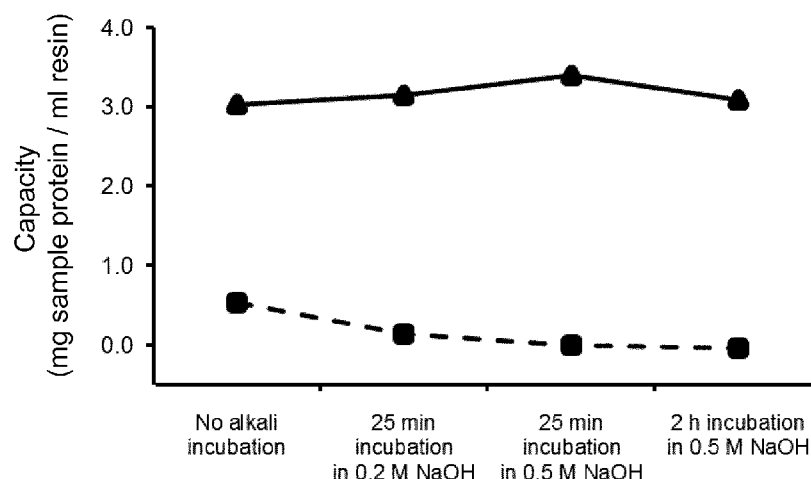
BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM,
DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT,
HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KN, KP, KR,
KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME,
MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ,
OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA,
SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM,
TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM,
ZW.

(84) Designated States (unless otherwise indicated, for every
kind of regional protection available): ARIPO (BW, GH,
GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, SZ, TZ,
UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ,
TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK,
EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV,
MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM,
TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW,
KM, ML, MR, NE, SN, TD, TG).

Published:

- with international search report (Art. 21(3))
- before the expiration of the time limit for amending the
claims and to be republished in the event of receipt of
amendments (Rule 48.2(h))
- with sequence listing part of description (Rule 5.2(a))

(54) Title: ABD BINDING POLYPEPTIDE

**FIGURE 7**

(57) Abstract: The disclosure provides an ABD binding polypeptide comprising an ABD binding motif BM, which motif consists of an amino acid sequence selected from EX₂X₃X₄AX₆X₇EIX₁₀X₁₁LPNLX₁₆X₁₇X₁₈QX₂₀X₂₁AFIX₂₅X₂₆LX₂₈D and amino acid sequences with at least 89 % identity thereto.



ALBUMIN BINDING POLYPEPTIDE

Field of the invention

This invention relates to polypeptides which bind to an albumin binding domain, ABD. The polypeptides have various industrial and pharmaceutical applications, for example use as an affinity ligand in separation technology or
5 as a detection agent in molecular diagnostics.

Background

Serum albumin is the most abundant protein in mammalian sera (35-50 g/l, i.e. 0.53-0.75 mM, in humans) and several strategies to covalently couple
10 a peptide or protein to carrier molecule that will allow *in vivo* association to serum albumin have been described e.g. in WO91/01743, in WO01/45746 and in Dennis *et al* (J Biol Chem 277:35035-43, 2002). WO91/01743 describes *inter alia* the use of albumin binding peptides or proteins derived from streptococcal protein G (SpG) for increasing the half-life of other
15 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 elimination from blood. The 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
20 peptide/protein. 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 *et al*, J Lab Clin Med 143:115, 2004).

Streptococcal protein G (SpG) is a bi-functional receptor present on the
25 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 domains and three serum albumin binding domains (Olsson *et al*, Eur J Biochem
30 168:319, 1987). The structure of one of the three serum albumin binding domains in SpG has been determined, showing a three-helix bundle fold

(Kraulis *et al*, FEBS Lett 378:190, 1996, Johansson *et al*, J. Biol. Chem. 277:8114-20, 2002). A 46 amino acid motif was defined as ABD (albumin binding domain) and has subsequently also been designated G148-GA3 (GA for protein G-related albumin binding and G148 from the strain of *Streptococcus* from which it is derived).

Artificial variants of GA3-G148 having a very much improved affinity for human serum albumin were developed (Jonsson *et al*, Prot Eng Des Sel 21:515-27, 2008; WO2009/016043), as well as engineered high affinity variants with reduced immune stimulatory properties (WO2012/004384). The latter were motivated by the fact that a few T- and B-cell epitopes were experimentally identified within GA3-G148 (Goetsch *et al*, Clin Diagn Lab Immunol 10:125-32, 2003), making this domain as such less suitable for use in pharmaceutical compositions for human administration. Throughout the present text, GA3-G148 as well as the various engineered derivatives thereof presented e.g. in WO2009/016043 and WO2012/004384, are collectively referred to as "ABD". Thus, in the present disclosure, "ABD" denotes these classes of albumin binding polypeptides, rather than a specified polypeptide with a specific amino acid sequence.

With the increased interest in incorporating an albumin binding domain into therapeutic or diagnostic compositions follows a growing need for inexpensive and efficient purification strategies for isolation of molecules covalently linked to ABD, produced for instance by recombinant expression in prokaryotic or eukaryotic systems, or by direct chemical conjugation to ABD. A general strategy for purification of recombinantly expressed proteins would be to include a commonly used affinity tag such as a polyhistidine tag, a chitin binding protein (CBP), a maltose binding protein (MBP), a glutathione-S-transferase (GST)-tag or a FLAG-tag, and perform a classic affinity separation using commercial resins developed specifically for each tag. However, for certain applications, and in particular for molecules to be used as therapeutics, the end product must be homogeneous. Because the tag would need to be removed, e.g. by enzymatic or chemical cleavage, it is necessary to ensure complete cleavage to obtain a homogeneous product, or to suffer a loss of yield when removing incompletely cleaved product. Both of

these issues serve to increase the production cost of the product. Therefore, a more motivated strategy would be to utilize the ABD moiety itself as a purification tag. One example of this would be to couple recombinant albumin to a solid support (see for example Jonsson *et al*, Prot Eng Des Sel 21:515-
5 27, 2008; Andersen *et al*, J Biol Chem 286:5234-41, 2011). From a crude solute, compounds comprising an ABD tag are captured by albumin, non-specifically adsorbed contaminants are removed and ABD-tagged compounds are subsequently recovered by disrupting the specific but reversible interaction with albumin. However, albumin is a large natural carrier
10 molecule with several interaction sites for different proteins, fatty acids, sterols, ions etc., and thus, the background binding of both specific and unspecific components may contaminate the recovered sample. Despite the fact that recombinant human albumin has been developed, it is still expensive to include as an affinity ligand in large-scale production of therapeutics, in
15 particular because it is incompatible with standard procedures for cleaning in place, that would have to be applied for the repeated use of the albumin-coupled matrix. Furthermore, harsher elution conditions may be required to recover molecules containing ABD variants with an exceptionally high affinity for albumin. Such conditions may be unfavorable also for the ABD-tagged
20 molecule.

Protein A from *Staphylococcus aureus* has long been used as an affinity ligand in the industrial production of monoclonal antibodies and Fc-fusion proteins, due to the native affinity of Protein A for the Fc portion of IgG. Protein A in its entirety, as well as the individual Fc-binding domains thereof,
25 have subsequently served as starting points for the rational design of engineered affinity ligands with improved properties. For an introduction, see the papers by Nord K and co-workers in Prot Eng (Nord *et al*, Prot Eng 8:601-608; 1995) and Nat Biotech (Nord *et al*, Nat Biotech 15:772-777; 1997).

30 It is an object of the present disclosure to provide new ABD binding agents. It is moreover an object of the disclosure to provide new ABD binding agents for use in biotechnology for example in protein purification and separation applications.

Description of the invention

These objects, and other objects apparent to the skilled person from the present disclosure, may be achieved by one or more of the various

5 inventive aspects disclosed below.

Thus, in a first aspect, the disclosure provides an ABD binding polypeptide, comprising an ABD binding motif BM, which motif consists of an amino acid sequence selected from:

10 i) $EX_2X_3X_4AX_6X_7EI\ X_{10}X_{11}LPNLX_{16}X_{17}X_{18}QX_{20}\ X_{21}AFIX_{25}X_{26}LX_{28}D$

wherein, independently from each other,

15 X_2 is selected from F, I and L;
 X_3 is selected from H, K, N, Q, R, S, T and V;
 X_4 is selected from A, D, F, G, H, I, K, L, M, N, Q, R, S, T, V, W and Y;
 X_6 is selected from F, I, L and Y;
 X_7 is selected from A, H, I, K, L, N, Q, R, S, T and V;
 X_{10} is selected from G, H, K, N, Q, R and S;
 20 X_{11} is selected from A, D, F, G, I, K, L, N, Q, R, S, T, V and Y;
 X_{16} is selected from N and T;
 X_{17} is selected from F, H, L, S and T;
 X_{18} is selected from D, E, H, I, K, L, M, N, Q, R, S, T and V;
 X_{20} is selected from H, K and R;
 25 X_{21} is selected from I, L and V;
 X_{25} is selected from F, I, L, V and Y;
 X_{26} is selected from K and S;
 X_{28} is selected from D and E;

30 and

ii) an amino acid sequence which has at least 89 % identity to the sequence defined in i).

The above definition of a class of sequence related, ABD binding polypeptides is based on a statistical analysis of a number of random polypeptide variants of a parent scaffold, that were selected for their
5 interaction with ABD in several different selection experiments. The identified ABD binding motif, or "BM", corresponds to the target binding region of the parent scaffold, which region constitutes two alpha helices within a three-helical bundle protein domain. In the parent scaffold, the varied amino acid residues of the two BM helices constitute a binding surface for interaction with
10 the constant Fc part of antibodies. In the present invention, the random variation of binding surface residues and subsequent selection of variants have replaced the Fc interaction capacity with a capacity for interaction with ABD.

The ABD binding polypeptide disclosed herein exhibits a set of
15 characteristics, which, for example when coupled to a solid support, makes it suitable as an affinity ligand for purification of molecules comprising ABD. For example, in comparison with using human albumin as affinity ligand, the disclosed ABD binding polypeptide demonstrates the following advantages:

- Higher purification capacity per ml ligand-coupled matrix.
- 20 • Compatibility with repeated procedures for cleaning in place, for example using 0.5 M sodium hydroxide, which makes the ABD binding polypeptide useful in the large scale production of therapeutics.
- Enablement of milder elution conditions, for example elution at a higher pH, which makes possible the purification of a wider range of different
25 molecules comprising ABD.

In the context of purification of an ABD-containing target protein, the ABD binding polypeptide of the present disclosure may be useful at one or more of the different stages of purification. Thus, it may be used without limitation at either one or more of a first capture step, in any intermediate
30 purification step or in a final polishing step.

The skilled person will appreciate that successful purification of a target protein, using a resin comprising an ABD binding polypeptide according to the present disclosure may be achieved regardless of where in the target protein

the ABD moiety is situated. Hence, the ABD moiety may be placed at the N-terminus or at the C-terminus of the target protein. Alternatively, the target protein may be a fusion protein which comprises an ABD moiety flanked by other protein moieties on both sides.

5

In the context of the present disclosure, "ABD" refers to three-helix albumin binding domains from streptococcal protein G and their derivatives. In particular, "ABD" may refer to GA3-G148 (Johansson *et al, supra*, hereby incorporated by reference) or variants of GA3-G148 having improved affinity
10 for albumin (WO2009/016043, hereby incorporated by reference), as well as high affinity variants with reduced immune stimulatory properties (WO2012/004384, hereby incorporated by reference). In other words, in the present disclosure, "ABD" denotes the classes of albumin binding polypeptides described in the referenced works, rather than a specified
15 polypeptide with a specific amino acid sequence.

As the skilled person will realize, the function of any polypeptide, such as the ABD binding capacity of the polypeptide of the present disclosure, 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
20 without affecting the function thereof. Thus, the invention encompasses polypeptides comprising modified variants of the BM which are such that the resulting sequence is at least 89 % identical to a sequence defined by i). In one embodiment of the present invention said modified variants of the BM are such that they are 93 % identical to a sequence defined by i). In yet another
25 embodiment, said BM variants are 96 % identical to a sequence defined by i).

In some embodiments, such changes may be made in all positions of the sequences of the ABD binding polypeptides as disclosed herein. In other embodiments, such changes may be made only in the non-variable positions, also denoted as scaffold amino acid residues. In such cases, changes are not
30 allowed in the variable positions, i.e. positions denoted with an "X" in sequence i). 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.

The term “% identity”, as used throughout, may be calculated as follows. The query sequence is aligned to the target sequence using the
 5 CLUSTAL W algorithm (Thompson *et al*, Nucleic Acids Research, 22: 4673-4680 (1994)). A comparison is made over the window corresponding to the shortest of the aligned sequences. The shortest of the aligned sequences may in some instances be the target sequence. In other instances, the query sequence may constitute the shortest of the aligned sequences. The amino
 10 acid residues at each position are compared, and the percentage of positions in the query sequence that have identical correspondences in the target sequence is reported as % identity.

In one embodiment, X_2 in sequence i) is selected from F and L.
 15 In one embodiment, X_2 in sequence i) is F.
 In one embodiment, X_2 in sequence i) is L.
 In one embodiment, X_3 in sequence i) is selected from H, K, R, T and V.
 In one embodiment, X_3 in sequence i) is selected from H, K, R and V.
 20 In one embodiment, X_3 in sequence i) is selected from H, K and V.
 In one embodiment, X_3 in sequence i) is selected from K and R.
 In one embodiment, X_3 in sequence i) is K.
 In one embodiment, X_3 in sequence i) is R.
 In one embodiment, X_3 in sequence i) is V.
 25 In one embodiment, X_4 in sequence i) is selected from A, H, I, L, N, V and W.
 In one embodiment, X_4 in sequence i) is selected from H, L, N and V.
 In one embodiment, X_4 in sequence i) is V.
 In one embodiment, X_4 in sequence i) is N.
 30 In one embodiment, X_4 in sequence i) is H.
 In one embodiment, X_4 in sequence i) is L.
 In one embodiment, X_4 in sequence i) is selected from A, L, N, V and W.

- In one embodiment, X_4 in sequence i) is selected from A, N, V and W.
In one embodiment, X_4 in sequence i) is selected from A, N and W.
In one embodiment, X_6 in sequence i) is selected from F and L.
In one embodiment, X_6 in sequence i) is F.
- 5 In one embodiment, X_6 in sequence i) is L.
In one embodiment, X_7 in sequence i) is selected from K, L, N, Q, R and S.
In one embodiment, X_7 in sequence i) is selected from K, N, Q, R and S.
- 10 In one embodiment, X_7 in sequence i) is selected from K, Q, R and S.
In one embodiment, X_7 in sequence i) is selected from K and R.
In one embodiment, X_7 in sequence i) is K.
In one embodiment, X_7 in sequence i) is R.
In one embodiment, X_{10} in sequence i) is selected from H, K, N, and R.
- 15 In one embodiment, X_{10} in sequence i) is selected from H, K and N.
In one embodiment, X_{10} in sequence i) is selected from K, N and R.
In one embodiment, X_{10} in sequence i) is selected from K and R.
In one embodiment, X_{10} in sequence i) is N.
In one embodiment, X_{10} in sequence i) is K.
- 20 In one embodiment, X_{10} in sequence i) is R.
In one embodiment, X_{10} in sequence i) is H.
In one embodiment, X_{11} in sequence i) is selected from A, F, L, R, T and Y.
In one embodiment, X_{11} in sequence i) is selected from A, F, T and Y.
- 25 In one embodiment, X_{11} in sequence i) is T.
In one embodiment, X_{11} in sequence i) is A.
In one embodiment, X_{11} in sequence i) is Y.
In one embodiment, X_{11} in sequence i) is F.
In one embodiment, X_{11} in sequence i) is selected from A, L, T and Y.
- 30 In one embodiment, X_{11} in sequence i) is selected from T and Y.
In one embodiment, X_{16} in sequence i) is T.
In one embodiment, X_{17} in sequence i) is selected from F, H and L.
In one embodiment, X_{17} in sequence i) is selected from F and H.

- In one embodiment, X_{17} in sequence i) is selected from H and L.
 In one embodiment, X_{17} in sequence i) is F.
 In one embodiment, X_{17} in sequence i) is H.
 In one embodiment, X_{17} in sequence i) is L.
 5 In one embodiment, X_{18} in sequence i) is selected from D, H, I, K and Q.
 In one embodiment, X_{18} in sequence i) is selected from D, H and Q.
 In one embodiment, X_{18} in sequence i) is selected from H and Q.
 In one embodiment, X_{18} in sequence i) is H.
 10 In one embodiment, X_{18} in sequence i) is Q.
 In one embodiment, X_{18} in sequence i) is D.
 In one embodiment, X_{20} in sequence i) is selected from K and R.
 In one embodiment, X_{20} in sequence i) is selected from H and R.
 In one embodiment, X_{20} in sequence i) is R.
 15 In one embodiment, X_{20} in sequence i) is K.
 In one embodiment, X_{21} in sequence i) is selected from I and L.
 In one embodiment, X_{21} in sequence i) is I.
 In one embodiment, X_{21} in sequence i) is L.
 In one embodiment, X_{25} in sequence i) is selected from I, L and V.
 20 In one embodiment, X_{25} in sequence i) is selected from V and I.
 In one embodiment, X_{25} in sequence i) is I.
 In one embodiment, X_{25} in sequence i) is V.
 In one embodiment, X_{25} in sequence i) is L.
 In one embodiment, X_{26} in sequence i) is K.
 25 In one embodiment, X_{28} in sequence i) is D.

In one embodiment, sequence i) is defined as follows: independently from each other,

- X_2 is selected from F and L;
 30 X_3 is selected from H, K, R, T and V;
 X_4 is selected from A, H, I, L, N, V and W;
 X_6 is selected from F and L;
 X_7 is selected from K, L, N, Q, R and S;

- X₁₀ is selected from H, K, N, and R;
 X₁₁ is selected from A, F, L, R, T, and Y;
 X₁₆ is T;
 X₁₇ is selected from F, H and L;
 5 X₁₈ is selected from D, H, I, K and Q;
 X₂₀ is selected from H and R;
 X₂₁ is selected from I and L;
 X₂₅ is selected from I, L and V;
 X₂₆ is K; and
 10 X₂₈ is D.

In one embodiment, sequence i) is defined as follows: independently from each other,

- X₂ is selected from F and L;
 15 X₃ is selected from H, K, T and V;
 X₄ is selected from H, L, N and V;
 X₆ is selected from F and L;
 X₇ is selected from K, R, Q, and S;
 X₁₀ is selected from H, K, and N;
 20 X₁₁ is selected from A, F, T, and Y;
 X₁₆ is T;
 X₁₇ is selected from F, H and L;
 X₁₈ is selected from D, H and Q;
 X₂₀ is R;
 25 X₂₁ is selected from I and L;
 X₂₅ is selected from I, L and V;
 X₂₆ is K; and
 X₂₈ is D.

- 30 In a more specific embodiment defining a sub-class of the ABD binding polypeptide, sequence i) fulfills at least four of the eight conditions I-VIII:

- I. X₂ is selected from F and L;

- II. X_6 is selected from F and L;
- III. X_{16} is T;
- IV. X_{20} is selected from H and R;
- V. X_{21} is selected from I and L;
- 5 VI. X_{25} is selected from I and V;
- VII. X_{26} is K; and
- VIII. X_{28} is D.

In some examples of an ABD binding polypeptide according to the first
 10 aspect, sequence i) fulfils at least five of the eight conditions I-VIII. More specifically, sequence i) may fulfill at least six of the eight conditions I-VIII, such at least seven of the eight conditions I-VIII, such as all of the eight conditions I-VIII.

15 As described in detail in the experimental section to follow, the selection of ABD binding polypeptide variants has led to the identification of a number of individual ABD binding motif (*BM*) sequences. These sequences constitute individual embodiments of sequence i) according to this aspect. The sequences of individual ABD binding motifs are presented in Figure 1
 20 and as SEQ ID NO:1-52. In some embodiments of this aspect, sequence i) is selected from any one of SEQ ID NO:1-52. More specifically, sequence i) may be selected from any one of SEQ ID NO:1-7, such as from SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3 and SEQ ID NO:4. In particular, sequence i) may be SEQ ID NO:1.

25 In some embodiments of the present disclosure, the *BM* as defined above “forms part of” a three-helix bundle protein domain. This is understood to mean that the sequence of the *BM* is “inserted” into or “grafted” onto the sequence of the original three-helix bundle domain, such that the *BM*
 30 replaces a similar structural motif in the original domain. For example, without wishing to be bound by theory, the *BM* 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 *BM* helices has to be performed so as not to affect the basic structure of the polypeptide. That is, the overall folding of the C α backbone of the polypeptide according to this embodiment of the invention is substantially the same as
 5 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, a *BM* 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
 10 properties are shared, those properties e.g. resulting in similar CD spectra. The skilled person is aware of other parameters that are relevant.

In particular embodiments, the ABD binding motif (*BM*) thus forms part of a three-helix bundle protein domain. For example, the *BM* may essentially constitute two alpha helices with an interconnecting loop, within said three-
 15 helix bundle protein domain. In particular embodiments, said three-helix bundle protein domain is selected from domains of bacterial receptor proteins. Non-limiting examples of such domains are the five different three-helical domains of Protein A from *Staphylococcus aureus*, such as domain B, and derivatives thereof. In some embodiments, the three-helical bundle protein
 20 domain is a variant of protein Z, which is derived from domain B of staphylococcal protein A.

In embodiments where the ABD binding polypeptide of the invention forms part of a three-helix bundle protein domain, the ABD binding polypeptide may comprise an amino acid sequence selected from:

25

iii) K-[*BM*]-DPSQS X_aX_bLLX_c EAKKL NDX_dQ;

wherein

[*BM*] is an ABD binding motif as defined above;

30 X_a is selected from A and S;

X_b is selected from N and E;

X_c is selected from A, S and C;

X_d is selected from A and S;
and

- iv) an amino acid sequence which has at least 81 % identity to a
5 sequence defined by iii).

As discussed above, polypeptides comprising minor changes as compared to the above amino acid sequences without largely affecting the tertiary structure and the function thereof are also within the scope of the present disclosure. Thus, in some embodiments, sequence iv) has at least
10 83 %, such as at least 85 %, such as at least 87 %, such as at least 89 %, such as at least 91 %, such as at least 93 %, such as at least 95 %, such as at least 97 % identity to a sequence defined by iii).

In one embodiment of the ABD binding polypeptide as defined above, X_a in sequence iii) is A. In an alternative embodiment, X_a in sequence iii) is S.

15 In one embodiment of the ABD binding polypeptide as defined above, X_b in sequence iii) is N. In an alternative embodiment, X_b in sequence iii) is E.

In one embodiment of the ABD binding polypeptide as defined above, X_c in sequence iii) is A. In an alternative embodiment, X_c in sequence iii) is S. In yet another alternative embodiment, X_c in sequence iii) is C.

20 In one embodiment of the ABD binding polypeptide as defined above, X_d in sequence iii) is A. In an alternative embodiment, X_d in sequence iii) is S.

In one embodiment of the ABD binding polypeptide as defined above, in sequence iii), X_a is A; X_b is N; X_c is A and X_d is A.

25 In a further embodiment of the ABD binding polypeptide as defined above, in sequence iii), X_a is A; X_b is N; X_c is C and X_d is A.

In a further embodiment of the ABD binding polypeptide as defined above, in sequence iii), X_a is S; X_b is E; X_c is S and X_d is S.

In a further embodiment of the ABD binding polypeptide as defined above, in sequence iii), X_a is S; X_b is E; X_c is C and X_d is S.

30 In a further embodiment of the ABD binding polypeptide as defined above, in sequence iii), X_a is S; X_b is E; X_c is A and X_d is A.

In a further embodiment of the ABD binding polypeptide as defined above, in sequence iii), X_a is S; X_b is E; X_c is C and X_d is A.

In yet a further embodiment, sequence iii) in the definition of ABD binding polypeptides above is selected from SEQ ID NO:53-104, in particular from SEQ ID NO:53-59. In a further embodiment, sequence iii) is selected
5 from SEQ ID NO:53, SEQ ID NO:54, SEQ ID NO:55 and SEQ ID NO:56. In particular, sequence iii) may be SEQ ID NO:53.

Also, in a further embodiment, there is provided an ABD binding polypeptide as defined above, which comprises an amino acid sequence
10 selected from:

v) YAK-[BM]-DPSQS SELLX_c EAKKL NDSQA P;

wherein [BM] is an ABD binding motif as defined above and X_c is selected from S and C; and

vi) an amino acid sequence which has at least 83 % identity to a
15 sequence defined by v).

In another embodiment, there is provided an ABD binding polypeptide as defined above, which comprises an amino acid sequence selected from:

vii) YAK-[BM]-DPSQS SELLX_c EAKKL NDAQA P;

wherein [BM] is an ABD binding motif as defined above and X_c is selected
20 from A and C; and

viii) an amino acid sequence which has at least 83 % identity to a sequence defined by vii).

Alternatively, there is provided an ABD binding polypeptide as defined above, which comprises an amino acid sequence selected from:

25 ix) FNK-[BM]-DPSQS ANLLX_c EAKKL NDAQA P;

wherein [BM] is an ABD binding motif as defined above and X_c is selected from A and C; and

x) an amino acid sequence which has at least 83 % identity to a sequence defined by ix).

30 As discussed above, polypeptides comprising minor changes as compared to the above amino acid sequences without largely affecting the tertiary structure and the function thereof are also within the scope of the present disclosure. Thus, in some embodiments, the ABD binding

polypeptides as defined above may for example have a sequence which is at least 84 %, at least 86 %, at least 88 %, at least 90 %, at least 92 %, at least 94 %, at least 96 %, or at least 98 % identical to a sequence defined by v), vii) or ix).

- 5 In some embodiments, the ABD binding motif may form part of a polypeptide comprising an amino acid sequence selected from

ADNNFNK-[BM]-DPSQSANLLSEAKKLNESQAPK;
 ADNKFNK-[BM]-DPSQSANLLAEAKKLNDAQAPK;
 ADNKFNK-[BM]-DPSVSKEILAEAKKLNDAQAPK;
 10 ADAQQNNFNK-[BM]-DPSQSTNVLGEAKKLNESQAPK;
 AQHDE-[BM]-DPSQSANVLGEAQKLNDSQAPK;
 VDNKFNK-[BM]-DPSQSANLLAEAKKLNDAQAPK;
 AEAKYAK-[BM]-DPSESSELLSEAKKLNKSQAPK;
 VDAKYAK-[BM]-DPSQSSELLAEAKKLNDAQAPK;
 15 VDAKYAK-[BM]-DPSQSSELLAEAKKLNDSQAPK;
 AEAKYAK-[BM]-DPSQSSELLSEAKKLNDSQAPK;
 VDAKYAK-[BM]-DPSQSSELLSEAKKLNDSQAPK;
 VDAKYAK-[BM]-DPSQSSELLAEAKKLNKAQAPK; and
 AEAKYAK-[BM]-DPSQSSELLAEAKKLNKAQAPK.

20

In one embodiment, the ABD binding polypeptide comprises an amino acid sequence selected from:

- xi) VDAKYAK-[BM]-DPSQSSELLAEAKKLNDAQAPK;
 wherein [BM] is an ABD binding motif as defined above; and
 25 xii) an amino acid sequence which has at least 84 % identity to the sequence defined in xi).

Again, polypeptides comprising minor changes as compared to the above amino acid sequences without largely affecting the tertiary structure and the function thereof are also within the scope of the present disclosure.

- 30 Thus, in some embodiments, the ABD binding polypeptides as defined above may for example have a sequence which is at least 86 %, at least 87 %, at least 89 %, at least 91 %, at least 93 %, at least 94 %, at least 96 %, or at least 98 % identical to the sequence defined by xi).

Sequence xi) in such a polypeptide may be selected from any one of SEQ ID NO:105-156. In particular, sequence xi) may be selected from any one of SEQ ID NO:105-111, such as selected from SEQ ID NO:105, SEQ ID NO:106, SEQ ID NO:107 and SEQ ID NO:108. In a specific embodiment of
5 this polypeptide, sequence xi) is SEQ ID NO:105.

The polypeptide of the invention may be in monomeric or multimeric forms. In one embodiment of the present invention the ABD binding polypeptide is present in multimeric form, comprising at least two ABD binding
10 polypeptide monomer units, whose amino acid sequences may be the same or different. Multimeric forms of the polypeptide may be advantageous in that they may have enhanced binding properties.

In one embodiment, said ABD binding polypeptide monomer units are covalently coupled together. In a particular embodiment, the ABD binding
15 polypeptide monomer units are expressed as a fusion protein.

The polypeptides may be joined 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, either directly or via a linker, for example an amino acid linker.
20 In one embodiment, said ABD binding polypeptide is in dimeric form. Possible multimeric forms also include trimeric forms. Multimeric forms of the polypeptide may comprise a suitable number of polypeptide sequences as defined above.

25 The skilled person will understand that various modifications and/or additions can be made to an ABD binding polypeptide according to any aspect disclosed herein in order to tailor the polypeptide to a specific application without departing from the scope of the present disclosure. For example, any ABD binding polypeptide disclosed herein may comprise further
30 C-terminal and/or N-terminal amino acids. Such a polypeptide should be understood as a polypeptide having additional amino acid residues at the very first and/or the very last position in the polypeptide chain, i.e. at the N- and/or C-terminus. Thus, an ABD binding polypeptide may comprise any suitable

number of additional amino acid residues, for example at least one additional amino acid residue. Each additional amino acid residue may individually or collectively be added in order to, for example, improve production, purification, stabilization *in vivo* or *in vitro*, coupling, or detection of the polypeptide. Such additional amino acid residues may comprise one or more amino acid residues added for the purpose of chemical coupling, which could for example allow coupling of the ABD binding polypeptide to a resin or matrix, for example a Sepharose-based resin or a SulfoLink coupling resin. One example of this is the addition of a cysteine residue as an additional amino acid residue, or as one of a number of additional amino acid residues. In one embodiment, the ABD binding polypeptide disclosed herein comprises a cysteine residue at the C-terminal end of the polypeptide, for example at the C-terminus. In one embodiment, said cysteine is a part of a C-terminal additional tripeptide VDC. In one embodiment, said cysteine is a lone C-terminal C.

In one particularly specific embodiment, the ABD binding polypeptide comprises a dimer of two ABD binding domains as defined above, as well as a C-terminal cysteine residue.

Such additional amino acid residues may also provide a "tag" for purification or detection of the polypeptide, such as a His₆ tag or a "myc" (c-myc) tag or a "FLAG" tag for interaction with antibodies specific to the tag or immobilized metal affinity chromatography (IMAC) in the case of the His₆-tag.

The further amino acids as discussed above may be coupled to the ABD binding polypeptide by means of chemical conjugation (using known organic chemistry methods) or by any other means, such as expression of the ABD binding polypeptide as a fusion protein.

In a further aspect, there is provided a polynucleotide encoding an ABD binding polypeptide as described above. An expression vector comprising such a polynucleotide may enable production of an ABD binding polypeptide, for example by expression in a host cell. Such tools for the expression of the ABD binding polypeptide disclosed herein are

encompassed by the present disclosure, as well as methods using them for recombinant production of the ABD binding polypeptide.

In another embodiment of the present invention there is provided a combination of the ABD binding polypeptide and a detectable agent. In a particular embodiment, the detectable agent is selected from a fluorescent agent and a radioactive agent. Fluorescent detectable agents include fluorescent polypeptides, such as green fluorescent protein, red fluorescent protein, luciferase and variants thereof. Radioactive detectable agents useful in this context include for example radionuclides such as ^3H and ^{125}I .

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

Brief description of the figures

Figure 1 is a listing of the amino acid sequences of examples of ABD binding motifs comprised in ABD binding polypeptides of the invention (SEQ ID NO:1-52), examples of 49-mer ABD binding polypeptides according to the invention (SEQ ID NO:53-104), examples of 58-mer ABD binding polypeptides according to the invention (SEQ ID NO:105-156) as well as the sequences of albumin binding domain variants (SEQ ID NO:157-162) used for selection and screening, or used in fusion proteins for illustration of the invention.

Figure 2 shows the response in ELISA for a selection of Z variants assayed with 2 µg/ml of biotinylated PEP07911, as described in Example 1.

The responses were normalized by dividing the absorbances with the signal from the positive control on the ELISA plate.

Figure 3 shows the response for a selection of Z variants in a blocking ELISA assay, performed as described in Example 1. Periplasm preparations
5 of Z variants were tested against 0.2 µg/ml PEP07911 with or without the addition of 10x excess HSA. Black bars correspond to samples without HSA and grey bars to samples with HSA added as blocking agent.

Figure 4 shows the result from column study I described in Example 3. The bars in the diagram represent the relative amounts of the sample
10 released from each of the ABD binding Z variant-coupled resins after the different wash and elution steps specified in Table 1.

Figure 5 shows the result from column study II described in Example 3. Figure 5A represents the relative amounts of sample released from each of the ABD binding Z variant-coupled resins, or from an HSA-Sepharose resin
15 included as a reference, after a first (a) or second (b) elution with 0.1 M sodium citrate, pH 3.0 or 0.5 M HAc, pH 2.5, respectively. Figure 5B shows the result of SDS-PAGE analysis of the corresponding fractions eluted from the Z variant-coupled resin in Figure 5A. "M" refers to Novex Sharp Pre-stained Protein standard (Invitrogen; Mw: 216, 160, 110, 80, 60, 50, 40, 30,
20 20, 15, 10, 3.5 kDa) and "Loaded sample" refers to the bacterial extract, containing PEP08515, initially loaded on each Z variant-coupled resin. 20 µl of the eluate from the Z variant-coupled resins, 5 µl of protein standard or bacterial extract were loaded in one lane each of the SDS-PAGE gel. Figure 5C shows the result of SDS-PAGE analysis of the corresponding fractions
25 eluted from HSA Sepharose resin in Figure 5A. Lane 1: First elution with 0.1 M sodium citrate, pH 3.0 (pH 3 (a)). Lane 2: Second elution with 0.1 M sodium citrate, pH 3.0 (pH 3 (b)). Lane 3: First elution with 0.5 M HAc, pH 2.5 (pH 2.5 (a)). Lane 4: Second elution with 0.5 M HAc, pH 2.5 (pH 2.5 (b)).
20 µl of each eluate were loaded on the SDS-PAGE gel. "M" refers to Novex
30 Sharp Pre-stained Protein standard (Invitrogen; Mw: 216, 160, 110, 80, 60, 50, 40, 30, 20, 15, 10, 3.5 kDa) of which 5 µl were loaded.

Figure 6 shows a chromatogram from affinity purification, carried out as described in Example 4, of an ABD-fusion protein on an EAH-Sepharose

resin coupled with His₆-(Z06677)₂-Cys (solid line). Purification of an identical sample on a Sepharose resin coupled with HSA (broken line), is included for reference. The sample injection point is indicated with an arrow. A and B indicate the flow-through fractions, and C and D the eluted fractions, from the His₆-(Z06677)₂-Cys-coupled resin and the HSA-coupled resin, respectively. Elution was performed with a linear pH gradient ranging from pH 5.5 to pH 2.3. The pH (dotted line) was monitored by the built-in pH meter of the HPLC system.

Figure 7 shows the dynamic binding capacity of His₆-(Z06677)₂-Cys coupled to EAH-Sepharose resin (solid line), measured after repeated alkali incubations carried out as indicated and as further described in Example 4. The binding capacity of HSA coupled to Sepharose resin (broken line) is included for reference.

Figure 8A shows a chromatogram from affinity purification of the ABD-fusion protein PEP10986 on anti-ABD agarose performed as described in Example 7. The sample injection point is indicated by an arrow. The absorbance signal at 280 nm is shown (solid line). FT and E refer to the flow-through fraction and eluted fraction, respectively. Elution was performed with 0.1 M HAc, pH 2.9 and the pH (dotted line) was monitored by the built-in pH meter of the HPLC system.

Figure 8B shows the result of SDS-PAGE analysis of selected fractions from the purification described in Example 7. Lane 1: Clarified bacterial extract loaded on the column, Lane 2: Fraction E1, Lane 3: Fraction E2 and Lane 4: Fraction E3. "M" refers to Novex Sharp Pre-stained Protein standard (Invitrogen; Mw: 216, 160, 110, 80, 60, 50, 40, 30, 20, 15, 10, 3.5 kDa). 5 µl of each sample were loaded in respective lanes of the SDS-PAGE gel, except for lane 2 where 1.25 µl was loaded.

Figure 9A shows a chromatogram from affinity purification of the ABD-fusion protein PEP03973 on anti-ABD agarose performed as described in Example 8. The sample injection point is indicated by an arrow. The absorbance signal at 280 nm is shown (solid line). FT and E refer to the flow-through fraction and eluted fraction, respectively. Elution was performed with

0.1 M HAc, pH 2.9 and the pH (dotted line) was monitored by the built-in pH meter of the HPLC system.

Figure 9B shows the result of SDS-PAGE analysis of selected fractions from the purification described in Example 8. Lane 1: Clarified bacterial
5 extract loaded on the column, Lane 2 and Lane 3: Flow-through, Lane 4: Fraction E1, Lane 5: Fraction E2, Lane 6: Fraction E3, Lane 7: Fraction E4, Lane 8: Fraction E5. "M" refers to Novex Sharp Pre-stained Protein standard (Invitrogen; Mw: 216, 160, 110, 80, 60, 50, 40, 30, 20, 15, 10, 3.5 kDa). 5 µl of each sample were loaded in respective lanes of the SDS-PAGE gel.

10 Figure 10A shows a chromatogram from affinity purification of the ABD-fusion protein PEP06548 on anti-ABD agarose performed as described in Example 9. The sample injection point is indicated by an arrow. The absorbance signal at 280 nm is shown (solid line). FT and E refer to the flow-through fraction and eluted fraction, respectively. Elution was performed with
15 0.1 M HAc, pH 2.9 and the pH (dotted line) was monitored by the built-in pH meter of the HPLC system.

Figure 10B shows the result of SDS-PAGE analysis of selected fractions from the purification described in Example 9. Lane 1: Clarified bacterial extract loaded on the column, Lane 2: Fraction E1. "M" refers to
20 Novex Sharp Pre-stained Protein standard (Invitrogen; Mw: 216, 160, 110, 80, 60, 50, 40, 30, 20, 15, 10, 3.5 kDa). 5 µl were loaded in lane M and lane 1 and 5.8 µl were loaded in lane 2 of the SDS-PAGE gel.

Figure 11A shows a chromatogram of the purification of the ABD-fusion protein PEP17081 on anti-ABD agarose performed as described in
25 Example 10. The absorbance signal at 280 nm (solid line) and the conductivity (dotted line) are shown. The sample injection point is indicated by an arrow. FT and E refer to the flow-through fraction and eluted fraction, respectively.

Figure 11B shows the result of SDS-PAGE analysis of selected
30 fractions from different stages of the purification described in Example 10. Lane M: 5 µl Novex Sharp Pre-stained Protein standard, Invitrogen (216, 160, 110, 80, 60, 50, 40, 30, 20, 15, 10, 3.5 kDa). Lane 1: 5 µl of the clarified, heat treated *E. coli* lysate that was loaded on the anti-ABD agarose column. Lane

2: 5 µl of the flow-through from the anti-ABD agarose column (FT in Figure 11A). Lane 3: 3.6 µl of the eluted pool from the anti-ABD agarose column (E in Figure 11A). Lane 4: 12 µl of PEP17081 further purified by RPC (reversed phase chromatography) and buffer exchanged.

5 Figure 12A shows a chromatogram from affinity purification of Protein 1, comprising ABD035 (SEQ ID NO:159) fused at its C-terminus to a protein hormone, on anti-ABD agarose performed as described in Example 11. The sample injection point is indicated by an arrow. The absorbance signal at 280 nm (solid line) and the conductivity (dotted line) are shown. FT, W and E
10 refer to the flow-through fraction, wash fractions and eluted fraction, respectively. First wash step, W1, was performed with 4 CV of TST and second wash step, W2, with 3 CV of 5 mM NH₄Ac, pH 5.5.

 Figure 12B shows the result of SDS-PAGE analysis of selected fractions from the purification described in Example 11. Lanes 1-3: SEC
15 purified protein: 10 µg (1), 5 µg (2) and 1 µg (3). Lane 4: Protein sample loaded on the anti-ABD agarose column, i.e. SEC purified protein diluted 1:10 in TST. Lane 5: FT concentrated 20 times. Lane 6: W1 concentrated 60 times. Lanes 7, 8 and 9: Eluted protein: 10 µg (7), 5 µg (8) and 1 µg (9), respectively. Lanes marked with "M" were loaded with a protein molecular
20 weight marker (Mw: 200, 116.3, 97.4, 66.3, 55.4, 36.5, 31, 21.5, 14.4, 6, 3.5, 2.5 kDa). Arrows indicate weak bands of contaminating proteins which were visible on the SDS-PAGE gel in samples purified by SEC only, but which were efficiently removed by purification on the anti-ABD agarose resin.

 Figure 13A shows a chromatogram from affinity purification of Protein
25 2, comprising ABD035 (SEQ ID NO:159) fused at its N-terminus to a peptide hormone, on anti-ABD agarose performed as described in Example 12. The sample injection point is indicated by an arrow. The absorbance signal at 280 nm (solid line) and the conductivity (dotted line) are shown. FT, W and E refer to the flow-through fraction, wash fractions and eluted fraction, respectively.
30 First wash step, W1, was performed with 8 CV of TST and second wash step, W2, with 3 CV of 5 mM NH₄Ac, pH 5.5.

 Figure 13B shows the result of SDS-PAGE analysis of selected fractions from the purification described in Example 12. Lane 1: Protein

sample loaded on the anti-ABD agarose column. Lane 2: FT. Lanes 4-6: Wash fractions. Lanes 7-13: Eluted protein fractions within the double peak marked in figure 13A. Fractions corresponding to the first peak within the double peak were loaded in lanes 7-10. Lanes marked with "M" were loaded with a protein molecular weight marker (Mw: 200, 116.3, 97.4, 66.3, 55.4, 36.5, 31, 21.5, 14.4, 6, 3.5, 2.5 kDa).

Examples

The following materials were used throughout this work except where otherwise noted:

- *Escherichia coli* strain XL1-Blue (Agilent Technologies, cat. no. 200268)
- Albumin binding domains ABD001 (SEQ ID NO:157), C-ABD001 (SEQ ID NO:158), ABD035 (SEQ ID NO:159), PP013 (SEQ ID NO:160), PEP07986 (SEQ ID NO:161) and PEP07911 (SEQ ID NO:162), produced essentially as described in WO2009/016043 or WO2012/004384.

Example 1

Selection and screening of ABD binding Z variants

Materials and methods

Biotinylation of target protein: ABD001 with an N-terminal cysteine (C-ABD001; SEQ ID NO:158) and PEP07911 (SEQ ID NO:162) were biotinylated using EZ-Link Maleimide PEG₂-Biotin (Pierce, cat. no. 21901). In brief, protein was dissolved in 50 mM sodium phosphate, 150 mM NaCl, 2 mM EDTA, pH 7.5. Dithiothreitol (DTT) was added to a final concentration of 20 mM and the samples were incubated for 1 h at 34 °C with end-over-end mixing. The buffer was exchanged to conjugation buffer (50 mM sodium phosphate, 150 mM NaCl, 1 mM EDTA, pH 7.0) using disposable PD-10 columns (GE Healthcare, cat. no. 17-0851-01). A five times (5x) molar excess of EZ-Link Maleimide PEG₂-Biotin (dissolved in conjugation buffer) was added to the protein samples and incubation proceeded for 2 h at room

temperature (RT) with end-over-end mixing. Subsequent buffer exchange to PBS (10 mM phosphate, 137 mM NaCl, 2.68 mM KCl, pH 7.4) was performed using PD-10 columns. PEP07986 (SEQ ID NO:161) was biotinylated according to the manufacturer's recommendations at RT for 30 min using No-
5 Weigh EZ-Link Sulfo-NHS-LC-Biotin (Pierce, cat. no. 21327) at a 10x molar excess. Subsequent buffer exchange to PBS was performed using a dialysis cassette (Slide-a-lyzer 3.5 K, 3500 MWCO, Pierce, cat. no. 66333) according to the manufacturer's instructions.

Phage display selection of ABD binding Z variants: A library of random
10 variants of protein Z displayed on bacteriophage, constructed in phagemid pAY02047 essentially as described in Grönwall *et al* (J Biotechnol, 128:162-183, 2007), was used to select ABD binding polypeptides. The library, Zlib004Naive.I, utilizes the *Taq* DNA polymerase binding molecule Z03639 (described in Gunneriusson *et al*, Protein Eng 12:873-878, 1999, where it was
15 denoted $Z_{TaqS1-1}$) as fusion partner. The library had an actual size of 1.4×10^{10} variants.

Phage stocks were prepared in a 20 l fermenter. Cells from a glycerol stock containing the phagemid library Zlib004Naive.I were inoculated in 20 l of TSB-YE (Tryptic Soy Broth-Yeast Extract; 30 g/l TSB, 5 g/l yeast extract)
20 supplemented with 2 % glucose and 100 µg/ml ampicillin. The cultivations were grown at 37 °C in a fermenter (Belach Bioteknik, BR20). When the cells reached an optical density (OD) of 0.7-0.8, approximately 2.6 l of the cultivation was infected using a 10x molar excess of M13K07 helper phage (New England Biolabs, cat. no. N0315S). The cells were incubated for 30
25 min, whereupon the fermenter was filled up to 20 l with TSB-YE supplemented with 0.1 mM IPTG (isopropyl-β-D-1-thiogalactopyranoside, for induction of expression), 25 µg/ml kanamycin and 12.5 µg/ml carbenicillin, and cells were grown at 30 °C for 22 h. The cells in the cultivation were pelleted by centrifugation at 15900 g and the phage particles remaining in the
30 medium were precipitated twice in PEG/NaCl (polyethylene glycol/sodium chloride), filtered and dissolved in PBS and glycerol as described in Grönwall *et al*, *supra*. Phage stocks were stored at -80 °C before use.

Selections were performed in three cycles against the different variants of biotinylated ABD. Phage stock preparation, selection procedure and amplification of phage between selection cycles were performed essentially as described for selection against another target in WO2009/077175. PBS supplemented with 0.1 % gelatine and 0.1 % Tween20 was used as selection buffer and the target-phage complexes were directly captured by Dynabeads® M-280 Streptavidin (Dyna, cat. no. 112.06). 1 mg beads per 0.25 µg ABD was used. *E. coli* strain XL1-Blue was used for phage amplification. Selection was performed in three cycles divided into three tracks: one track using PEP07911, one track using C-ABD001 and one track alternating between C-ABD001 and PEP07986. In cycle 1 of the selections, 100 nM PEP07911 or C-ABD001 was used in the different selection tracks, and two washes with PBST 0.1 % (PBS supplemented with 0.1 % Tween-20) were performed. An increased stringency, using a lowered target concentration and an increased number of washes, was applied in the subsequent two cycles. For the selection tracks with only one target, 50 nM followed by 25 nM PEP07911 or C-ABD001, were used in cycle 2 and 3, respectively. In the track with alternating target, 75 nM PEP07986 was used in cycle 2 and 40 nM C-ABD001 was used in cycle 3. For all tracks, 4 and 8 washes were performed in cycle 2 and 3, respectively, using PBST 0.1 %. After the wash, the bound phages were eluted with 500 µl 0.1 M glycine-HCl, pH 2.2 followed by immediate neutralization with 50 µl Tris-HCl, pH 8.0 and 450 µl PBS. In the last selection cycle, all tracks were first eluted with 0.5 M HAc, pH 4.0, followed by elution with glycine-HCl as described above. The different eluates were thereafter treated separately.

ELISA screening of Z variants: To verify that the selected Z variant molecules could indeed interact with different variants of ABD, ELISA assays were performed. The Z variants were produced by inoculating single colonies from the selections into 1 ml TSB-YE medium supplemented with 100 µg/ml ampicillin and 0.1 mM IPTG in deep-well plates (Nunc, cat. no. 278752). The plates were incubated for 18-24 h at 37 °C. Cells were pelleted by centrifugation, re-suspended in 400 µl PBST 0.05 % and frozen at -80 °C to release the periplasmic fraction of the cells. Frozen samples were

subsequently thawed in a water bath and the freeze-thawing was repeated eight times. 400 µl PBST 0.05 % was added to the samples and cells were pelleted by centrifugation. The periplasmic supernatant contained the Z variants as fusions to the *Taq* DNA polymerase binding molecule Z03639, expressed as AQHDEALE-[Z#####]-VDYV-[Z03639]-YVPG. Z##### refers to individual 58 amino acid residue Z variants.

Half-area 96-well ELISA plates (Costar, cat. no. 3690) were coated with 50 µl/well of coating buffer (50 mM sodium carbonate, pH 9.6) containing 4 µg/ml of an antibody specific for Z variants (Affibody, cat. no. 20.1000.01.0005) and incubated over-night at 4 °C. The antibody solution was poured off and the wells were blocked with 100 µl of PBSC (PBS supplemented with 0.5 % casein; Sigma, cat. no. C8654) for 2 h at RT. The blocking solution was discarded and 50 µl periplasmic solution was added to the wells and incubated for 1.5 h at RT under slow shaking. The supernatants were poured off and the wells were washed 4 times with PBST 0.05 %. Then, 50 µl of biotinylated PEP07986 or PEP07911, at a concentration of 2 µg/ml in PBSC, was added to each well. The plates were incubated for 1 h at RT followed by washes as described above. Streptavidin-HRP (Horseradish peroxidase; Thermo Scientific, cat. no. N100) diluted 1:30 000 in PBSC, was added to the wells and the plates were incubated for 45 min. After washing as described above, 50 µl ImmunoPure TMB substrate (Thermo Scientific, cat. no. 34021) was added to the wells and the plates were treated according to the manufacturer's recommendations. Absorbance of the wells was measured at 450 nm using a multi-well plate reader, Victor³ (Perkin Elmer).

As positive control, a periplasmic fraction containing a Z variant binding to an irrelevant but specified target protein and expressed as above was assayed against 5 µg/ml of this target protein, biotinylated. As negative control, the same periplasmic preparation was assayed against PEP07986 or PEP07911. Sequencing was performed for the clones with positive absorbance values against PEP07986 or PEP07911.

Sequencing: PCR fragments were amplified in two steps from single colonies using a standard PCR program and the primers AFFI-21 (5'-tgcttccggctcgtatgtgtgtg; SEQ ID NO:163) and AFFI-22 (5'-

- cggaaccagagccaccaccgg; SEQ ID NO:164). Sequencing of amplified fragments was performed using the biotinylated oligonucleotide AFFI-72 (5'-biotin-cggaaccagagccaccaccgg; SEQ ID NO:165) and a BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, cat. no. 4336919), used in accordance with the manufacturer's protocol. The sequencing reactions were purified by binding to magnetic streptavidin coated beads (Detach Streptavidin Beads, Nordiag, cat. no. 2012-01) using a Magnatrix 8000 (Magnetic Biosolution) and analyzed on ABI PRISM® 3130xl Genetic Analyzer (PE Applied Biosystems).
- 10 Blocking ELISA: A subset of clones from the initial ELISA screen were subjected to an ELISA blocking assay in order to elucidate if their target binding was affected by the presence of HSA. For the selected Z variants, the same periplasmic fractions as above were used. The ELISA blocking assay was run as the ELISA screening assay, with the following protocol
- 15 modification introduced at the target step: HSA was mixed with the target protein before addition to the assay plate. 0.2 µg/ml of biotinylated PEP07911 was mixed with a 10x molar excess of HSA and then incubated for 15 min at RT to allow complex formation before addition to the plate. As positive control, a periplasmic fraction containing a molecule binding an irrelevant
- 20 target protein and expressed as above was assayed against 5 µg/ml biotinylated specific target protein. As negative control, the same periplasmic preparation was assayed against biotinylated PEP07911. As blank, PBSC was added instead of periplasmic preparation and biotinylated PEP07911 added as target. All controls were prepared as samples with and without
- 25 addition of HSA in the same manner as above.

Results

- 30 Phage display selection of ABD binding Z variants: Individual clones were obtained after three cycles of phage display selections against different variants of biotinylated ABD. The phage particle yield (phage particles out/phage particles in) increased for each cycle, indicating enrichment in target binding clones.

ELISA screening of Z variants: The clones obtained after three cycles of selection were produced in 96-well plates and screened for ABD binding activity in ELISA. The results of a selection of the positive clones (signals corresponding to at least 2x the negative control) assayed against PEP07911 are presented in Figure 2. The control molecule specific for an irrelevant protein gave a positive signal for the specific protein, whereas no signal was obtained against PEP07986 or PEP07911.

Blocking ELISA: Clones positive for PEP07986 or PEP07911 were subjected to a blocking assay including HSA, to see if the Z variants had overlapping binding sites with the natural ligand HSA. For all tested clones, the binding signal to PEP07911 was completely extinguished by the presence of HSA, reaching the same level as the background (Figure 3). The binding of the positive control was not affected by addition of an excess of HSA and the blank showed no background signal.

Sequencing: Sequencing was performed for the clones with positive absorbance values against ABD in the ELISA screening. Each variant was given a unique identification number #####, and individual variants are referred to as Z#####. The amino acid sequences of the 58 amino acid residues long Z variants are listed in Figure 1 and in the sequence listing as SEQ ID NO:105-156. The deduced ABD binding motifs of these Z variants are denoted BM##### and listed in Figure 1 and in the sequence listing as SEQ ID NO:1-52. The amino acid sequences of the 49 amino acid residues long polypeptides predicted to constitute the complete three-helix bundle within each of these Z variants are denoted P##### and listed in Figure 1 and in the sequence listing as SEQ ID NO:53-104.

Example 2

Cloning and production of ABD binding Z variants

Materials and methods

5 Subcloning of Z variants: The DNA of seven ABD binding Z variants, Z06608 (SEQ ID NO:107), Z06620 (SEQ ID NO:110), Z06638 (SEQ ID NO:106), Z06650 (SEQ ID NO:108), Z06677 (SEQ ID NO:105), Z06678 (SEQ ID NO:109) and Z06695 (SEQ ID NO:111), were amplified from the library vector pAY02047. A subcloning strategy for construction of dimeric Z variant
10 molecules with N-terminal His₆ tag and C-terminal Cys was applied using standard molecular biology techniques and as described in detail in WO 2009/077175 for Z variants binding another target. The Z gene fragments were subcloned into the expression vector pAY01449 resulting in the encoded sequence MGSSHHHHHLQ-[Z#####][Z#####]-VDC.

15 Cultivation and purification: *E. coli* BL21(DE3) cells (Novagen) were transformed with plasmids containing the dimeric gene fragment of each respective Z variant and cultivated at 37 °C in 1 l of TSB + YE medium (Tryptic Soy Broth with Yeast Extract) supplemented with 50 µg/ml kanamycin. At OD₆₀₀ = 1, IPTG was added to induce protein expression at a
20 final concentration of 0.17 mM and the cultivation was incubated at 37 °C for another 5 h. The cells were harvested by centrifugation.

4.8 g of each cell pellet were re-suspended in 30 ml of denaturing binding buffer (20 mM sodium phosphate, 0.5 M NaCl, 20 mM imidazole, 8 M urea, pH 7.4) supplemented with 29 U/ml Benzonase® (Merck, cat. no.
25 1.01654.0001) and incubated with agitation at RT for 1 h to release the expressed protein. Cell debris was removed by centrifugation and each supernatant was applied on a 1 ml His GraviTrap IMAC column (GE Healthcare, cat. no. 11-0033-99). Contaminants were removed by washing with denaturing binding buffer, native binding buffer (20 mM sodium
30 phosphate, 0.5 M NaCl, 20 mM imidazole, pH 7.4) and wash buffer (20 mM sodium phosphate, 0.5 M NaCl, 60 mM imidazole, pH 7.4) and the ABD binding Z variants were subsequently eluted with elution buffer (20 mM sodium phosphate, 0.5 M NaCl, 250 mM imidazole, pH 7.4). Each purified

ABD binding Z variant was transferred to 10 mM NH_4HCO_3 by size exclusion chromatography. Protein concentrations were determined by measuring the absorbance at 280 nm, using a NanoDrop® ND-1000 spectrophotometer, and using the extinction coefficient of the respective protein. The ABD binding Z variants were lyophilized and the purity of the final products was analyzed by SDS-PAGE stained with Coomassie Blue. The identity of each purified ABD binding Z variant was confirmed using HPLC-MS analysis.

Results

Cultivation and purification: The seven ABD binding Z variants Z06608 (SEQ ID NO:107), Z06620 (SEQ ID NO:110), Z06638 (SEQ ID NO:106), Z06650 (SEQ ID NO:108), Z06677 (SEQ ID NO:105), Z06678 (SEQ ID NO:109) and Z06695 (SEQ ID NO:111), constructed as dimers and with an N-terminal His₆ tag and a C-terminal Cys, expressed well in *E. coli*. The amount of IMAC-purified protein from 4.8 g bacterial pellets, determined spectrophotometrically by measuring the absorbance at 280 nm, ranged from 3 mg to 19 mg for the different ABD binding Z variants.

SDS-PAGE analysis of each final protein preparation showed that these predominantly contained respective ABD binding Z variant. The correct molecular weight of each ABD binding Z variant was confirmed by HPLC-MS.

Example 3

Assessment of binding and elution characteristics of ABD binding Z variants

In this Example, the binding and elution characteristics of a set of ABD binding polypeptides, selected and produced as described in Example 1 and 2, respectively, were studied in a small-scale format using spin columns.

Materials and methods

Coupling of the ABD binding Z variants to resin: 1 mg of each lyophilized ABD binding Z variant Z06608, Z06620, Z06638, Z06650, Z06677, Z06678 and Z06695, in the format His₆-(Z#####)₂-Cys produced as described in Example 2, was resuspended in a reducing buffer (50 mM Tris-HCl, 5 mM

EDTA, 20 mM DTT, pH 8.5) and incubated at RT for 2 h. Reduced protein solutions were transferred to a coupling buffer (50 mM Tris-HCl, 5 mM EDTA, pH 8.5) including 50 mM DTT, using size exclusion chromatography, and then mixed with portions of 0.2 ml SulfoLink Coupling Resin (Thermo Fisher Scientific, cat. no. 20401). This procedure enables site-directed reaction between the thiol groups of the C-terminal cysteine residues of the ABD binding Z variants and the iodoacetyl groups of the resin, forming a covalent thioether bond. The coupling reaction was carried out according to the instructions by the manufacturer. The degree of coupling, defined as mg of coupled ABD binding Z variant per ml of resin, was determined as follows: The amount of coupled ABD binding Z variant was calculated by subtracting the amount of uncoupled material from the original amount of sample, based on concentrations obtained by spectrophotometric measurements of the absorbance at 280 nm. The amount of coupled ABD binding Z variant in mg was then divided with the volume of the SulfoLink Coupling Resin.

Column study I: Assessment of ABD binding Z variant-coupled resins using a pure sample: A first column study was performed to test binding, wash and elution properties of each of the seven ABD binding Z variants coupled to resin and by using a previously purified sample of the ABD fusion protein PEP08517. PEP08517 comprises the albumin binding domain PP013 (SEQ ID NO:160) fused at its N-terminus to a cytokine binding variant of protein Z. 0.1 ml portions of the resins were transferred to test tubes and 0.1 ml of a pure sample of PEP08517 at 2 mg/ml in coupling buffer was added. The tubes were incubated on a rotating wheel for 1 h at RT. Each sample-resin mix was transferred to an empty spin column. Unbound protein was collected by centrifugation (flow-through, FT). 0.1 ml of different solutions, specified in Table 1, were thereafter added to wash and subsequently elute bound PEP08517 molecules. Each wash and elution fraction was collected by centrifugation.

Collected fractions were analyzed in a spectrophotometer by measuring the absorbance at 280 nm.

Table 1: Specification of the sample and solutions in column study I

Step	Sample / Solution	Abbreviation
1	Pure sample of PEP08517 in 50 mM Tris-HCl, 5 mM EDTA, pH 8.5	PEP08517
2	Unbound PEP08517	FT
3	2.68 mM KCl, 1.47 mM KH ₂ PO ₄ , 137 mM NaCl, 8.1 mM Na ₂ HPO ₄ , pH 7.4	PBS
4	5 mM Tris-HCl, pH 8	Tris
5	0.5 M HAc / NaAc, pH 4	pH 4 (a)
6	0.5 M HAc / NaAc, pH 4	pH 4 (b)
7	0.5 M HAc, pH 2.5	pH 2.5 (a)
8	0.5 M HAc, pH 2.5	pH 2.5 (b)

Column study II: Assessment of ABD binding Z variant-coupled resins

using a bacterial extract: A second column study was performed to further

- 5 test binding, wash and elution properties of four ABD binding Z variant-coupled resins from column study I. The ligands of these resins were His₆-(Z06608)₂-Cys, His₆-(Z06638)₂-Cys, His₆-(Z06677)₂-Cys and His₆-(Z06650)₂-Cys. An additional column packed with HSA Sepharose (human serum albumin coupled, according to the instructions by the manufacturer, via free
- 10 amines to CNBr-activated Sepharose 4 Fast Flow, GE Healthcare, cat. no. 17-0981) was included as a reference. A clarified bacterial extract, prepared from an *E. coli* pellet by sonication and centrifugation, was used as sample in this experiment. 0.17 ml of the bacterial extract, containing approximately 0.5 mg of PEP08515 (comprising the albumin binding domain PP013 (SEQ ID
- 15 NO:160) fused at its N-terminus to a PDGFR- β (Platelet Derived Growth Factor Receptor beta) binding variant of protein Z) was transferred to spin columns packed with each respective ABD binding Z variant-coupled resin or HSA Sepharose. Flow-through was collected by centrifugation and reapplied twice to the columns. Different solutions, specified in Table 2, were added to
- 20 remove bacterial host proteins and to subsequently elute bound PEP08515 molecules. Wash and elution fractions were collected by centrifugation and analyzed in a spectrophotometer by measuring the absorbance at 280 nm, and by SDS-PAGE.

Table 2: Specification of the sample and solutions in column study II

Step	Sample / Solution	Abbreviation	Volume (ml)
1	Sample: bacterial extract containing PEP08515	PEP08515	0.17
2	Flow-through I collected after sample application	FT I	0.17
3	Flow-through II collected after re-application of flow-through I	FT II	0.17
4	2.68 mM KCl, 1.47 mM KH ₂ PO ₄ , 137 mM NaCl, 8.1 mM Na ₂ HPO ₄ , pH 7.4	PBS	4 x 0.2
5	5 mM NH ₄ Ac, pH 5.5	NH ₄ Ac	2 x 0.2
6	0.1 M sodium citrate, pH 3.0	pH 3.0 (a)	0.1
7	0.1 M sodium citrate, pH 3.0	pH 3.0 (b)	0.1
8	0.5 M HAc, pH 2.5	pH 2.5 (a)	0.1
9	0.5 M HAc, pH 2.5	pH 2.5 (b)	0.1

Results

Coupling efficiency of ABD binding polypeptides: The degree of

- 5 coupling of each ABD binding polypeptide to the SulfoLink Coupling Resin is shown in Table 3.

Table 3: The degree of coupling of ABD binding Z variants to SulfoLink Resin

ABD binding polypeptide	Amount polypeptide coupled per ml resin (mg)	Amount polypeptide coupled per ml resin (μmol)
His ₆ -(Z06608) ₂ -Cys	2.8	0.19
His ₆ -(Z06620) ₂ -Cys	2.7	0.19
His ₆ -(Z06638) ₂ -Cys	2.9	0.20
His ₆ -(Z06650) ₂ -Cys	3.4	0.23
His ₆ -(Z06677) ₂ -Cys	2.2	0.15
His ₆ -(Z06678) ₂ -Cys	2.1	0.14
His ₆ -(Z06695) ₂ -Cys	3.9	0.27

- 10 Column study I: Assessment of ABD binding Z variant-coupled resins using a pure sample: The amount of the sample PEP08517 released from each of the seven ABD binding Z variant-coupled resins after the different wash and elution steps specified in Table 1, and as determined by measuring the absorbance at 280 nm, is shown in Figure 4.
- 15 Under the conditions studied, the resins coupled with the ABD binding Z variants (His₆-(Z06608)₂-Cys, His₆-(Z06638)₂-Cys, His₆-(Z06650)₂-Cys) and

His₆-(Z06677)₂-Cys, respectively, showed the most favorable elution profile with minor sample leakage during sample application and wash and with the majority of sample being released at elution with pH 2.5 buffer.

None of the ABD binding Z variant-coupled resins eluted much of the sample at pH 4. Interestingly, some resins, for example His₆-(Z06608)₂-Cys, eluted most of the sample at the second addition of pH 2.5 buffer ("pH 2.5 (b)"), while for example His₆-(Z06677)₂-Cys eluted the majority of the sample at the first pH 2.5 buffer addition ("pH 2.5 (a)"), indicating that a higher elution pH could be used with the affinity ligand His₆-(Z06677)₂-Cys as compared with the other candidates.

Column study II: Assessment of ABD binding Z variant-coupled resins using a bacterial extract: The four best performing ABD binding Z variant-coupled resins from column study I, including the affinity ligands His₆-(Z06608)₂-Cys, His₆-(Z06638)₂-Cys, His₆-(Z06650)₂-Cys and His₆-(Z06677)₂-Cys, were selected for further binding, wash and elution studies in which a bacterial extract containing PEP08515 was used as a sample. The amount of PEP08515 released from each of the four ABD binding Z variant-coupled resins, or from an HSA-Sepharose resin included as a reference, after the different elution steps specified in Table 2, and as determined by measuring the absorbance at 280 nm, is shown in Figure 5A.

The SDS-PAGE analysis of the same eluted fractions is shown in Figure 5B. The results from the two analyses agreed well, i.e. for a certain affinity ligand, an eluted fraction with a high absorbance reading also gave a thick band on the SDS-PAGE gel, and vice versa for fractions with a lower absorbance reading. As in column study I, the resin coupled with His₆-(Z06677)₂-Cys released a majority of its bound protein at the higher pH range of elution ("pH 3 (a)" and "pH 3 (b)").

The purity of all eluted samples was generally very high (exceeding 95 %, as estimated from the SDS-PAGE analysis), but slight differences could be observed for the different ABD binding Z variants.

In comparison to the HSA Sepharose resin (SDS-PAGE analysis shown in Figure 5C), the ABD binding Z variant-coupled resins appear to have a higher binding capacity as well as more favorable elution properties as

judged both from the absorbance readings and the SDS-PAGE analysis. The difference in capacity cannot be due to differences in ligand density; in fact, the number of coupled HSA molecules on HSA Sepharose was approximately 1.5 - 2.2 times higher compared to the ABD binding Z variant-coupled resins.

5

Example 4

Further characterization and column studies of an ABD binding Z variant

Based on the results presented in Example 3, His₆-(Z06677)₂-Cys was
10 selected for further characterization and studies on columns connected to a chromatography system.

Materials and methods

Determination of the melting point by circular dichroism analysis:

15 Circular dichroism (CD) analysis was carried out to determine the melting point (T_m) of His₆-(Z06677)₂-Cys. The purified sample was diluted to 0.5 mg/ml in PBS. A variable temperature measurement (VTM) was performed, in which the absorbance at 220 nm was monitored during heating of the sample from 20 °C to 90 °C, with a temperature slope of 5 °C/min. T_m was
20 determined from the midpoint of the transition in the CD signal vs. temperature plot. The CD measurement was performed on a Jasco J-810 spectropolarimeter (Jasco Scandinavia AB) using a cell with an optical path length of 1 mm.

Chromatography study of elution pH: His₆-(Z06677)₂-Cys was
25 immobilized on activated EAH-Sepharose 4B (GE Healthcare, cat. no. 17-0569-01). Activation and immobilization was carried out according to the manufacturer's instructions, and the degree of coupling was determined as described in Example 3. Activated EAH Sepharose contains an iodoacetyl group that enables site-directed coupling via the C-terminal cysteine of the
30 affinity ligand. 0.43 ml of the resin was packed in a Tricorn 5/50 column (GE Healthcare). As a reference, 0.47 ml HSA Sepharose (see Example 3) was packed in the same kind of column.

The elution experiment was performed using an ÄKTAexplorer 10 chromatography system (GE Healthcare). The two columns were attached to the system and equilibrated with PBS. 2 ml of a sample containing 1 mg PEP08519 (comprising the albumin binding domain PP013 (SEQ ID NO:160) fused at its N-terminus to a *Taq* polymerase binding variant of protein Z) was loaded on each column followed by wash with PBS and 0.1 M sodium citrate, 0.9 % NaCl, pH 5.5. Bound protein was eluted by a linear pH gradient ranging from pH 5.5 to pH 2.3 (using 0.1 M sodium citrate, 0.9 % NaCl, pH 5.5, and 0.1 M sodium citrate, 0.9 % NaCl, pH 2.3) over 16 column volumes (CV). The pH was monitored by the built-in pH meter of the ÄKTAexplorer system.

Chromatography study of the binding capacity and alkali resistance of an ABD binding polypeptide: In this experiment, the dynamic binding capacity and resistance to alkali was tested for the His₆-(Z06677)₂-Cys resin and an HSA Sepharose resin packed in the Tricorn 5/50 columns used in the chromatography study described in the previous section.

The experiment was performed using an ÄKTAexplorer 10 chromatography system. The two columns were attached to the system and equilibrated with PBS. For the capacity study, a sample containing 0.25 mg/ml of PEP08519 in PBS was loaded on each column at a flow-rate of 0.2 ml/min (corresponding to a residence time of 2.4 min) until the absorbance at 280 nm of the solution emerging from the columns reached 10 % of the absorbance of the original sample (i.e. 10 % breakthrough). The dead volume (i.e. tubing and column volume, measured by running a sample through a column comprising no affinity ligand) was subtracted from the sample volume. Bound protein was eluted with 0.1 M sodium citrate, 0.9 % NaCl, pH 2.3. The columns were then re-equilibrated with PBS.

After the capacity runs, the columns were subjected to alkali treatment by applying 5 CV of 0.2 M NaOH at a flow-rate of 1 ml/min, followed by an incubation period without flow. The total incubation time in alkali was 25 min. Neutralization of the columns was performed by washing with 5 CV of PBS at 1 ml/min, followed by a new capacity measurement as described above.

Two more alkali treatments followed by capacity measurements were carried out as above, but with the NaOH concentration increased to 0.5 M and, in the last alkali treatment, the incubation time was extended to 2 h.

5 Results

Circular dichroism analysis: The melting point of His₆-(Z06677)₂-Cys was determined to be 54°C.

Chromatography study of elution pH of an ABD binding Z variant: The coupling degree of His₆-(Z06677)₂-Cys to the activated EAH Sepharose resin was determined to be 2.8 mg polypeptide per ml resin.

As shown in Figure 6, the elution of the ABD-fusion molecule PEP08519 from the column containing the His₆-(Z06677)₂-Cys resin appeared earlier in the gradient (i.e. at a higher pH) than from the column with the HSA Sepharose resin, which also showed lower binding capacity with a larger fraction emerging in the flow through.

pH at peak maximum for the eluted peak from the His₆-(Z06677)₂-Cys column was 3.3, while the corresponding pH for the HSA Sepharose column was 2.6.

Chromatography study of the binding capacity and alkali resistance of an ABD binding Z variant: The dynamic binding capacity was determined by measuring the amount of sample loaded until 10 % breakthrough. The dynamic binding capacity of the His₆-(Z06677)₂-Cys column was determined to be 3.0 mg PEP08519 per ml resin. The corresponding capacity for the HSA Sepharose column was determined to 0.5 mg per ml resin. As in Example 3, the difference in capacity cannot be explained by the difference in ligand density, since the number of immobilized HSA molecules were estimated to be twice the number of His₆-(Z06677)₂-Cys molecules on their respective resins.

The dynamic binding capacity was re-measured after each alkali incubation. The results are shown in Figure 7. The His₆-(Z06677)₂-Cys column maintained its original capacity after all incubations. This indicates a high resistance to relatively harsh alkali conditions, which is an important feature for cleaning in place (CIP) procedures enabling repeated use of the

column. In contrast, the HSA Sepharose column, included as a reference, lost most of its capacity after 25 min in 0.2 M NaOH.

5

Example 5

Column studies of an ABD binding Z variant immobilized on different resins

In this example, the ABD binding Z variant His₆-(Z06677)₂-Cys was immobilized on four different types of resins, and the binding capacity and alkali resistance were assessed chromatographically.

10

Materials and methods

Coupling of His₆-(Z06677)₂-Cys to different resins: 4 mg of the lyophilized His₆-(Z06677)₂-Cys (produced as described in Example 2) was dissolved in 1 ml 0.1 M NaHCO₃, 0.5 M NaCl, pH 8.3. One half of the sample was immobilized on 0.67 ml NHS-activated Sepharose 4 Fast Flow (GE Healthcare, cat. no. 17-0906) and one half of the sample was immobilized on 0.67 ml CNBr-activated Sepharose 4 Fast Flow (GE Healthcare, cat. no. 17-0981). Both coupling reactions were carried out according to the instructions by the manufacturer.

15

2 mg of lyophilized His₆-(Z06677)₂-Cys was dissolved in 0.5 ml 50 mM Tris-HCl, 5 mM EDTA, 20 mM DTT, pH 8.5 and incubated for 2 h. DTT was removed by size exclusion chromatography and the reduced polypeptide was mixed with 0.67 ml SulfoLink Coupling Resin. The coupling reaction was performed according to the instructions by the manufacturer.

20

The fourth resin assessed in this experiment was EAH Sepharose immobilized with His₆-(Z06677)₂-Cys as described in Example 4.

The degree of coupling for each resin was determined as described in Example 3.

Chromatography study of the binding capacity and alkali resistance of

four ABD binding resins: The four His₆-(Z06677)₂-Cys-coupled resins were packed in Tricorn 5/50 columns. The dynamic binding capacity was tested, after which the resins were subjected to 0.5 M NaOH for 2 h, followed by a

30

second dynamic binding capacity test. The capacity tests and alkali incubations were carried out as described in Example 4.

Results

- 5 Coupling of the ABD binding Z variant His₆-(Z06677)₂-Cys to different resins: The degree of immobilization of His₆-(Z06677)₂-Cys to each of the four different resins assessed in this experiment are shown in Table 5.

Table 5: The degree of coupling of His₆-(Z06677)₂-Cys to different resins

Type of resin	His ₆ -(Z06677) ₂ -Cys coupled per ml resin (mg)
NHS-activated Sepharose 4 Fast Flow	2.6
CNBr-activated Sepharose 4 Fast Flow	2.6
SulfoLink Coupling Resin	1.9
Activated EAH Sepharose	2.8

10

Chromatography study of the binding capacity and alkali resistance of four ABD binding resins: The dynamic binding capacity determined after coupling of His₆-(Z06677)₂-Cys to each of the four resins is presented in Table 6.

15

Table 6: The dynamic binding capacity of His₆-(Z06677)₂-Cys coupled to different resins

Type of resin	Dynamic binding capacity (mg sample/ ml resin)	(mol sample/ mol ligand)
NHS-activated Sepharose 4 Fast Flow	0.9	0.4
CNBr-activated Sepharose 4 Fast Flow	2.0	0.9
SulfoLink Coupling Resin	2.3	1.4
Activated EAH Sepharose	3.4	1.4

- 20 The dynamic binding capacities of the two iodoacetyl-coupled resins, SulfoLink Coupling Resin and Activated EAH Sepharose, were determined to 2.3 and 3.4 mg sample per ml resin, respectively. However, when comparing the molar amount of sample that can bind per mole of affinity ligand, these resins show the same dynamic binding capacity (1.4 mol of sample / mol of His₆-(Z06677)₂-Cys ligand). In other words, for His₆-(Z06677)₂-Cys coupled to the SulfoLink resin, the two Z molecules within the dimer bind a sample

molecule each to a higher extent compared to His₆-(Z06677)₂-Cys coupled to EAH Sepharose resin.

The dynamic binding capacities of the two amine-coupled resins, i.e. NHS-activated Sepharose and CNBr-activated Sepharose, were lower than
 5 for the two iodoacetyl-coupled resins. This indicates that the site-directed immobilization through a single cysteine, as used for coupling to the iodoacetyl resins, makes the binding sites of the ABD binding Z variant more accessible to the sample molecules in the mobile phase.

The dynamic binding capacity of the NHS resin was less than half
 10 compared with the CNBr resin, despite the fact that the NHS resin holds a spacer arm that would theoretically improve the capacity, by making the affinity ligands more accessible.

The dynamic binding capacities of the four different resins after alkali treatment with 0.5 M NaOH for 2 h are presented in Table 7. The dynamic
 15 binding capacities of the iodoacetyl-coupled resins were unaffected by the alkali incubation, while the capacities of the amine-coupled resins dropped by approximately 10 %.

20 Table 7. Comparison of the dynamic binding capacities before and after alkali treatment.

Type of resin	Original dynamic binding capacity (mol sample / mol affinity ligand)	Dynamic binding capacity after alkali incubation (mol sample / mol affinity ligand)
NHS-activated Sepharose 4 Fast Flow	0.41	0.38
CNBr-activated Sepharose 4 Fast Flow	0.92	0.81
SulfoLink Coupling Resin	1.44	1.44
Activated EAH Sepharose	1.44	1.44

Example 6

Production of anti-ABD agarose

This Example describes a larger-scale production of the ABD binding Z variant Z006677 in a dimeric format and with an C-terminal Cys, i.e.

- 5 (Z06677)₂-Cys, and its subsequent immobilization on a SulfoLink Coupling Resin.

Materials and methods

- Cultivation and purification: The ABD binding Z variant Z06677 was
10 subcloned as a dimer into an expression vector in which expression is regulated by a T7 promoter. The ABD binding polypeptide was expressed with the additional N-terminal amino acid sequence GSSLQ and the additional C-terminal amino acid sequence VDC. Thus, the expressed polypeptide has the sequence GSSLQ-[Z06677][Z06677]-VDC. *E. coli*
15 BL21(DE3) cells (Novagen) were transformed with the plasmid and cultivated at 37 °C in 20 l of TSB + YE medium (Tryptic Soy Broth with Yeast Extract) supplemented with 50 µg/ml kanamycin. At OD₆₀₀ = 1, IPTG, at a final concentration of 0.17 mM, was added to induce protein expression and the cultivation was incubated at 37 °C for another 5 h. The cells were harvested
20 by centrifugation.

- As seen in Example 4 and Example 5, His₆-(Z06677)₂-Cys was proven to be resistant to alkali. This property was taken advantage of in the purification of (Z06677)₂-Cys, as high concentrations of NaOH contribute to both cell disruption and purification, and a large part of *E. coli* host proteins
25 denature at high pH, whereas (Z06677)₂-Cys remains in solution.

- 165 g of cell pellet was re-suspended in 1.2 l of 0.5 M NaOH by use of Ultra-Turrax T-50 basic (IKA WERKE). The suspension was incubated for 1 h at RT under stirring and then frozen at -20 °C. The frozen sample was thawed and titrated to pH 8.2 by addition of 300 ml of 2 M HCl and Tris base to a final
30 concentration of 20 mM. Cell debris, denatured protein and DNA were removed by centrifugation. To the clarified sample, 800 ml of [20 mM Tris-HCl, pH 8.0, 4.6 g DTT] and Benzonase® (Merck, cat. no. 1.01654.0001) to a final concentration of 10 U/ml were added and incubation proceeded for 1 h at

RT. Thereafter, 300 g of ammonium sulphate was added to a concentration of 1 M, and the sample was incubated at RT under stirring for 40 min. Protein precipitated after the addition of ammonium sulphate was removed by centrifugation, followed by filtration through a Nalgene bottle top filter (0.45 μm).

Acetonitrile (ACN) was added to the sample to a concentration of 2 % (v/v). The sample was loaded onto a FineLine 35 column (GE Healthcare) packed with 125 ml SOURCE 30 RPC (GE Healthcare) by use of an ÄKTAexplorer 100 chromatography system (GE Healthcare). The column was washed with 2 % ACN in Milli-Q water containing 0.1 % TFA (tri-fluoro acetic acid) followed by wash with an identical buffer containing 4 % of ACN. Bound protein was eluted by increasing the ACN concentration from 4 % to 25 % ACN over 15 CV. Eluted fractions were collected and analyzed by SDS-PAGE and LC-MS. Relevant fractions were pooled and transferred to coupling buffer (50 mM Tris-HCl, 5 mM EDTA, pH 8.5) by size exclusion chromatography using an XK-50 column (GE Healthcare) packed with 500 ml Sephadex G-25 (GE Healthcare). The buffer exchanged sample was analyzed spectrophotometrically by measuring the absorbance at 280 nm, and by SDS-PAGE, loading several different quantities of (Z06677)₂-Cys, and subsequent staining with Comassie Blue.

Coupling of the ABD binding polypeptide (Z06677)₂-Cys to resin: The sample containing (Z06677)₂-Cys at a concentration of 3.4 mg/ml in coupling buffer was reduced by addition of DTT to a concentration of 20 mM, followed by incubation under stirring for 1 h at RT. DTT was removed by size exclusion chromatography using an XK-50 column packed with 500 ml Sephadex G-25, pre-equilibrated with coupling buffer. The reduced (Z06677)₂-Cys was mixed with SulfoLink Coupling Resin (Thermo Scientific, cat. no. 20404) at a ratio of 6 mg (Z06677)₂-Cys per ml resin. The subsequent coupling reaction was carried out according to the instructions by the manufacturer, but the incubation of the protein-resin mixture was extended from 15 to 35 min. The coupling efficiency was estimated by SDS-PAGE analysis of the uncoupled protein.

Results

Cultivation and purification: The purified dimeric ABD binding Z variant (Z06677)₂-Cys was analyzed in a spectrophotometer by measuring the absorbance at 280 nm and by SDS-PAGE. (Z06677)₂-Cys lacks tryptophans, resulting in low absorbance readings at 280 nm. The concentration of the Z variant molecule was therefore estimated from the SDS-PAGE analysis, which included several different quantities of (Z06677)₂-Cys. Visual inspection of the SDS-PAGE gel estimated the concentration of (Z06677)₂-Cys to 3.4 mg/ml at a purity of approximately 95 %.

10 Coupling of the ABD binding polypeptide (Z06677)₂-Cys to resin: The ligand density was estimated to 5.8 mg (Z06677)₂-Cys / ml SulfoLink Coupling Resin by visually inspecting an SDS-PAGE gel run on the uncoupled protein fraction of the coupling reaction.

 The produced (Z06677)₂-Cys-coupled resin is further referred to as
15 “anti-ABD agarose”, and in the following Examples it was used to demonstrate the wide applicability of the resin, in terms of binding to different albumin binding domain moieties in fusion with different proteins of varying size and functions. It is shown that successful purification is achieved regardless of where in the target protein the ABD moiety is situated. In other
20 words, the ABD moiety may be placed at the N-terminus, at the C-terminus or even within a fusion protein such that it is flanked by other protein moieties on either side.

Example 7

25 Purification of PEP10986 using anti-ABD agarose

 In this example, PEP10986 (12.5 kDa), comprising an albumin binding domain (PP013, SEQ ID NO:160) fused at its N-terminus to a cytokine binding variant of protein Z, was purified using a column packed with anti-ABD agarose.

30

Materials and methods

 Pelleted *E. coli* cells harboring soluble PEP10986 were suspended in TST-buffer (Tris, Saline, Tween: 25 mM Tris-HCl, 1 mM EDTA, 200 mM NaCl,

0.05 % Tween 20, pH 8.0) supplemented with 20 U/ml Benzonase®. Cells were disrupted by sonication and the cell debris removed by centrifugation. Chromatography was performed using an ÄKTAexplorer 100 system. The clarified sample was applied on an XK-50 column packed with 100 ml anti-

5 ABD agarose (prepared as described in Example 6) and pre-equilibrated with TST. The column was washed with 8 CV of TST followed by 8 CV of 5 mM NH₄Ac pH 5.5. Bound protein was eluted by applying 3 CV of 0.1 M HAc, pH 2.9. Flow-through fractions, wash fractions and eluted fractions were collected for further analyses by SDS-PAGE and HPLC-MS.

10 To investigate the robustness and alkali stability of the anti-ABD agarose resin, several purifications, each followed by a CIP (Cleaning In Place) cycle of 2-3 column volumes of 0.5 M NaOH, were subsequently performed using the same column as above.

15 *Results*

The chromatogram from purification of PEP10986 on the anti-ABD agarose resin is presented in Figure 8A and the SDS-PAGE analysis of selected fractions is shown in Figure 8B. The eluted fractions E1, E2 and E3, corresponding to lane 2, 3 and 4 respectively in Figure 8B, were pooled. The
20 correct mass of PEP10986 was verified by HPLC-MS analysis and the purity was estimated to be higher than 95 % as judged from SDS-PAGE analysis. According to spectrophotometrical analysis, the pool contained 908 mg of purified PEP10986, corresponding to a yield of approximately 90 %.

Robustness and alkali stability of the anti-ABD agarose resin was
25 confirmed by additional purifications of both PEP10986 and other ABD fusion molecules, each followed by a CIP (Cleaning In Place) cycle of 2-3 column volumes of 0.5 M NaOH, using the same column as in this Example. After 23 CIP cycles, no significant decrease in capacity had yet been observed.

Example 8

Purification of PEP03973 using anti-ABD agarose

In this example, PEP03973 (12.4 kDa), comprising the wild-type albumin binding domain (ABD001, SEQ ID NO:157) fused at its N-terminus to
5 a HER2 (Human Epidermal growth factor Receptor 2) binding variant of protein Z, was purified using a column packed with anti-ABD agarose.

Materials and methods

Pelleted *E. coli* cells harboring soluble PEP03973 were suspended in
10 TST-buffer supplemented with 20 U/ml Benzonase®. Cells were disrupted by sonication and the cell debris removed by centrifugation. Chromatography was performed using an ÄKTAexplorer 100 system. The clarified sample was applied on an XK-50 column packed with 100 ml anti-ABD agarose (prepared as described in Example 6) and pre-equilibrated with TST. The column was
15 washed with 5 CV of TST followed by 3 CV of 5 mM NH₄Ac pH 5.5. Bound protein was eluted by applying 3 CV of 0.1 M HAc, pH 2.9. Flow-through fractions, wash fractions and eluted fractions were collected for further analyses by SDS-PAGE and HPLC-MS.

20 *Results*

The chromatogram from purification of PEP03973 on the anti-ABD agarose resin is presented in Figure 9A and the SDS-PAGE analysis of selected fractions is shown in Figure 9B. The eluted fractions E2, E3 and E4, corresponding to lane 5, 6 and 7 respectively in Figure 9B, were pooled. The
25 correct mass of PEP03973 was verified by HPLC-MS analysis and the purity was estimated to be higher than 95 % as judged from SDS-PAGE analysis. According to spectrophotometrical analysis, the pool contained 1036 mg of purified PEP03973, corresponding to a yield of approximately 97 %.

Example 9

Purification of PEP06548 using anti-ABD agarose

In this example, PEP06548 (20.3 kDa), comprising an albumin binding domain (ABD035, SEQ ID NO:159, with the C-terminal extension VDC) fused at its N-terminus to two moieties (i.e. a dimer) of a variant of protein Z binding to TNF- α (tumor necrosis factor alpha), was purified using a column packed with anti-ABD agarose. PEP06548 also comprises an N-terminal hexahistidine tag and a C-terminal cysteine residue.

10 *Materials and methods*

Pelleted *E. coli* cells harboring soluble PEP06548 were suspended in TST-buffer supplemented with 27 U/ml Benzonase®. Cells were disrupted by sonication and the cell debris removed by centrifugation. The C-terminal cysteines of the PEP06548 sample molecules were reduced by addition of DTT to a final concentration of 20 mM, followed by incubation for 30 min at RT. Chromatography was performed using an ÄKTAexplorer 100 system. The clarified and reduced sample was applied on an XK-16 column packed with 9 ml anti-ABD agarose and pre-equilibrated with TST. This batch of anti-ABD agarose was prepared essentially as described in Example 6, except for a lower ligand density of approximately 2.3 mg (Z06677)₂-Cys per ml of resin. The column was washed with 5.5 CV of TST followed by 3.3 CV of 5 mM NH₄Ac pH 5.5. Bound protein was eluted by applying 2.9 CV of 0.1 M HAc, pH 2.9. Fractions were collected for further analyses by SDS-PAGE and HPLC-MS.

25

Results

The chromatogram from purification of PEP06548 on the anti-ABD agarose resin is presented in Figure 10A and the SDS-PAGE analysis of the eluted fraction E1 is shown in Figure 10B. The purity was estimated to be higher than 95 % as judged from the SDS-PAGE analysis. Bands at around 40 kDa were most probably derived from dimers formed by reaction between the C-terminal cysteines of the sample molecules. The correct masses of

30

PEP06548, as well as a minor portion of the dimeric variant, were also identified in HPLC-MS analysis.

According to absorbance measurements, 3.7 mg purified PEP06548 per ml of resin was obtained, or approximately one PEP06548 molecule per
5 (Z06677)₂-Cys ligand.

Example 10

Purification of PEP17081 using anti-ABD agarose

10 In this example, PEP17081 (18.6 kDa), comprising an albumin binding domain (PEP07986, SEQ ID NO:161) fused to two moieties of a cytokine binding variant of protein Z in the format Z-PEP07986-Z, was purified using a column packed with anti-ABD agarose. Subsequent purification is also described.

15

Materials and methods

Pelleted *E. coli* cells harboring soluble PEP17081 were suspended in TST-buffer. Cells were disrupted by heat treatment in a water bath (83 °C, 10 min), followed by cooling on ice. Benzonase® was added to a final
20 concentration of 15 U/ml to reduce viscosity caused by nucleic acids. Cell debris was removed by centrifugation and filtration (0.45 µm filter).

A first purification step was performed by anti-ABD agarose affinity chromatography using an ÄKTApurifier 100 system. The clarified sample was applied on an XK-26 column packed with 25 ml anti-ABD agarose and pre-
25 equilibrated with 2x TST (i.e. TST buffer with all components at double concentration). The column was washed with 5 CV of 2x TST followed by 8 CV of 5 mM NH₄Ac, pH 5.5. Bound protein was eluted by applying 3 CV of 0.1 M HAc, pH 2.9. Fractions were collected for further analyses by SDS-PAGE and HPLC-MS.

30 A second purification step was performed by RPC, using an ÄKTAexplorer 100 system. The protein eluted from the anti-ABD agarose column was supplemented with acetonitrile to a final concentration of 10 % and loaded on an HR-16 column (GE Healthcare) packed with 24 ml

SOURCE 15 RPC (GE Healthcare) and pre-equilibrated with 10 % acetonitrile, 0.1 % trifluoroacetic acid (TFA) in Milli-Q water (Solvent A). The column was washed with 3.9 CV Solvent A. Bound material was eluted with a 15 CV linear gradient of increasing concentration of 80 % acetonitrile, 0.1 %
5 TFA in Milli-Q water (Solvent B), ending at 50 % Solvent B. Fractions were collected for further analyses by SDS-PAGE and HPLC-MS.

A third purification and buffer exchange step was performed by size exclusion chromatography (SEC) using an ÄKTAexplorer 100 system. Selected fractions eluted from the RPC column were pooled and buffer
10 exchanged to 1x PBS on a XK-50 column packed with 500 ml Sephadex G-25 (GE Healthcare). Fractions were collected for further analyses by SDS-PAGE and HPLC-MS.

Results

15 The chromatogram from purification of PEP17081 on the anti-ABD agarose resin is presented in Figure 11A, and the SDS-PAGE analysis is shown in Figure 11B.

Heat treatment of the *E. coli* cells served both as a cell disruption step and a purification step. PEP17081 is resistant to heat while many other *E. coli*
20 proteins are not and consequently precipitated upon heating. As a result, the material loaded on the anti-ABD agarose column was relatively pure (lane 1, Figure 11B).

The vast majority of PEP17081 bound to the anti-ABD agarose resin, which is demonstrated by the apparent absence of PEP17081 in the flow
25 through sample (lane 2, Figure 11B). According to spectrophotometric analysis, the eluted pool contained 330 mg PEP17081, corresponding to a binding capacity of 13 mg PEP17081 per ml resin and a yield exceeding 95 %. The purity of eluted PEP17081 was estimated to be higher than 95 % as judged from the SDS-PAGE analysis (lane 3, Figure 11B). A further
30 increase in purity was obtained after RPC and buffer exchange by SEC (lane 4, Figure 11B). The correct mass of PEP17081 was verified by HPLC-MS analysis.

Example 11

Purification of a protein hormone using anti-ABD agarose

In this example, Protein 1 (22.6 kDa; pI 10), comprising an albumin
5 binding domain (ABD035, SEQ ID NO: 159) fused at its C-terminus to a
protein hormone, was purified on an ÄKTAexplorer system using a column
packed with anti-ABD agarose.

Materials and methods

10 Protein 1 was expressed in *E.coli* in the form of inclusion bodies. After
solubilization and refolding of the inclusion bodies the material was run three
times on size-exclusion chromatography (SEC) for buffer exchange and
purification. 8 mg of the SEC purified Protein 1, in PBS diluted 1:10 in TST,
was loaded at 0.5 ml/min on an XK 16/20 column (GE Healthcare) packed
15 with 10-12 ml anti-ABD agarose and pre-equilibrated with TST. The column
was washed with 4 CV of TST, followed by an additional 3 CV wash with 5
mM NH₄Ac, pH 5.5, at a flow rate of 1 ml/min. Bound protein was eluted with
3 CV of 0.1 M HAc, pH 2.9, and then neutralized to pH 7 with 1 M Tris-HCl,
pH 9. Flow-through fractions, wash fractions and eluted fractions were
20 collected for further analysis by SDS-PAGE. 4-12 % Bis-Tris NuPAGE gels
(Invitrogen) were run non-reduced and the protein stained with Simply Blue
(Life Technologies). The column was re-equilibrated with TST after elution
and cleaned in place (CIP) with 3 CV of 0.5 M NaOH followed by 3 CV of
TST. Between purifications, the resin was stored in TST + 20 % ethanol.

25

Results

The chromatogram from purification of Protein 1 on the anti-ABD
agarose resin is presented in Figure 12A and the SDS-PAGE analysis of
selected fractions is shown in Figure 12B. The purity of the SEC purified
30 Protein 1 loaded on the anti-ABD agarose column was high. However, weak
bands of contaminating protein were visible on the SDS-PAGE gel, see
arrows in Figure 12B. These contaminants were effectively eliminated by
purification on the anti-ABD agarose column. A sample from the wash step

concentrated 60 times was analyzed on SDS-PAGE and revealed contaminants (as well as leakage of Protein 1; lane 6, Figure 12B). No contaminants were seen in the eluted protein sample (lanes 7-9, Figure 12B). The purity of the eluted sample was estimated to be higher than 98 % as
5 judged from SDS-PAGE analysis. The correct mass of the eluted Protein 1 was verified by HPLC-MS analysis.

Of the initial 8 mg of SEC purified Protein 1, 5.4 mg was collected in the eluted fractions from the anti-ABD agarose column, resulting in a yield of approximately 68 %. Although no protein was detected in the flow through
10 sample loaded in lane 5 (Figure 12B), there was some leakage of Protein 1 upon washing with TST as well as removal of dimers and contaminating proteins.

15

Example 12

Purification of a peptide hormone using anti-ABD agarose

In this example, Protein 2 (9.5 kDa, pI 6.6), comprising an albumin binding domain (ABD035, SEQ ID NO: 159) fused at its N-terminus to a peptide hormone, was expressed in *Pichia Pink* and purified in a single step
20 on an ÄKTAexplorer system using a column packed with anti-ABD agarose.

Materials and methods

100 ml of *Pichia Pink* supernatant containing secreted Protein 2 was lyophilized and resuspended in 2 ml DMSO (dimethyl sulfoxide) and
25 subsequently diluted with 200 ml of TST. The sample was loaded at 1 ml/min on an XK 16/20 column packed with 10-12 ml anti-ABD agarose and pre-equilibrated with TST. The column was washed with 8 CV of TST, followed by an additional 3 CV wash with 5 mM NH₄Ac, pH 5.5. The usual wash of 4 CV TST was increased due to the high density of contaminating proteins from the
30 media. Bound protein was eluted with 3 CV of 0.1 M HAc, pH 2.9 and then adjusted to pH 4 with 1 M Tris-HCl, pH 9. The column was re-equilibrated with TST after elution followed by CIP and storage in TST + 20 % ethanol. Flow-

through fractions, wash fractions and eluted fractions were collected for further analysis by SDS-PAGE.

Results

- 5 The chromatogram from purification of Protein 2 on the anti-ABD agarose resin is presented in Figure 13A and the SDS-PAGE analysis of selected fractions is shown in Figure 13B. The elution peak from the anti-ABD agarose column contained 3-5 mg of Protein 2, corresponding to a yield of approximately 75 % (estimated from a Western blot). The correct mass of the
- 10 eluted Protein 2 was verified by HPLC-MS analysis. The purity was estimated to be higher than 98 % as judged from SDS-PAGE analysis followed by Simply Blue staining. No contaminating media proteins could be detected in the elution fractions (lanes 7-13, Figure 13B). It is recognized that Protein 2 runs smaller on SDS-PAGE gels as indicated by the molecular weight
- 15 standards included on the gels.

ITEMIZED LISTING OF EMBODIMENTS

1. ABD binding polypeptide, comprising an ABD binding motif BM, which motif consists of an amino acid sequence selected from:

5

i) $EX_2X_3X_4AX_6X_7EIX_{10}X_{11}LPNLX_{16}X_{17}X_{18}QX_{20}X_{21}AFIX_{25}X_{26}LX_{28}D$

wherein, independently from each other,

10

X_2 is selected from F, I and L;

X_3 is selected from H, K, N, Q, R, S, T and V;

X_4 is selected from A, D, F, G, H, I, K, L, M, N, Q, R, S, T, V, W and Y;

X_6 is selected from F, I, L and Y;

X_7 is selected from A, H, I, K, L, N, Q, R, S, T and V;

15

X_{10} is selected from G, H, K, N, Q, R and S;

X_{11} is selected from A, D, F, G, I, K, L, N, Q, R, S, T, V and Y;

X_{16} is selected from N and T;

X_{17} is selected from F, H, L, S and T;

X_{18} is selected from D, E, H, I, K, L, M, N, Q, R, S, T and V;

20

X_{20} is selected from H, K and R;

X_{21} is selected from I, L and V;

X_{25} is selected from F, I, L, V and Y;

X_{26} is selected from K and S;

X_{28} is selected from D and E;

25

and

ii) an amino acid sequence which has at least 89 % identity to the sequence defined in i).

30

2. ABD binding polypeptide according to item 1, wherein X_2 in sequence i) is selected from F and L.

3. ABD binding polypeptide according to item 2, wherein X_2 in sequence i) is F.

4. ABD binding polypeptide according to item 2, wherein X_2 in
5 sequence i) is L.

5. ABD binding polypeptide according to any one of the preceding items, wherein X_3 in sequence i) is selected from H, K, R, T and V.

10 6. ABD binding polypeptide according to item 5, wherein X_3 in sequence i) is selected from H, K, R and V.

7. ABD binding polypeptide according to item 6, wherein X_3 in sequence i) is selected from K and R.

15

8. ABD binding polypeptide according to item 7, wherein X_3 in sequence i) is K.

9. ABD binding polypeptide according to item 7, wherein X_3 in
20 sequence i) is R.

10. ABD binding polypeptide according to any one of the preceding items, wherein X_4 in sequence i) is selected from A, H, I, L, N, V and W.

25 11. ABD binding polypeptide according to item 10, wherein X_4 in sequence i) is selected from H, L, N and V.

12. ABD binding polypeptide according to item 11, wherein X_4 in sequence i) is V.

30

13. ABD binding polypeptide according to any one of the preceding items, wherein X_6 in sequence i) is selected from F and L.

14. ABD binding polypeptide according to item 13, wherein X_6 in sequence i) is F.

5 15. ABD binding polypeptide according to item 13, wherein X_6 in sequence i) is L.

16. ABD binding polypeptide according to any one of the preceding items, wherein X_7 in sequence i) is selected from K, L, N, Q, R and S.

10 17. ABD binding polypeptide according to item 16 wherein X_7 in sequence i) is selected from K, N, Q, R and S.

18. ABD binding polypeptide according to item 17, wherein X_7 in sequence i) is selected from K, Q, R and S.

15 19. ABD binding polypeptide according to item 18, wherein X_7 in sequence i) is selected from K and R.

20 20. ABD binding polypeptide according to item 19, wherein X_7 in sequence i) is K.

21. ABD binding polypeptide according to item 19, wherein X_7 in sequence i) is R.

25 22. ABD binding polypeptide according to any one of the preceding items, wherein X_{10} in sequence i) is selected from H, K, N, and R.

23. ABD binding polypeptide according to item 22, wherein X_{10} in sequence i) is selected from H, K and N.

30 24. ABD binding polypeptide according to item 22, wherein X_{10} in sequence i) is selected from K, N and R.

25. ABD binding polypeptide according to item 23 or 24, wherein X_{10} in sequence i) is N.

26. ABD binding polypeptide according to item 24, wherein X_{10} in
5 sequence i) is selected from K and R.

27. ABD binding polypeptide according item 26, wherein X_{10} in sequence i) is K.

10 28. ABD binding polypeptide according to item 26, wherein X_{10} in sequence i) is R.

29. ABD binding polypeptide according to any one of the preceding items, wherein X_{11} in sequence i) is selected from A, F, L, R, T and Y.

15 30. ABD binding polypeptide according to item 29, wherein X_{11} in sequence i) is selected from A, F, T and Y.

31. ABD binding polypeptide according to item 30, wherein X_{11} in
20 sequence i) is T.

32. ABD binding polypeptide according to any one of the preceding items, wherein X_{16} in sequence i) is T.

25 33. ABD binding polypeptide according to any one of the preceding items, wherein X_{17} in sequence i) is selected from F, H and L.

34. ABD binding polypeptide according to item 33, wherein X_{17} in sequence i) is selected from F and H.

30 35. ABD binding polypeptide according to item 33, wherein X_{17} in sequence i) is selected from H and L.

36. ABD binding polypeptide according to item 34, wherein X_{17} in sequence i) is F.

37. ABD binding polypeptide according to item 34 or 35, wherein X_{17} in
5 sequence i) is H.

38. ABD binding polypeptide according to item 35, wherein X_{17} in sequence i) is L.

10 39. ABD binding polypeptide according to any one of the preceding items, wherein X_{18} in sequence i) is selected from D, H, I, K and Q.

40. ABD binding polypeptide according to item 39, wherein X_{18} in sequence i) is selected from D, H and Q.

15 41. ABD binding polypeptide according to item 40, wherein X_{18} in sequence i) is selected from H and Q.

42. ABD binding polypeptide according to item 41, wherein X_{18} in
20 sequence i) is H.

43. ABD binding polypeptide according to item 41, wherein X_{18} in sequence i) is Q.

25 44. ABD binding polypeptide according to any one of the preceding items, wherein X_{20} in sequence i) is selected from H and R.

45. ABD binding polypeptide according to any one of items 1-43, wherein X_{20} in sequence i) is selected from K and R.

30 46. ABD binding polypeptide according to item 44 or 45, wherein X_{20} in sequence i) is R.

47. ABD binding polypeptide according to item 45, wherein X_{20} in sequence i) is K.

48. ABD binding polypeptide according to any one of the preceding
5 items, wherein X_{21} in sequence i) is selected from I and L.

49. ABD binding polypeptide according to item 48, wherein X_{21} in sequence i) is I.

10 50. ABD binding polypeptide according to item 48, wherein X_{21} in sequence i) is L.

51. ABD binding polypeptide according to any one of the preceding
items, wherein X_{25} in sequence i) is selected from I, L and V.

15 52. ABD binding polypeptide according to item 51, wherein X_{25} in sequence i) is selected from V and I.

53. ABD binding polypeptide according to item 52, wherein X_{25} in
20 sequence i) is I.

54. ABD binding polypeptide according to item 52, wherein X_{25} in sequence i) is V.

25 55. ABD binding polypeptide according to any one of the preceding items, wherein X_{26} in sequence i) is K.

56. ABD binding polypeptide according to any one of the preceding
items, wherein X_{28} in sequence i) is D.

30 57. ABD binding polypeptide according to any one of the preceding items, wherein sequence i) fulfills at least four of the eight conditions I-VIII:

58

- 5
- I. X_2 is F or L;
 - II. X_6 is F or L;
 - III. X_{16} is T;
 - IV. X_{20} is H or R;
 - V. X_{21} is I or L;
 - VI. X_{25} is I or V;
 - VII. X_{26} is K; and
 - VIII. X_{28} is D.

10 58. ABD binding polypeptide according to item 57, wherein sequence i) fulfills at least five of the eight conditions I-VIII.

59. ABD binding polypeptide according to item 58, wherein sequence i) fulfills at least six of the eight conditions I-VIII.

15

60. ABD binding polypeptide according to item 59, wherein sequence i) fulfills at least seven of the eight conditions I-VIII.

20 61. ABD binding polypeptide according to item 60, wherein sequence i) fulfills all of the eight conditions I-VIII.

62. ABD binding polypeptide according to any one of the preceding items, wherein sequence i) is selected from SEQ ID NO:1-52.

25 63. ABD binding polypeptide according to item 62, wherein sequence i) is selected from SEQ ID NO:1-7.

64. ABD binding polypeptide according to item 63, wherein sequence i) is selected from SEQ ID NO:1-4.

30

65. ABD binding polypeptide according to item 64, wherein sequence i) is SEQ ID NO:1.

66. ABD binding polypeptide according to any preceding item, wherein said ABD binding motif forms part of a three-helix bundle protein domain.

67. ABD binding polypeptide according to item 66, wherein said ABD
5 binding motif essentially forms part of two helices with an interconnecting loop, within said three-helix bundle protein domain.

68. ABD binding polypeptide according to any one of items 66-67,
wherein said three-helix bundle protein domain is selected from bacterial
10 receptor domains.

69. ABD binding polypeptide according to item 68, wherein said three-
helix bundle protein domain is selected from domains of protein A from
Staphylococcus aureus or derivatives thereof.
15

70. ABD binding polypeptide according to any preceding item, which
comprises an amino acid sequence selected from:

iii) K-[BM]-DPSQS X_aX_bLLX_c EAKKL NDX_dQ;
20
wherein

[BM] is an ABD binding motif as defined in any one of items 1-65;
X_a is selected from A and S;
25 X_b is selected from N and E;
X_c is selected from A, S and C;
X_d is selected from A and S;

and
30

iv) an amino acid sequence which has at least 81 % identity to a
sequence defined by iii).

71. ABD binding polypeptide according to item 70, wherein X_a in sequence iii) is A.

5 72. ABD binding polypeptide according to item 70, wherein X_a in sequence iii) is S.

73. ABD binding polypeptide according to any one of items 70-72, wherein X_b in sequence iii) is N.

10

74. ABD binding polypeptide according to any one of items 70-72, wherein X_b in sequence iii) is E.

75. ABD binding polypeptide according to any one of items 70-74,
15 wherein X_c in sequence iii) is A.

76. ABD binding polypeptide according to any one of items 70-74, wherein X_c in sequence iii) is S.

20 77. ABD binding polypeptide according to any one of items 70-74, wherein X_c in sequence iii) is C.

78. ABD binding polypeptide according to any one of items 70-77, wherein X_d in sequence iii) is A.

25

79. ABD binding polypeptide according to any one of items 70-77, wherein X_d in sequence iii) is S.

80. ABD binding polypeptide according to item 70, wherein, in
30 sequence iii), X_a is A; X_b is N; X_c is A and X_d is A.

81. ABD binding polypeptide according to item 70, wherein, in sequence iii), X_a is A; X_b is N; X_c is C and X_d is A.

82. ABD binding polypeptide according to item 70, wherein, in sequence iii), X_a is S; X_b is E; X_c is S and X_d is S.

5 83. ABD binding polypeptide according to item 70, wherein, in sequence iii), X_a is S; X_b is E; X_c is C and X_d is S.

84. ABD binding polypeptide according to item 70, wherein, in sequence iii), X_a is S; X_b is E; X_c is C and X_d is A.

10

85. ABD binding polypeptide according to item 70, wherein, in sequence iii), X_a is S; X_b is E; X_c is A and X_d is A.

86. ABD binding polypeptide according to item 85, wherein sequence
15 iii) is selected from any one of SEQ ID NO:53-104.

87. ABD binding polypeptide according to item 86, wherein sequence iii) is selected from any one of SEQ ID NO:53-59.

20 88. ABD binding polypeptide according to item 87, wherein sequence iii) is selected from any one of SEQ ID NO:53-56.

89. ABD binding polypeptide according to item 88, wherein sequence iii) is SEQ ID NO:53.

25

90. ABD binding polypeptide according to any one of items 1-70, which comprises an amino acid sequence selected from:

v) YAK-[BM]-DPSQS SELLX_c EAKKL NDSQA P;

30

wherein [BM] is an ABD binding motif as defined in any one of items 1-65 and X_c is selected from S and C; and

- vi) an amino acid sequence which has at least 83 % identity to a sequence defined by v).

91. ABD binding polypeptide according to any one of items 1-70, which
5 comprises an amino acid sequence selected from:

- vii) YAK-[BM]-DPSQS SELLX_c EAKKL NDAQA P;

wherein [BM] is an ABD binding motif as defined in any one of items 1-65 and
10 X_c is selected from A and C; and

- viii) an amino acid sequence which has at least 83 % identity to a sequence defined by vii).

92. ABD binding polypeptide according to any one of items 1-70, which
15 comprises an amino acid sequence selected from:

- ix) FNK-[BM]-DPSQS ANLLX_c EAKKL NDAQA P;

wherein [BM] is an ABD binding motif as defined in any one of items 1-65 and
20 X_c is selected from A and C; and

- x) an amino acid sequence which has at least 83 % identity to a sequence defined by ix).

25

93. ABD binding polypeptide according to item 69, which comprises an amino acid sequence selected from:

- ADNNFNK-[BM]-DPSQSANLLSEAKKLNESQAPK;
ADNKFNK-[BM]-DPSQSANLLAEAKKLNDQAQAPK;
30 ADNKFNK-[BM]-DPSVSKEILAEAKKLNDQAQAPK;
ADAQQNNFNK-[BM]-DPSQSTNVLGEAKKLNESQAPK;
AQHDE-[BM]-DPSQSANVLGEAQKLNDQAQAPK;
VDNKFNK-[BM]-DPSQSANLLAEAKKLNDQAQAPK;

AEAKEYAK-[BM]-DPSESSELLSEAKKLNKSQAPK;
 VDAKYAK-[BM]-DPSQSSELLAEAKKLNDAQAPK;
 VDAKYAK-[BM]-DPSQSSELLAEAKKLNDSQAPK;
 AEAKEYAK-[BM]-DPSQSSELLSEAKKLNDSQAPK;
 5 VDAKYAK-[BM]-DPSQSSELLSEAKKLNDSQAPK;
 VDAKYAK-[BM]-DPSQSSELLAEAKKLNKAQAPK; and
 AEAKEYAK-[BM]-DPSQSSELLAEAKKLNKAQAPK;

wherein [BM] is an ABD binding motif as defined in any one of items 1-65.

10

94. ABD binding polypeptide according to any preceding item, which comprises an amino acid sequence selected from:

xi) VDAKYAK-[BM]-DPSQSSELLAEAKKLNDAQAPK;

15

wherein [BM] is an ABD binding motif as defined in any one of items 1-65; and

xii) an amino acid sequence which has at least 84 % identity to the sequence defined in xi).

20

95. ABD binding polypeptide according to item 94, in which sequence xi) is selected from SEQ ID NO:105-156.

25 96. ABD binding polypeptide according to item 95, in which sequence xi) is selected from SEQ ID NO 105-111.

97. ABD binding polypeptide according to item 96, in which sequence xi) is selected from SEQ ID NO:105-108.

30

98. ABD binding polypeptide according to item 97, in which sequence xi) is SEQ ID NO:105.

99. ABD binding polypeptide according to any one of the preceding items in multimeric form, comprising at least two ABD binding polypeptide monomer units, whose amino acid sequences may be the same or different.

5 100. ABD binding polypeptide according to item 99, wherein said ABD binding polypeptide monomer units are covalently coupled together.

101. ABD binding polypeptide according to item 99, wherein the ABD binding polypeptide monomer units are expressed as a fusion protein.

10

102. ABD binding polypeptide according to any preceding items which further comprises a cysteine residue at the C-terminal end of the polypeptide, for example at the C-terminus.

15 103. A polynucleotide encoding a polypeptide according to any one of the preceding items.

104. Combination of an ABD binding polypeptide according to any one of items 1-102 and a detectable agent.

20

105. Combination according to item 104, wherein the detectable agent is a fluorescent agent or a radioactive agent.

CLAIMS

1. ABD binding polypeptide, comprising an ABD binding motif BM,
which motif consists of an amino acid sequence selected from:

5

i) $EX_2X_3X_4AX_6X_7EIX_{10}X_{11}LPNLX_{16}X_{17}X_{18}QX_{20}X_{21}AFIX_{25}X_{26}LX_{28}D$

wherein, independently from each other,

10

X_2 is selected from F, I and L;

X_3 is selected from H, K, N, Q, R, S, T and V;

X_4 is selected from A, D, F, G, H, I, K, L, M, N, Q, R, S, T, V, W and Y;

X_6 is selected from F, I, L and Y;

X_7 is selected from A, H, I, K, L, N, Q, R, S, T and V;

15

X_{10} is selected from G, H, K, N, Q, R and S;

X_{11} is selected from A, D, F, G, I, K, L, N, Q, R, S, T, V and Y;

X_{16} is selected from N and T;

X_{17} is selected from F, H, L, S and T;

X_{18} is selected from D, E, H, I, K, L, M, N, Q, R, S, T and V;

20

X_{20} is selected from H, K and R;

X_{21} is selected from I, L and V;

X_{25} is selected from F, I, L, V and Y;

X_{26} is selected from K and S;

X_{28} is selected from D and E;

25

and

ii) an amino acid sequence which has at least 89 % identity to the
sequence defined in i).

30

2. ABD binding polypeptide according to claim 1, wherein sequence i)
fulfills at least four of the eight conditions I-VIII:

- 5
- I. X_2 is F or L;
 - II. X_6 is F or L;
 - III. X_{16} is T;
 - IV. X_{20} is H or R;
 - V. X_{21} is I or L;
 - VI. X_{25} is I or V;
 - VII. X_{26} is K; and
 - VIII. X_{28} is D.

10 3. ABD binding polypeptide according to any one of the preceding claims, wherein sequence i) is selected from SEQ ID NO:1-52.

4. ABD binding polypeptide according to claim 3, wherein sequence i) is selected from SEQ ID NO:1-7.

15 5. ABD binding polypeptide according to any preceding claim, wherein said ABD binding motif forms part of a three-helix bundle protein domain.

20 6. ABD binding polypeptide according to claim 5, wherein said three-helix bundle protein domain is selected from domains of protein A from *Staphylococcus aureus* or derivatives thereof.

7. ABD binding polypeptide according to any preceding claim, which comprises an amino acid sequence selected from:

25 iii) K-[BM]-DPSQS $X_a X_b LLX_c$ EAKKL NDX_dQ;

wherein

30 [BM] is an ABD binding motif as defined in any one of claims 1-4;
 X_a is selected from A and S;
 X_b is selected from N and E;
 X_c is selected from A, S and C;

X_d is selected from A and S;

and

- 5 iv) an amino acid sequence which has at least 81 % identity to a sequence defined by iii).

8. ABD binding polypeptide according claim 7, wherein sequence iii) is selected from any one of SEQ ID NO:53-104.

10

9. ABD binding polypeptide according to claim 8, wherein sequence iii) is selected from any one of SEQ ID NO:53-59.

10. ABD binding polypeptide according to claim 6, which comprises an amino acid sequence selected from:

ADNNFNK-[BM]-DPSQSANLLSEAKKLNESQAPK;
 ADNKFNK-[BM]-DPSQSANLLAEAKKLNDQAQAPK;
 ADNKFNK-[BM]-DPSVSKEILAEAKKLNDQAQAPK;
 ADAQQNNFNK-[BM]-DPSQSTNVLGEAKKLNESQAPK;
 20 AQHDE-[BM]-DPSQSANVLGEAQKLNDQAQAPK;
 VDNKFNK-[BM]-DPSQSANLLAEAKKLNDQAQAPK;
 AEAKEYAK-[BM]-DPSESSELLSEAKKLNKSQAPK;
 VDAKYAK-[BM]-DPSQSSELLAEAKKLNDQAQAPK;
 VDAKYAK-[BM]-DPSQSSELLAEAKKLNDQAQAPK;
 25 AEAKEYAK-[BM]-DPSQSSELLSEAKKLNDQAQAPK;
 VDAKYAK-[BM]-DPSQSSELLSEAKKLNDQAQAPK;
 VDAKYAK-[BM]-DPSQSSELLAEAKKLNKAQAPK; and
 AEAKEYAK-[BM]-DPSQSSELLAEAKKLNKAQAPK;

30 wherein [BM] is an ABD binding motif as defined in any one of claims 1-4.

11. ABD binding polypeptide according to any preceding claim, which comprises an amino acid sequence selected from:

xi) VDAKYAK-[BM]- DPSQSSELLAEAKKLNDAQAPK;

wherein [BM] is an ABD binding motif as defined in any one of claims 1-4; and

5

xii) an amino acid sequence which has at least 84 % identity to the sequence defined in ix).

12. ABD binding polypeptide according to claim 11, in which sequence
10 xi) is selected from SEQ ID NO:105-156.

13. ABD binding polypeptide according to claim 12, in which sequence xi) is selected from SEQ ID NO 105-111.

15 14. A polynucleotide encoding a polypeptide according to any one of the preceding claims.

15. Combination of an ABD binding polypeptide according to any one of claims 1-13 and a detectable agent.

20

Designation	Amino acid sequence	SEQ ID NO
BM06677	ELKVAFKKEINTLPNLTTHQQRIAFIIKLDD	1
BM06638	ELVNAFSEIKALPNLTTLHQRLAFIVKLDD	2
BM06608	ELHHAFREIKFLPNLTTFHQRLAFIVKLDD	3
BM06650	EFKLALQEIHYLPNLTLDQRLAFILKLDD	4
BM06678	ELRWALNEIRRLPNLTFFKQHIAFIVKLDD	5
BM06620	EFRAALLEIKLLPNLTFFIQRlafIVKLDD	6
BM06695	EFTIALREIHALPNLTTLHQRLAFIIKLDD	7
BM06681	ELRKAFHEIQILPNLTLSQRLAFIVKLDD	8
BM06655	EFKDALDEIKDLPNLTSMQRIAFIVKLDD	9
BM06602	ELTTAFAEIQKLPNLTTFEQKIAFIIKLDD	10
BM06670	EFREAIIEIRRLPNLTTLHQRLAFIMKLDD	11
BM06668	EIKTAFAEIRVLPNLTTFaQRLAFIIKLDD	12
BM06690	ERRYAFREIRLLPNLTFSQRIAFIVKLDD	13
BM06667	ELKAAFREINDLPNLTHTQRIAFILKLDD	14
BM06641	ELKMAFQEIRYLPNLTRLQRIAFIVKLDD	15
BM06686	EWKKALREIHYLPNLTTLTQRLAFIVKLDD	16
BM06664	EFEKALVEIKTLPNLTALQRIAFIVKLDD	17
BM06583	EFIFALSEIKVLPNLTTHVQKIAFIVKLDD	18
BM06687	EFKYAIQEIKDLPNLTSGQRIAFIVKLDD	19
BM06700	ELNQALWEIRQLPNLTFFNQRVAFIVKLDD	20
BM06661	ELQGALTEIKNLPNLTGHQRIAFIVKLDD	21
BM06669	ELADALYEIKNLPNLTHEQHIAFIVKLDD	22
BM06676	ELGVALKEIGQLPNLTHTQRIAFIIKLDD	23
BM06680	ELSIALNEIKGLPNLTSLQKIAFIVKLDD	24
BM06531	EIRSAKYKEINVLPNLTFFSQKIAFIYKLED	25
BM06665	ELSSALLEISHLPNLTTHQQRIAFIVKLDD	26
BM06683	ELVHAFGEIRYLPNLTTHSQRIAFIIKLDD	27
BM06698	ELHNAFSEIKQLPNLTTHQRIAFIIKLDD	28
BM06692	EGVNAFNEIKGLPNLTFFHQKIAFIVKLDD	29

FIGURE 1A

Designation	Amino acid sequence	SEQ ID NO
BM06660	ELKYALMEIRYLPNLTTHRQKIAFILKLLDD	30
BM06645	ELRWAVSEIRHLPNLTFFHQRIAFIVKLLDD	31
BM06508	ELQRAFAEIQSLPNLTINQHIAFIYKLED	32
BM06649	ELKAAFREIRTLPNLTFGQRTAFIVKLLDD	33
BM06666	ELTTAMKEIQALPNLTTHQQRIAFIVKLLDD	34
BM06549	EDMRAFHEINKLPNLTIAQRVAFIYKLED	35
BM06552	ELNAAFTTEISSLPNLTLDQRLAFIFKLLDD	36
BM06672	ELRWALNEIHILPNLTLEQKVAFIVKLLDD	37
BM06484	ELKNAFIEIKNLPNLTNTQTAFIVKLLDD	38
BM06652	ELSLAFVEIHKLPNLTTHHQRLAFIVKLLDD	39
BM06480	ELQWAFNEIHNLPNLTYYQRIAFIVKLLDD	40
BM06512	EEQTAMQEINSLPNLTLEQRIAFIFKLED	41
BM06648	ELGWAFREIRNLPNLTTHYQRIAFIMKLLDD	42
BM06691	ENLWAFNEIKGLPNLTTHDQRIAFIVKLLDD	43
BM06501	ELSFASFSEINVLPNLTFFHQKIAFIYKLED	44
BM06544	EFRGAI AEIRDLPNLTLEQKYAFIFKLED	45
BM06487	EEENAYKEIGSLPNLTIAQKVAFILKLED	46
BM06688	ELRQALQEIHILPNLTTHSQRVAFIVKLLDD	47
BM06503	ENHAAFQEILSLPNLTINQRLAFITKLLDD	48
BM06491	ETNYAFKEIDL LPNLTLMQKLA FIVKLLDD	49
BM06675	EISLAFKEIKALPNLTGQQRF AFILKLLDD	50
BM06584	ELSKALTEIRMLPNLTFRQRIAFI IKLLDD	51
BM06540	ELDMAYTEIGLLPNLTFSQLLAFI IKLLDD	52
P06677	KELKVAFKEINTLPNLTTHQQRIAFI IKLDDDPQSSELLAEAKKLNDAAQ	53
P06638	KELVNAFSEIKALPNLT LHQRLAFIVKLLDDDPQSSELLAEAKKLNDAAQ	54
P06608	KELHHAFREIKFLPNLTFFHQRLAFIVKLLDDDPQSSELLAEAKKLNDAAQ	55
P06650	KEFKLAIQEIHYLPNLTLDQRLAFI IKLDDDPQSSELLAEAKKLNDAAQ	56
P06678	KELRWALNEIRRLPNLTFFKQHI AFIVKLLDDDPQSSELLAEAKKLNDAAQ	57
P06620	KEFRAAILLEIKLLPNLTFFIQR LAFIVKLLDDDPQSSELLAEAKKLNDAAQ	58

FIGURE 1B

Designation	Amino acid sequence	SEQ ID NO
P06695	KEFTIALREIHALPNLTTLHQRLAFIIKLDDDDPSQSSELLAEAKKLINDAQ	59
P06681	KELRKAFHEIQIILPNLTLSQRLAFIVKLDLDDPSQSSELLAEAKKLINDAQ	60
P06655	KEFKDALDEIKDLPNLTSMQRIAFIVKLDLDDPSQSSELLAEAKKLINDAQ	61
P06602	KELTTAFAEIQKLPNLTTFEQKIAFIIKLDDDDPSQSSELLAEAKKLINDAQ	62
P06670	KEFREAIIEIRRLPNLTTLHQRLAFIMKLDLDDPSQSSELLAEAKKLINDAQ	63
P06668	KEIKTAFAEIRVLPNLTTFARLAFIIKLDDDDPSQSSELLAEAKKLINDAQ	64
P06690	KERYAFREIRLLPNLTTFSQRIAFIVKLDLDDPSQSSELLAEAKKLINDAQ	65
P06667	KELKAAFREINDLPNLTTHTQRIAFIILKLDLDDPSQSSELLAEAKKLINDAQ	66
P06641	KELKMAFQEIRYLPNLTTRLQRIAFIVKLDLDDPSQSSELLAEAKKLINDAQ	67
P06686	KWKKALREIHYLPNLTTLTQRLAFIVKLDLDDPSQSSELLAEAKKLINDAQ	68
P06664	KEFEKALVEIKTLPNLTALQRIAFIVKLDLDDPSQSSELLAEAKKLINDAQ	69
P06583	KEFI FALSEIKVLPNLTTHVQKIAFIVKLDLDDPSQSSELLAEAKKLINDAQ	70
P06687	KEFKYAIQEIKDLPNLTSGQRIAFIVKLDLDDPSQSSELLAEAKKLINDAQ	71
P06700	KELNQALWEIRQLPNLTTFNQRFIVKLDLDDPSQSSELLAEAKKLINDAQ	72
P06661	KELQGALTEIKNLPNLTGHQRIAFIVKLDLDDPSQSSELLAEAKKLINDAQ	73
P06669	KELADALYEIKNLPNLTTHQKIAFIVKLDLDDPSQSSELLAEAKKLINDAQ	74
P06676	KELGVALKEIGQLPNLTTHTQRIAFIILKLDLDDPSQSSELLAEAKKLINDAQ	75
P06680	KELSIAlNEIKGLPNLTSLQKIAFIVKLDLDDPSQSSELLAEAKKLINDAQ	76
P06531	KEIRSAFKEINVLPNLTTFSQKIAFIYKLEDDPSQSSELLAEAKKLINDAQ	77
P06665	KELSSALLEISHLPNLTTHQKIAFIVKLDLDDPSQSSELLAEAKKLINDAQ	78
P06683	KELVHAFGEIRYLPNLTTHSQRIAFIILKLDLDDPSQSSELLAEAKKLINDAQ	79
P06698	KELHNAFSEIKQLPNLTTLTQRIAFIILKLDLDDPSQSSELLAEAKKLINDAQ	80
P06692	KEGVNAFNEIKGLPNLTTFHQKIAFIVKLDLDDPSQSSELLAEAKKLINDAQ	81
P06660	KELKYALMEIRYLPNLTTHRQKIAFIVKLDLDDPSQSSELLAEAKKLINDAQ	82
P06645	KELRWAVSEIRHLPNLTTFHQRIAFIVKLDLDDPSQSSELLAEAKKLINDAQ	83
P06508	KELQRAFAEIQSLPNLTNLNQHIAFIYKLEDDPSQSSELLAEAKKLINDAQ	84
P06649	KELKAAFREIRTLPNLTTFGQRTAFIVKLDLDDPSQSSELLAEAKKLINDAQ	85
P06666	KELTTAMKEIQALPNLTTHQKIAFIVKLDLDDPSQSSELLAEAKKLINDAQ	86
P06549	KEDMRAFHEINKLPNLTALQRFIVKLEDDPSQSSELLAEAKKLINDAQ	87

FIGURE 1C

Designation	Amino acid sequence	SEQ ID NO
P06552	KELNAAFTEISSLPNLTLDQRLAFIFKLDLDDPSQSSELLAEAKKLNDAAQ	88
P06672	KELRWALNEIHIILPNLTLEQKVAFIVKLDLDDPSQSSELLAEAKKLNDAAQ	89
P06484	KELKNAFIEIKNLPNLTNTQTAFIVKLDLDDPSQSSELLAEAKKLNDAAQ	90
P06652	KELSLAFVEIHKLPNLTTHQRLAFIVKLDLDDPSQSSELLAEAKKLNDAAQ	91
P06480	KELQWAFNEIHNLPNLTIVQRIAFIVKLDLDDPSQSSELLAEAKKLNDAAQ	92
P06512	KEEQTAMQEINSLPNLTLEQRIAFIFKLEDDPSQSSELLAEAKKLNDAAQ	93
P06648	KELGWAFREIRNLPNLTTHYQRIAFIMKLDLDDPSQSSELLAEAKKLNDAAQ	94
P06691	KENLWAFNEIKGLPNLTTHDQRIAFIVKLDLDDPSQSSELLAEAKKLNDAAQ	95
P06501	KELSFASFSEINVLPNLTTFHQKIAFIYKLEDDPSQSSELLAEAKKLNDAAQ	96
P06544	KEFRGAIAEIRDLPNLTLEQKYAFIFKLEDDPSQSSELLAEAKKLNDAAQ	97
P06487	KEENAYKEIGSLPNLTLAQKVAFIIKLEDDPSQSSELLAEAKKLNDAAQ	98
P06688	KELRQALQEIHIILPNLTTHSQRVAFIVKLDLDDPSQSSELLAEAKKLNDAAQ	99
P06503	KENHAAFQEIISLPNLTLNQRLAFITKLDLDDPSQSSELLAEAKKLNDAAQ	100
P06491	KETNYAFKEIDLPLNLTLMQKLAFIGKLDLDDPSQSSELLAEAKKLNDAAQ	101
P06675	KEISLAFKEIKALPNLTGQQRFAFIIKLDLDDPSQSSELLAEAKKLNDAAQ	102
P06584	KELSKALTIEIRMLPNLTFRQRIAFIIKLDLDDPSQSSELLAEAKKLNDAAQ	103
P06540	KELDMAYTEIGLLPNLTFSQLLAFIIKLDLDDPSQSSELLAEAKKLNDAAQ	104
Z06677	VDKAYAKELKVAFKEINTLPNLTTHQQRIFAIIKLDLDDPSQSSELLAEAKKLNDAAQAPK	105
Z06638	VDKAYAKELVNAFSEIKALPNLTILHQRLAFIVKLDLDDPSQSSELLAEAKKLNDAAQAPK	106
Z06608	VDKAYAKELHHAFREIKFLPNLTTFHQRLAFIVKLDLDDPSQSSELLAEAKKLNDAAQAPK	107
Z06650	VDKAYAKEFKLALQEIHYLPNLTLDQRLAFIILKLDLDDPSQSSELLAEAKKLNDAAQAPK	108
Z06678	VDKAYAKELRWALNEIRRLPNLTFFKQHIAFIVKLDLDDPSQSSELLAEAKKLNDAAQAPK	109
Z06620	VDKAYAKEFRAALIEIKLLPNLTTFIQRLAFIVKLDLDDPSQSSELLAEAKKLNDAAQAPK	110
Z06695	VDKAYAKEFTIALREIHALPNLTILHQRLAFIILKLDLDDPSQSSELLAEAKKLNDAAQAPK	111
Z06681	VDKAYAKELRKAFHEIQIILPNLTLSQRLAFIVKLDLDDPSQSSELLAEAKKLNDAAQAPK	112
Z06655	VDKAYAKEFKDALDEIKDLPNLTSMQRIAFIVKLDLDDPSQSSELLAEAKKLNDAAQAPK	113
Z06602	VDKAYAKELTTFATAFAEIQKLPNLTTFEQKIAFIIKLDLDDPSQSSELLAEAKKLNDAAQAPK	114
Z06670	VDKAYAKEFREAIIEIRRLPNLTILHQRLAFIMKLDLDDPSQSSELLAEAKKLNDAAQAPK	115
Z06668	VDKAYAKEIKTAFAEIRVLPNLTFFAQRLAFIILKLDLDDPSQSSELLAEAKKLNDAAQAPK	116

FIGURE 1D

Designation	Amino acid sequence	SEQ ID NO
Z06690	VDKYAKERRYAFREIRLLPNLTFSSQRIAFIVKLDLDDPSQSSELLAEAKKLNDQAQPK	117
Z06667	VDKYAKE LKA AFREINDLPNLTHTRIAFILKLDLDDPSQSSELLAEAKKLNDQAQPK	118
Z06641	VDKYAKE LKMAFQEIRYLPNLTRLQRIAFIVKLDLDDPSQSSELLAEAKKLNDQAQPK	119
Z06686	VDKYAKEWKKALREIHYPNLTLTQRLAFIVKLDLDDPSQSSELLAEAKKLNDQAQPK	120
Z06664	VDKYAKEFEKALVEIKTLPNLTAIQRIAFIVKLDLDDPSQSSELLAEAKKLNDQAQPK	121
Z06583	VDKYAKEFIFALSEIKVLPNLTHVQKIAFIVKLDLDDPSQSSELLAEAKKLNDQAQPK	122
Z06687	VDKYAKEFEKYA IQEIKDLPNLTSQRIAFIVKLDLDDPSQSSELLAEAKKLNDQAQPK	123
Z06700	VDKYAKE LQALWEIRQLPNLTFNQRFIVKLDLDDPSQSSELLAEAKKLNDQAQPK	124
Z06661	VDKYAKE LQALTEIKNLPNLTGHRIAFIVKLDLDDPSQSSELLAEAKKLNDQAQPK	125
Z06669	VDKYAKE LADALYEIKNLPNLTHEQHIAFIVKLDLDDPSQSSELLAEAKKLNDQAQPK	126
Z06676	VDKYAKE LGVALKEIGQLPNLTHTRIAFIIKLDLDDPSQSSELLAEAKKLNDQAQPK	127
Z06680	VDKYAKE LSI ALNEIKGLPNLTSIQKIAFIVKLDLDDPSQSSELLAEAKKLNDQAQPK	128
Z06531	VDKYAKE IRSAYKEINVLPNLTFSSQRIAFIYKLEDDPSQSSELLAEAKKLNDQAQPK	129
Z06665	VDKYAKE LSSALLEI SHLPNLTHQQRIAFIVKLDLDDPSQSSELLAEAKKLNDQAQPK	130
Z06683	VDKYAKE LVHAFGEIRYLPNLTHSQRIAFIIKLDLDDPSQSSELLAEAKKLNDQAQPK	131
Z06698	VDKYAKE LHNAFSEIKQLPNLTTQRIAFIIKLDLDDPSQSSELLAEAKKLNDQAQPK	132
Z06692	VDKYAKE GVNAFNEIKGLPNLTFHQKIAFIVKLDLDDPSQSSELLAEAKKLNDQAQPK	133
Z06660	VDKYAKE LKYALMEIRYLPNLTHRQKIAFILKLDLDDPSQSSELLAEAKKLNDQAQPK	134
Z06645	VDKYAKE LRWAVSEIRHLPNLTFHQRIAFIVKLDLDDPSQSSELLAEAKKLNDQAQPK	135
Z06508	VDKYAKE LQRAFAEIQSLPNLTLNQHIAFIYKLEDDPSQSSELLAEAKKLNDQAQPK	136
Z06649	VDKYAKE LKA AFREIRTLPNLTFGQRTAFIVKLDLDDPSQSSELLAEAKKLNDQAQPK	137
Z06666	VDKYAKE LTTAMKEIQALPNLTHQQRIAFIVKLDLDDPSQSSELLAEAKKLNDQAQPK	138
Z06549	VDKYAKE DMRAFHEINKLPNLTLAQRFVFIYKLEDDPSQSSELLAEAKKLNDQAQPK	139
Z06552	VDKYAKE LNAAFTEISSLPNLTLDQRLAFIFKLDLDDPSQSSELLAEAKKLNDQAQPK	140
Z06672	VDKYAKE LRWALNEIHILPNLTLIEQKVFIVKLDLDDPSQSSELLAEAKKLNDQAQPK	141
Z06484	VDKYAKE LKNAFIEIKNLPNLTTNQTLAFIVKLDLDDPSQSSELLAEAKKLNDQAQPK	142
Z06652	VDKYAKE LSLAFVEIHKLPNLTHHQRLAFIVKLDLDDPSQSSELLAEAKKLNDQAQPK	143
Z06480	VDKYAKE LQWAFNEIHNLPNLTYVQRIAFIVKLDLDDPSQSSELLAEAKKLNDQAQPK	144
Z06512	VDKYAKEEQTAMQEI NSLPNLTLQRIAFIFKLEDDPSQSSELLAEAKKLNDQAQPK	145

FIGURE 1E

Designation	Amino acid sequence	SEQ ID NO
Z06648	VDKYYAKELGWAFREIRNLPLNLTHYQRIAFIMKLDLDDPSQSSELLAEAKKLNDQAQPK	146
Z06691	VDKYYAKENLWAFNEIKGLPNLTHDQRIAFIVKLDLDDPSQSSELLAEAKKLNDQAQPK	147
Z06501	VDKYYAKELSFASFSEINVLPLNLTFHQKIAFIYKLEDDPSQSSELLAEAKKLNDQAQPK	148
Z06544	VDKYYAKEFRGAIAEIRDLPLNLTLQKYAFIFKLEDDPSQSSELLAEAKKLNDQAQPK	149
Z06487	VDKYYAKEEENAYKEIGSLPNLTLAQKVAFILKLEDDPSQSSELLAEAKKLNDQAQPK	150
Z06688	VDKYYAKELRQALQEIHIPLNLTHSQRVAFIVKLDLDDPSQSSELLAEAKKLNDQAQPK	151
Z06503	VDKYYAKENHAAFAQEII LSLPNLTLNQRLAFITKLDLDDPSQSSELLAEAKKLNDQAQPK	152
Z06491	VDKYYAKETNYAFKEIDLPLNLTIMQKLA FIVKLDLDDPSQSSELLAEAKKLNDQAQPK	153
Z06675	VDKYYAKEISLAFKEIKALPNLTGQQRFAFILKLDLDDPSQSSELLAEAKKLNDQAQPK	154
Z06584	VDKYYAKELSKALTEIRMLPNLTFRQRIAFIIKLDLDDPSQSSELLAEAKKLNDQAQPK	155
Z06540	VDKYYAKELDMAYTEIGLLPNLTFSQLLA FIIKLDLDDPSQSSELLAEAKKLNDQAQPK	156
ABD001	LAEAKVLANRELDKYGVSDYYKNLINNAKTVEGVKALIDEILAAALP	157
C-ABD001	CLAEAKVLANRELDKYGVSDYYKNLINNAKTVEGVKALIDEILAAALP	158
ABD035	LAEAKVLANRELDKYGVSDFYKRLINKAKTVEGVKALHILAAALP	159
PP013	LAEAKEAANAELDSYGVSDFYKRLIDKAKTVEGVKALKDAILAALP	160
PEP07986	GSLAEAKEAANAELDSYGVSDFYKRLIDKAKTVEGVKALKDAILAALP	161
PEP07911	GLSAKEAANAELDCYGVSDFYKRLIDKAKTVEGVKALKDAILAALP	162

FIGURE 1F

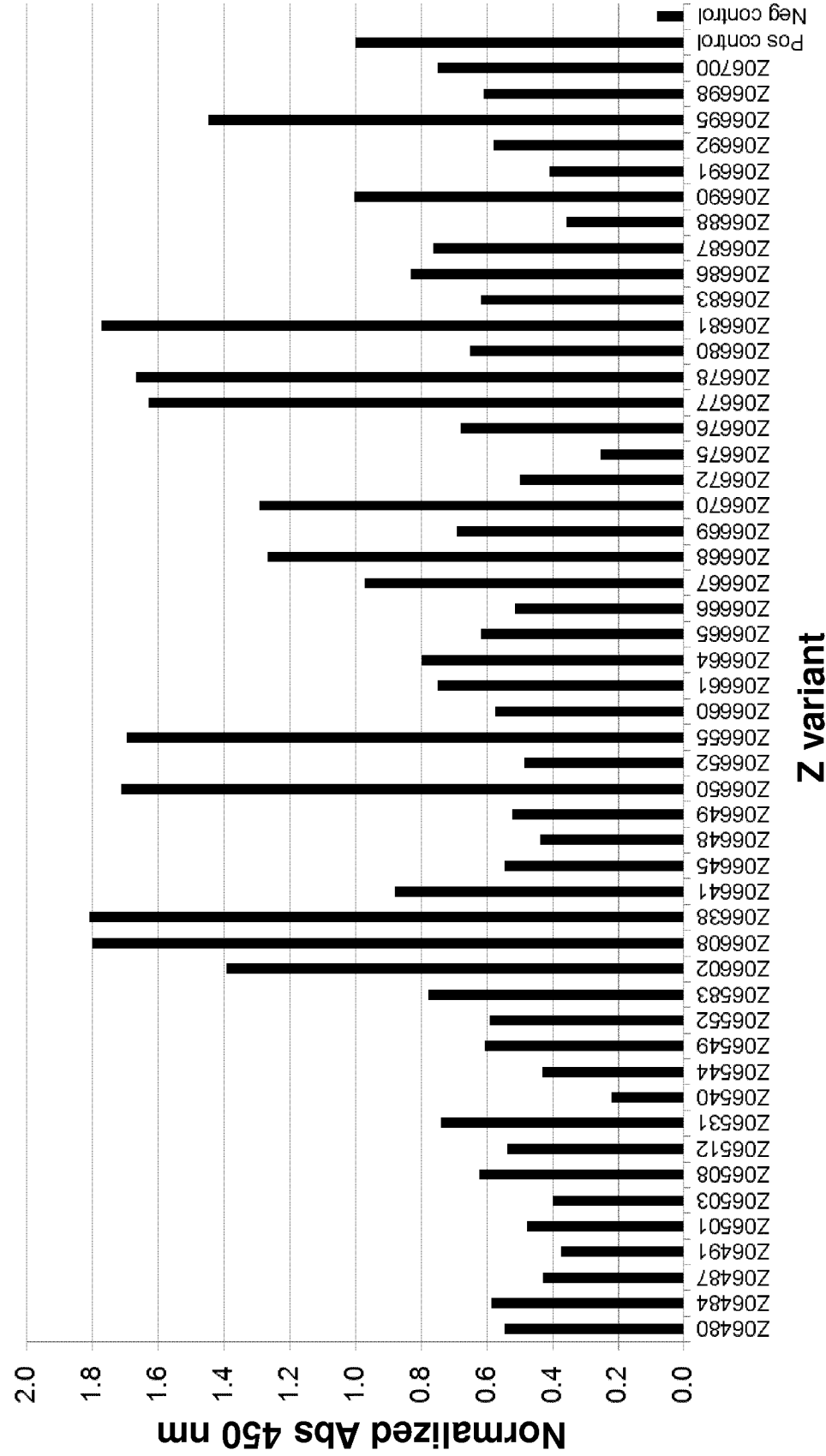


FIGURE 2

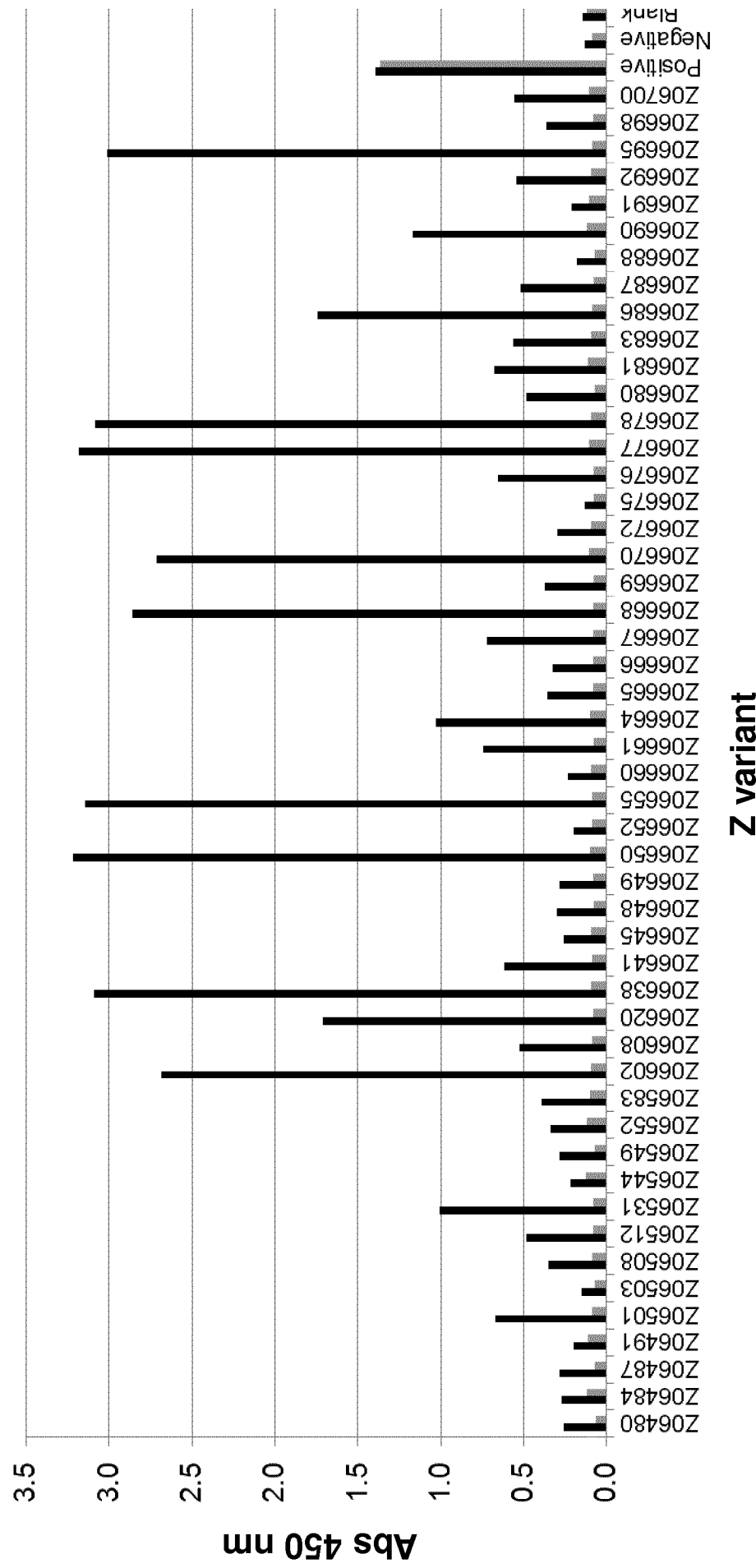


FIGURE 3

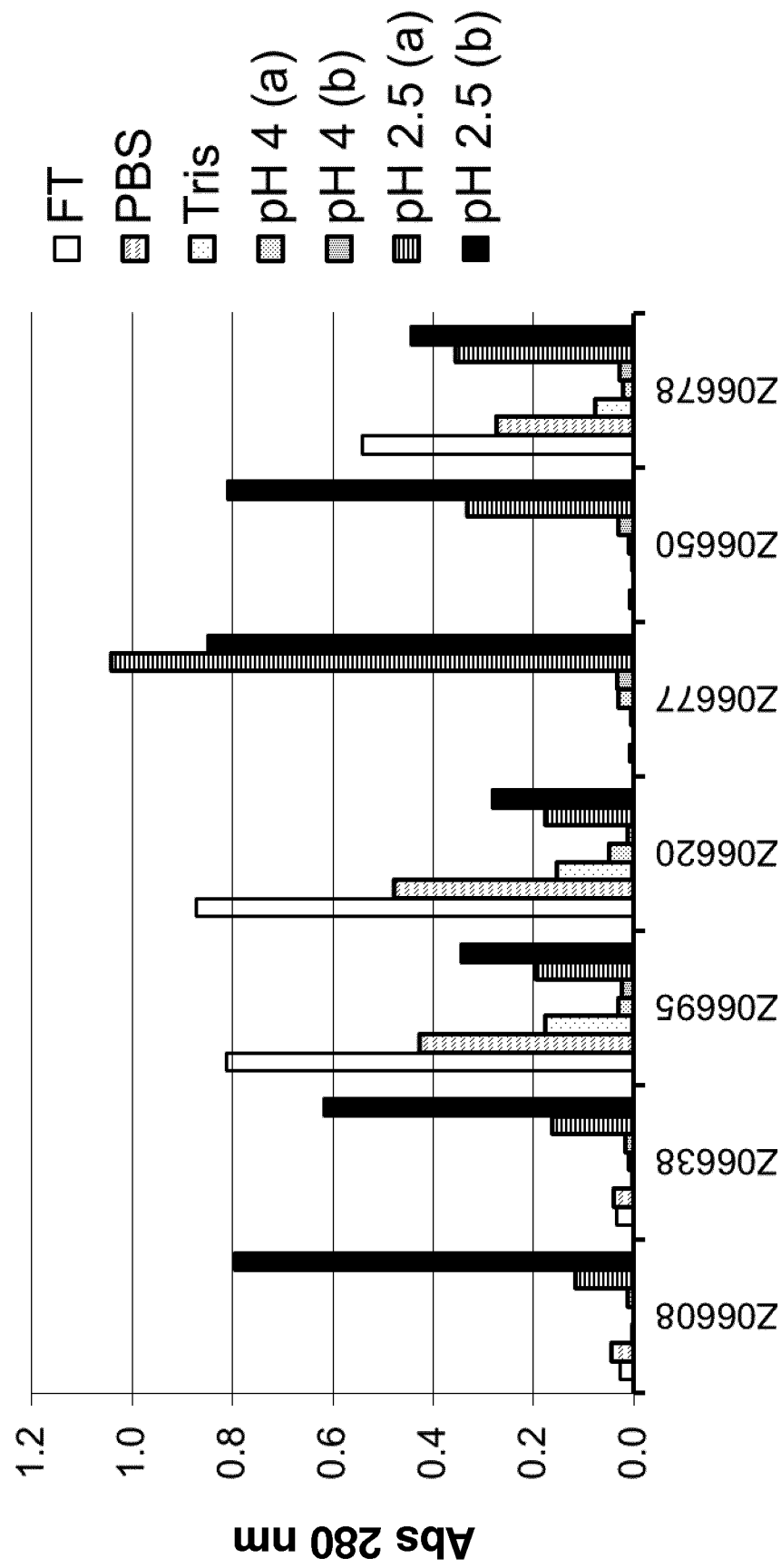


FIGURE 4

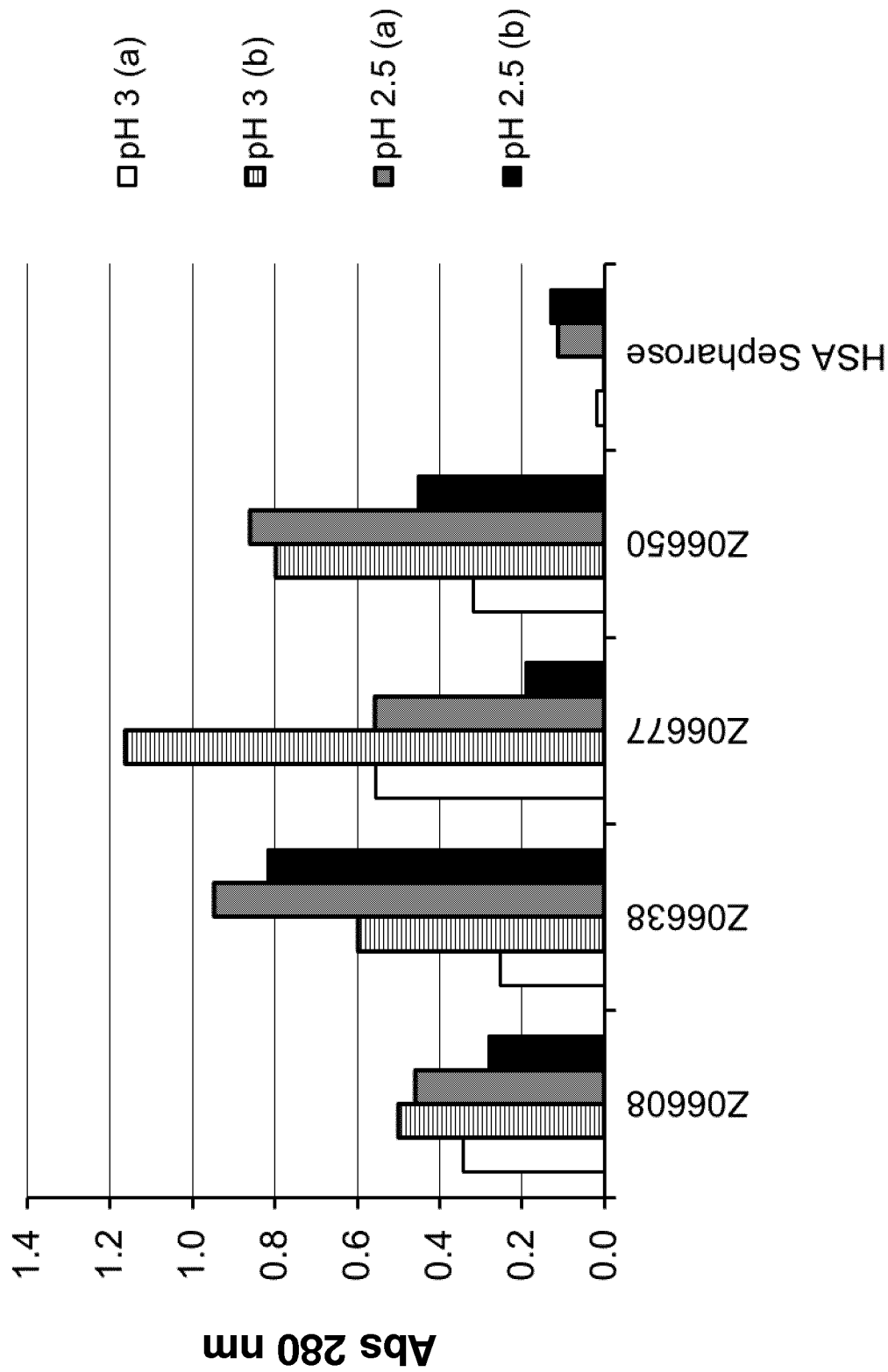


FIGURE 5A

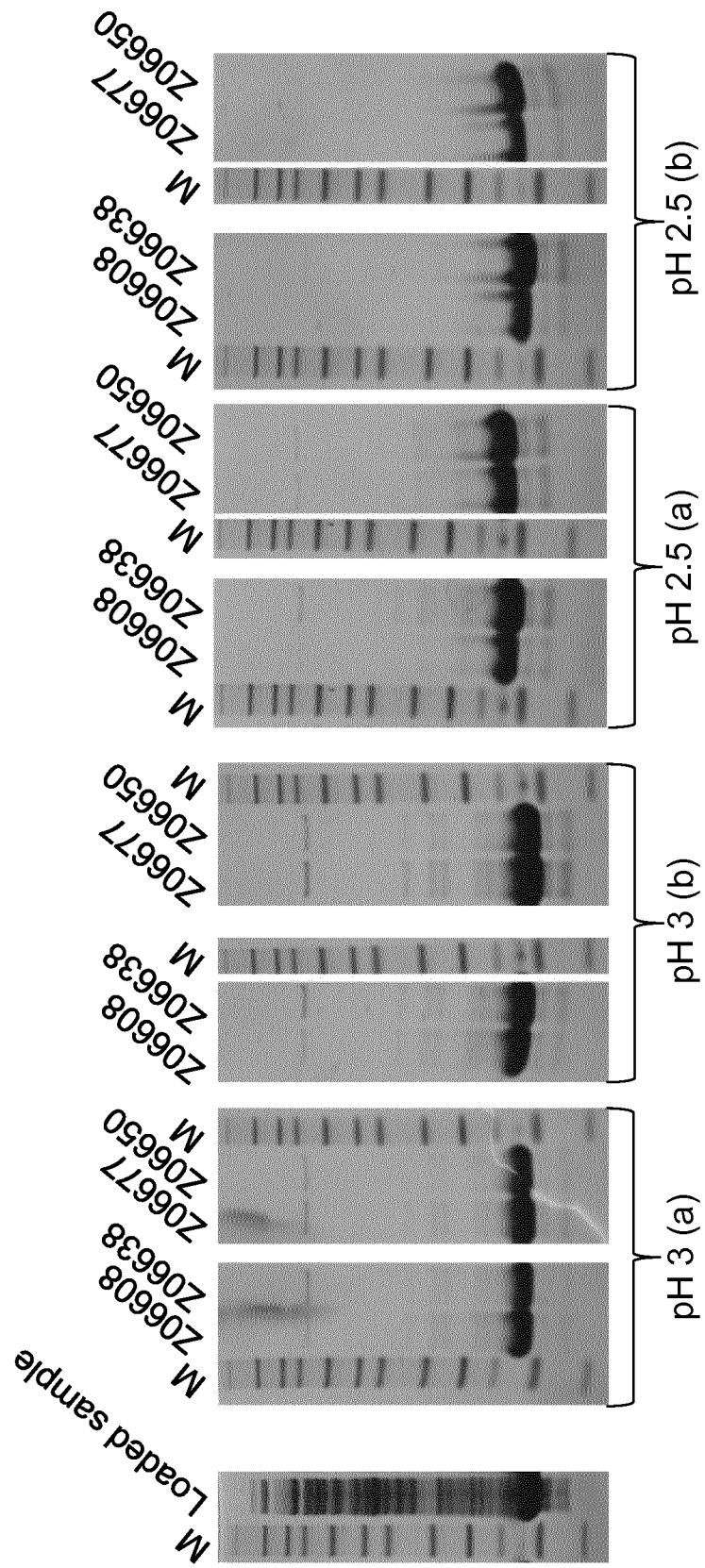


FIGURE 5B

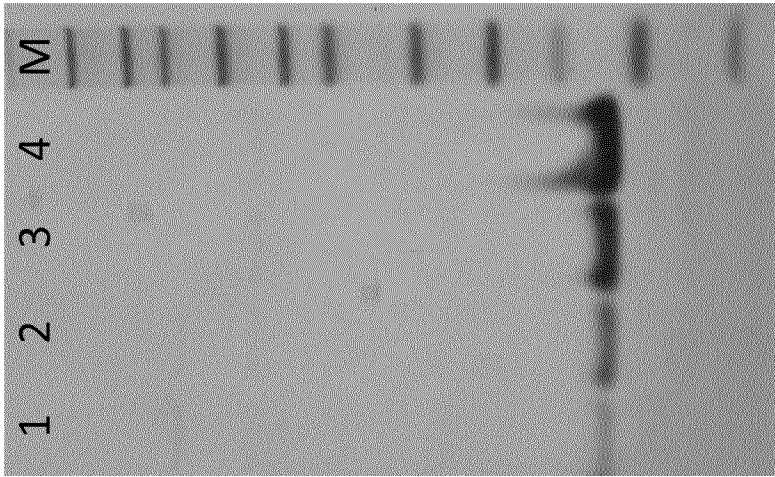


FIGURE 5C

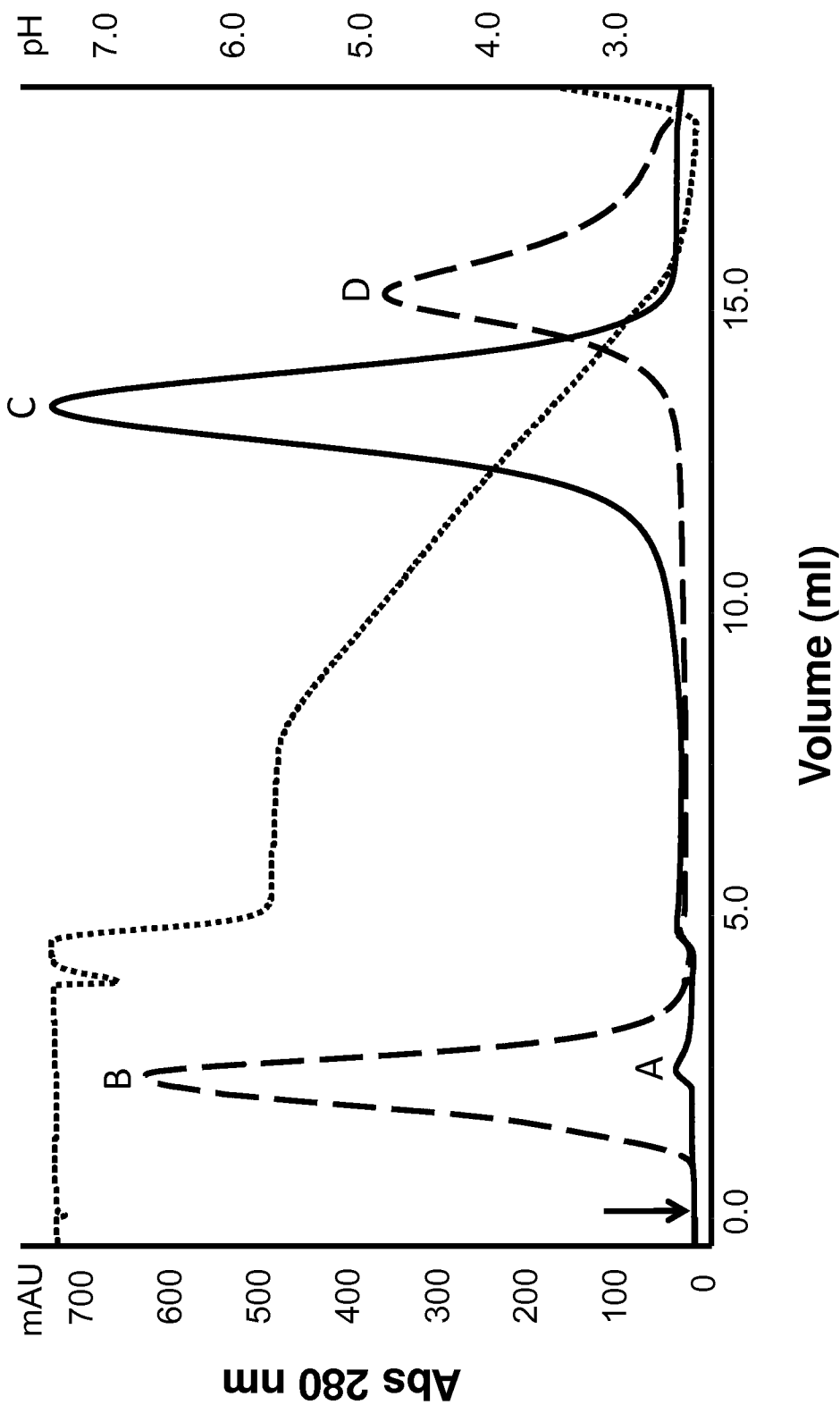


FIGURE 6

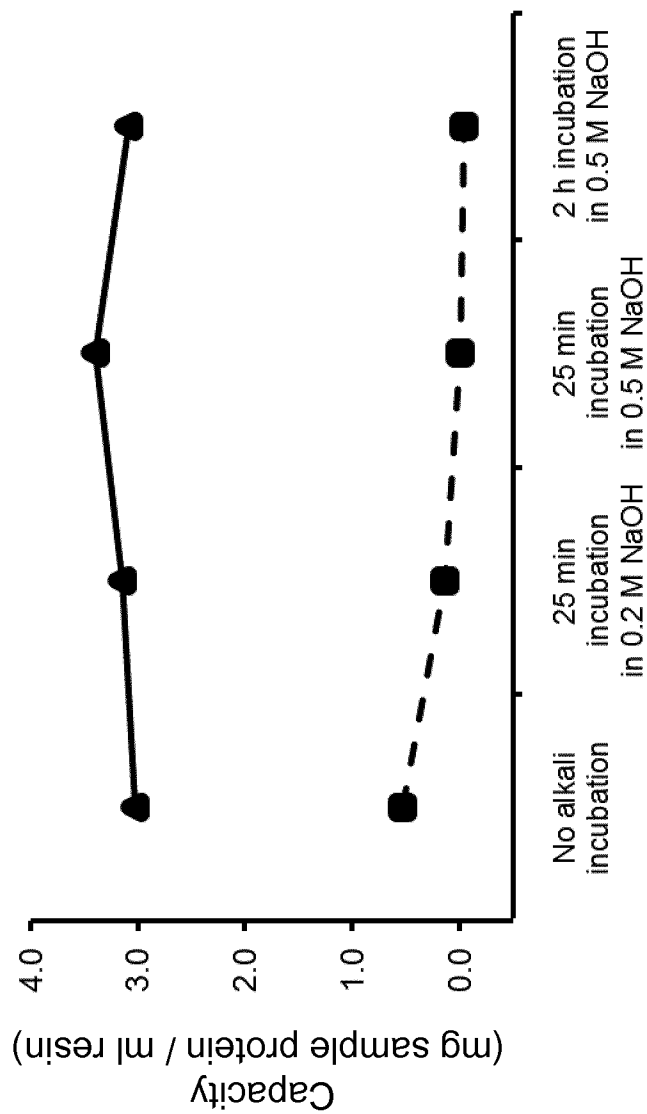


FIGURE 7

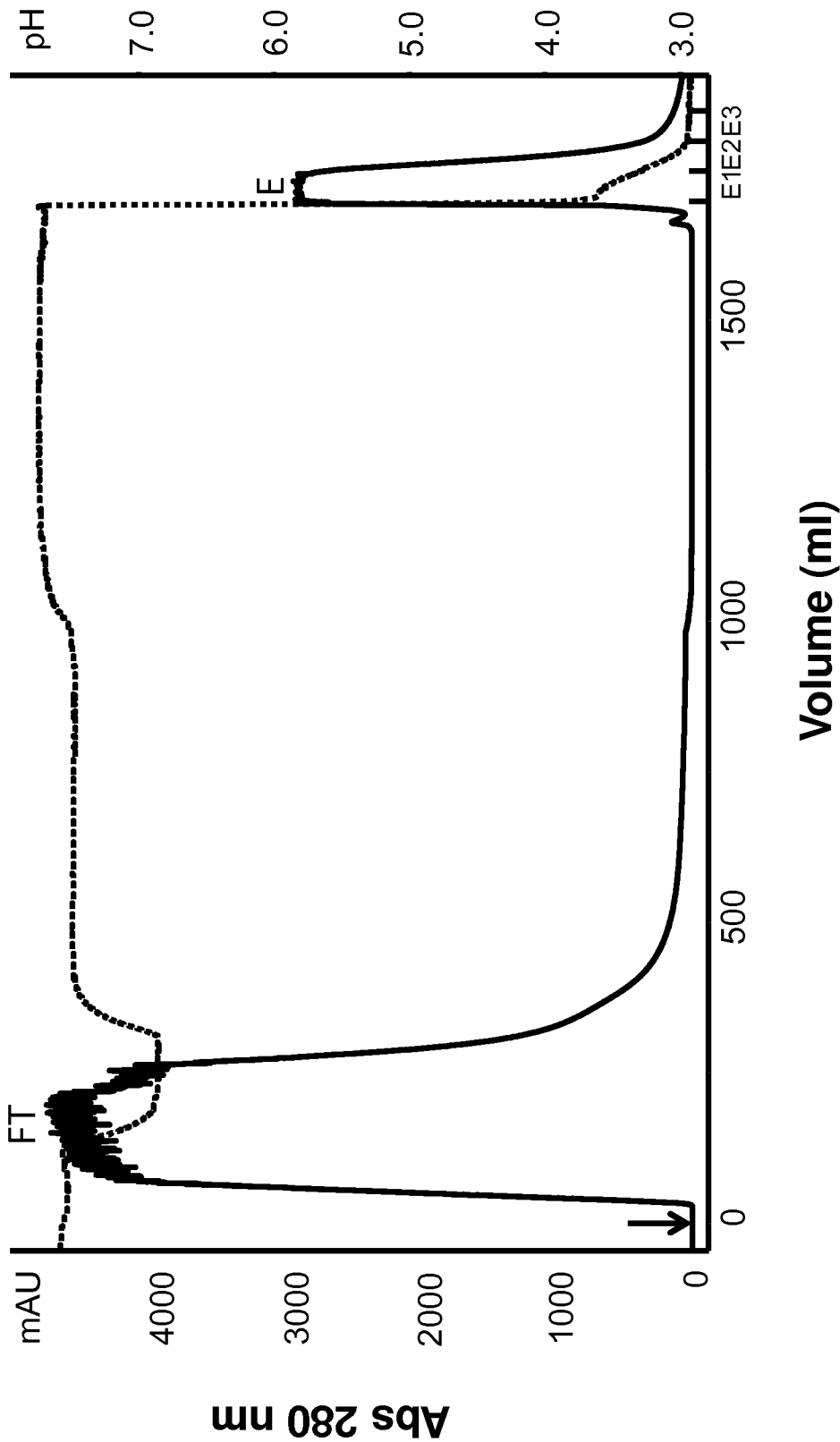


FIGURE 8A

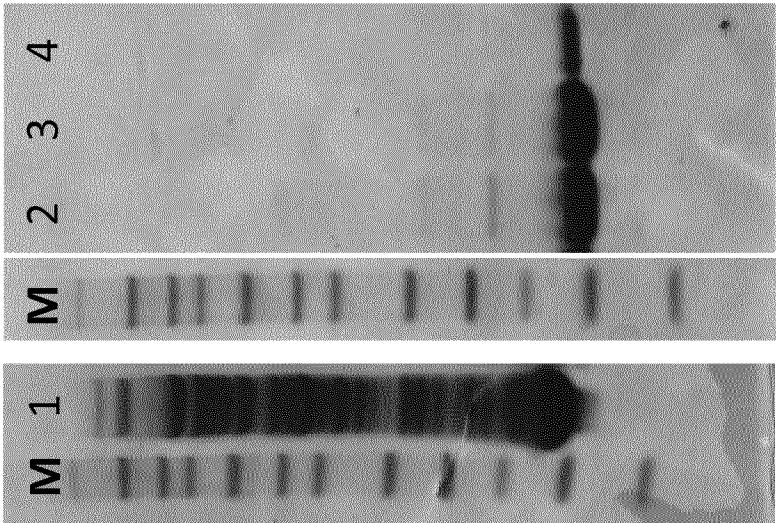


FIGURE 8B

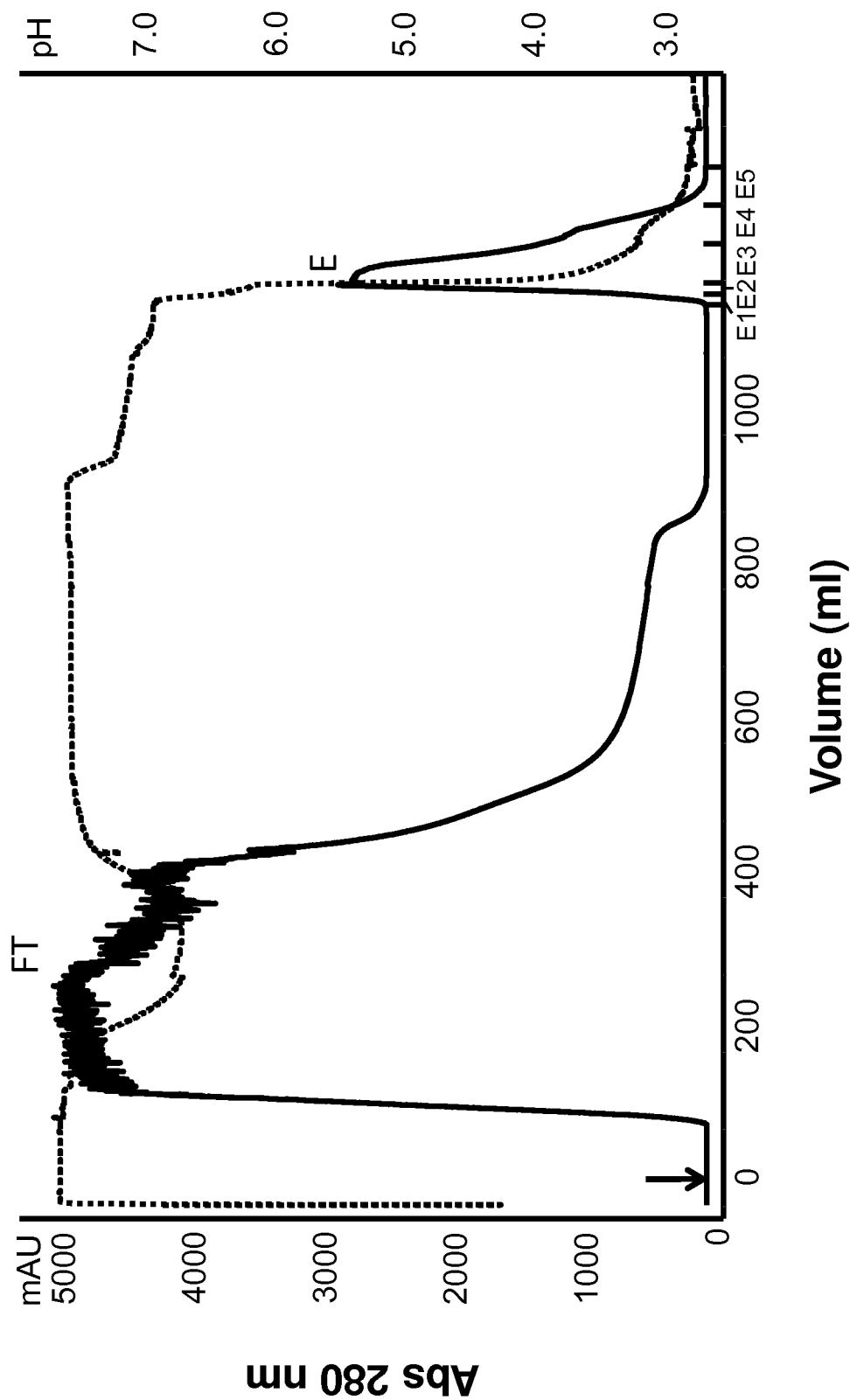


FIGURE 9A

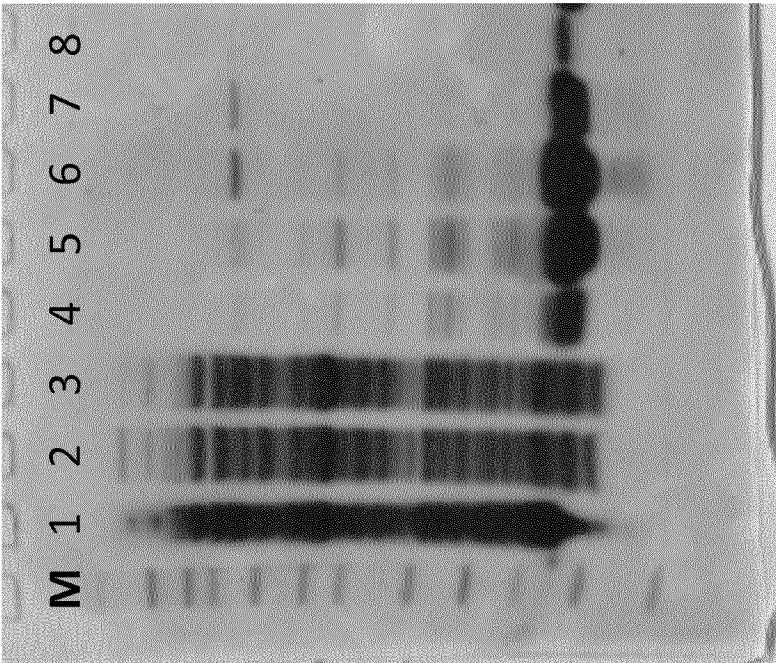


FIGURE 9B

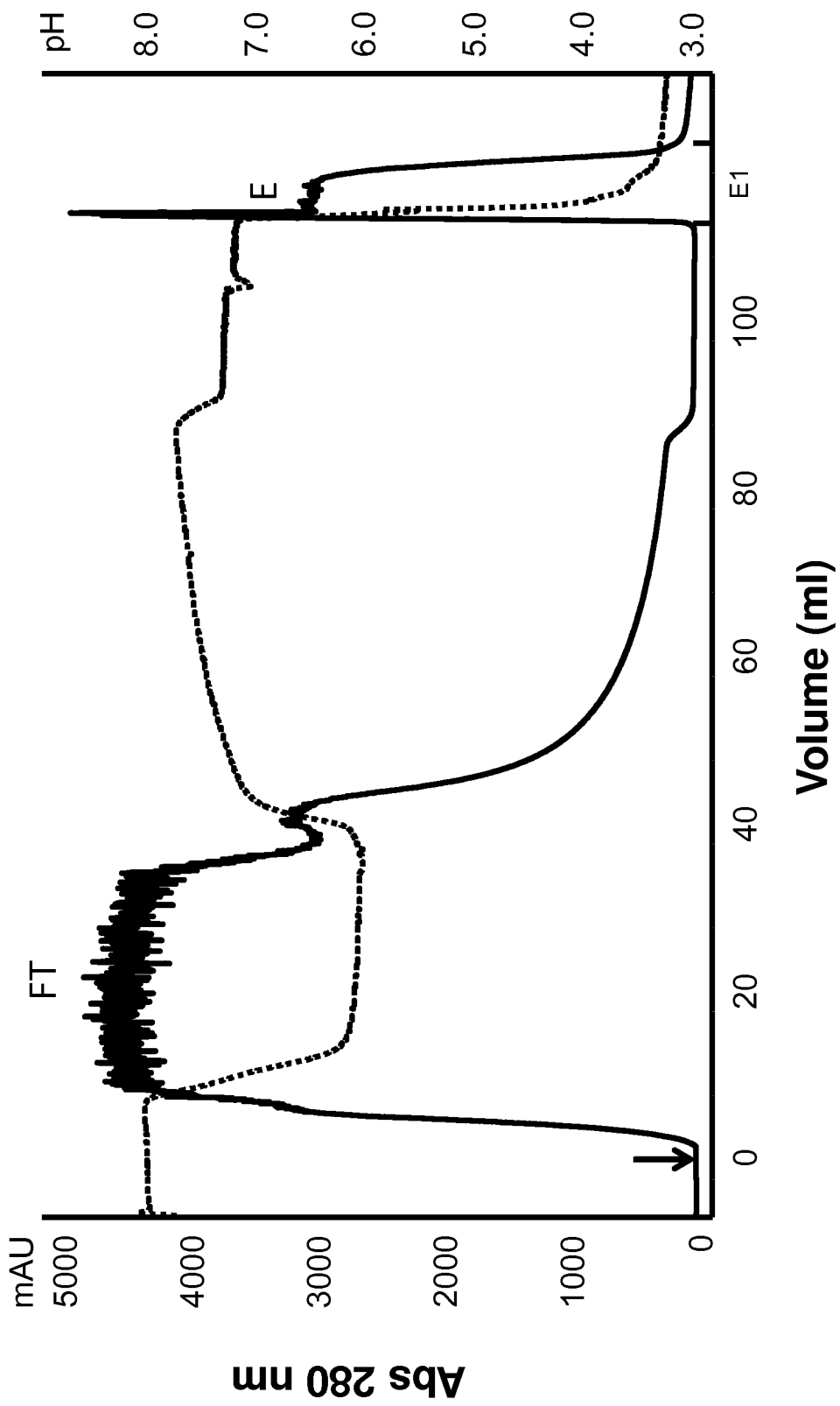


FIGURE 10A

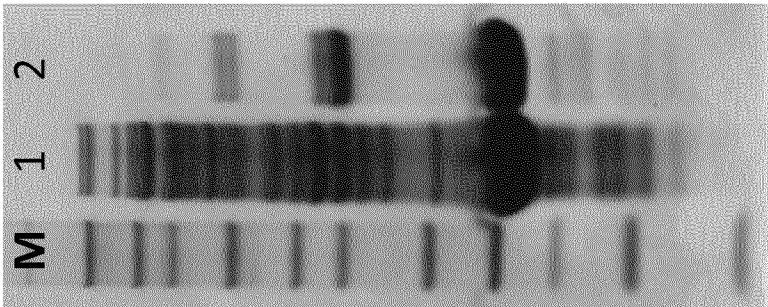


FIGURE 10B

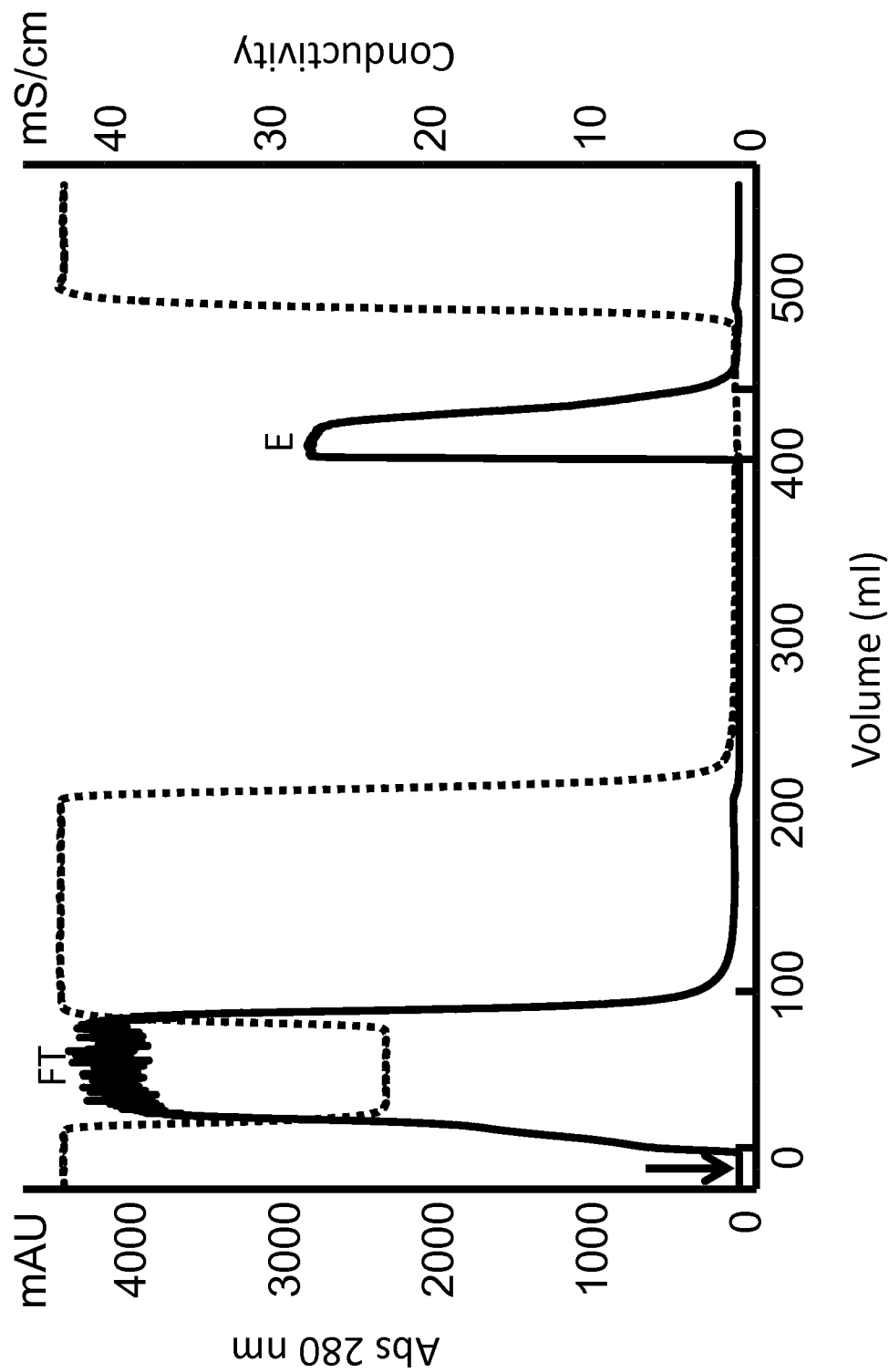


FIGURE 11A

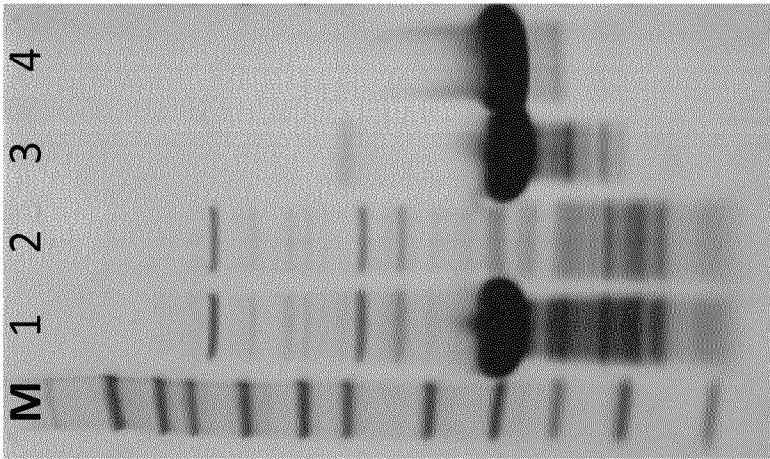


FIGURE 11B

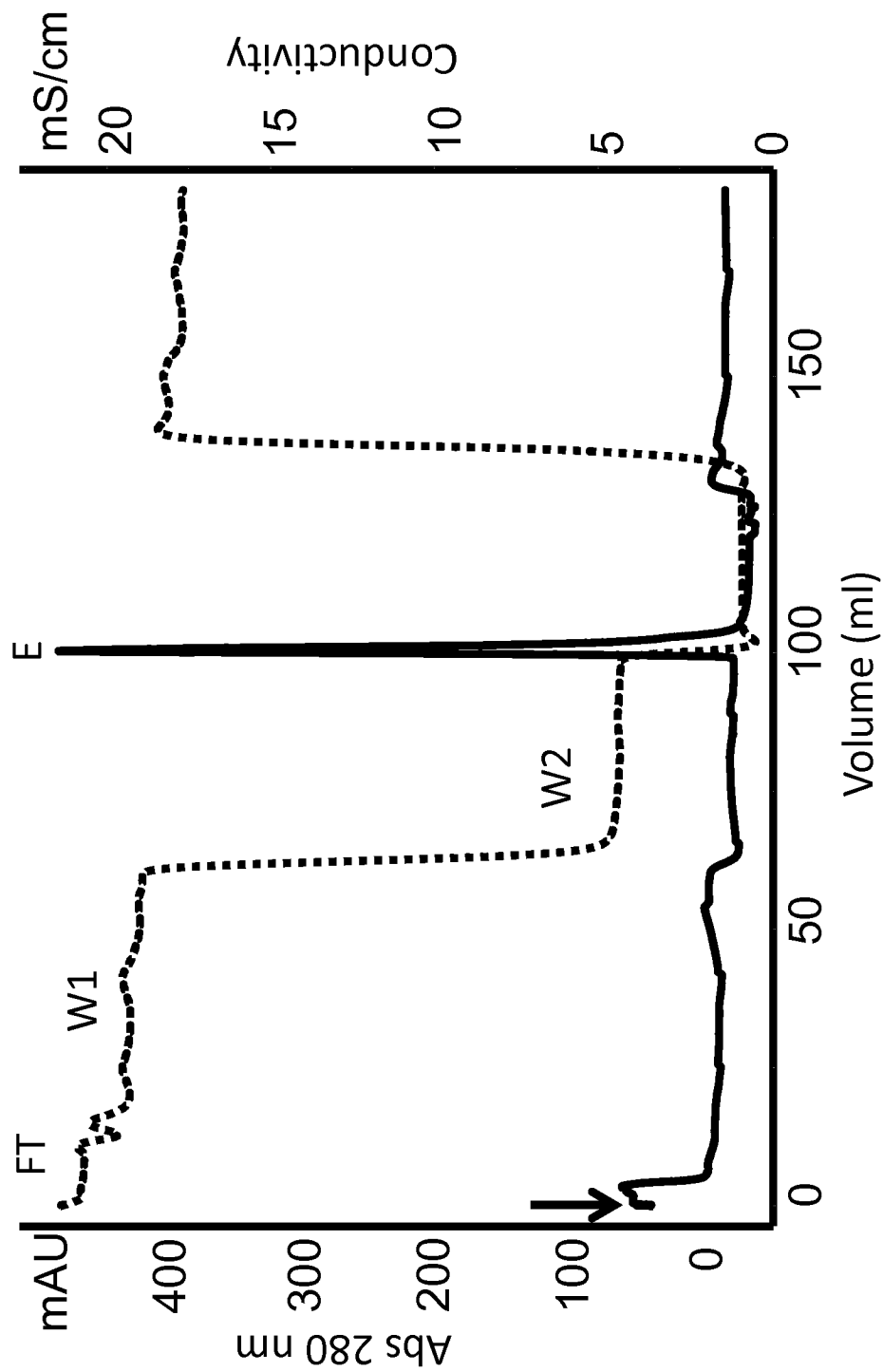


FIGURE 12A

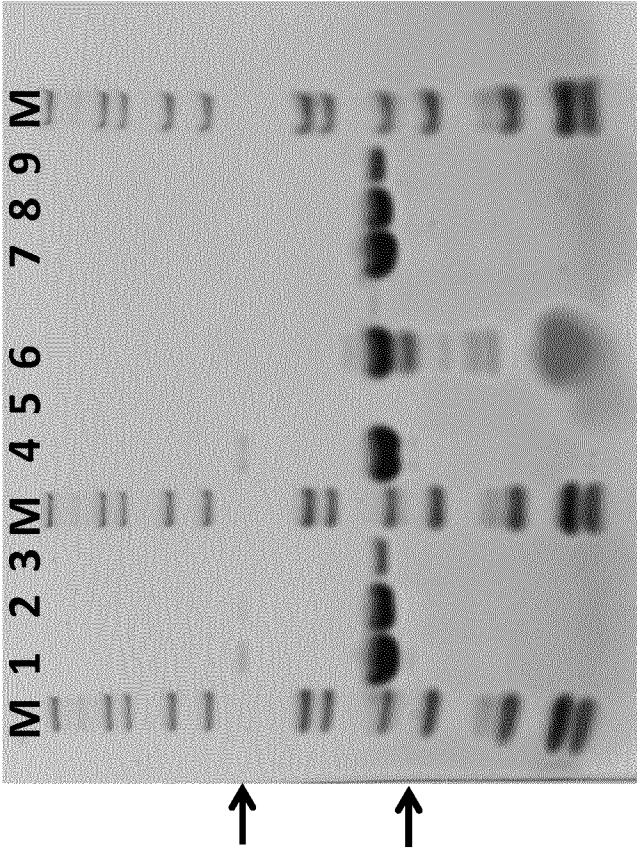


FIGURE 12B

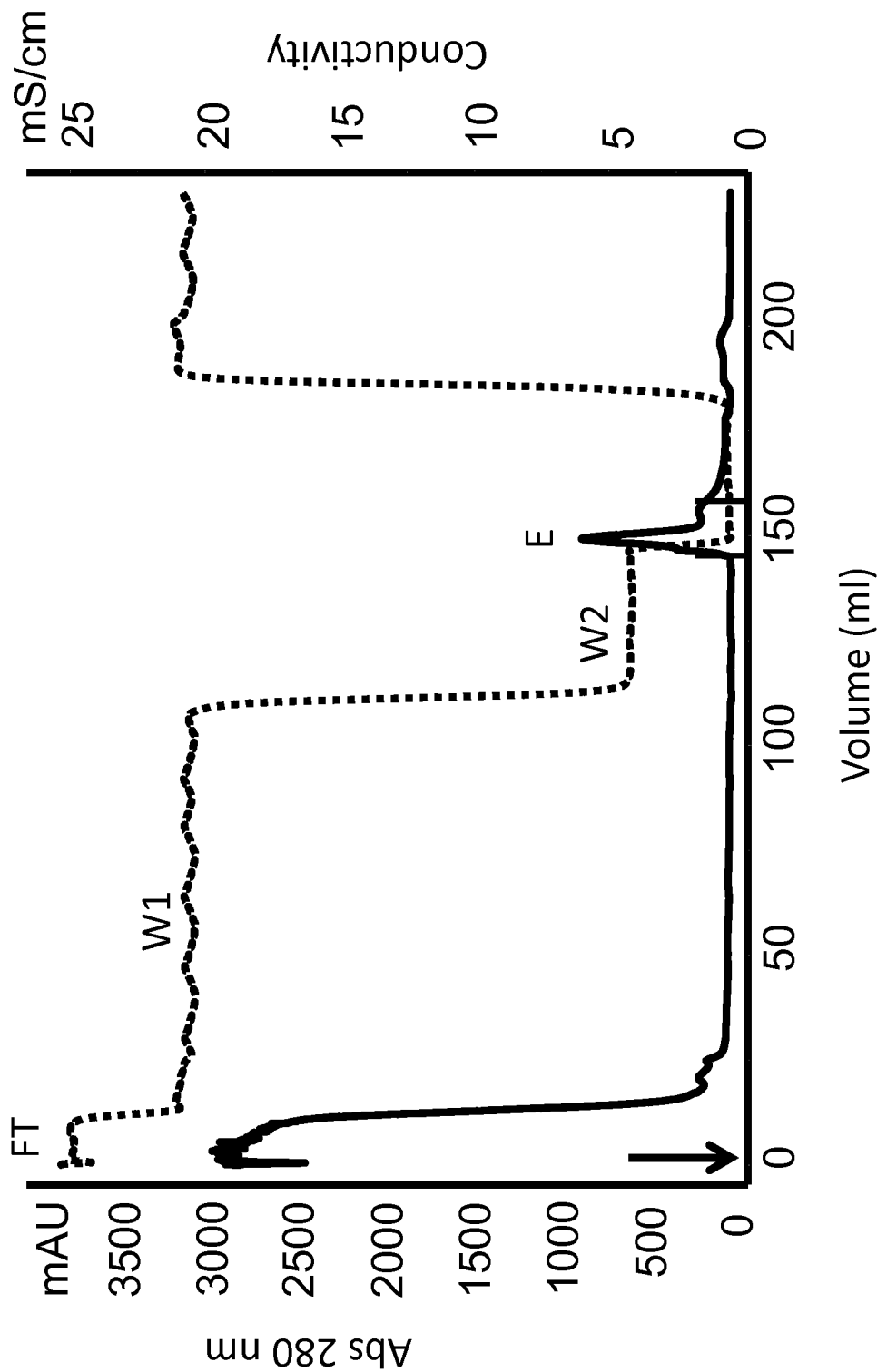


FIGURE 13A

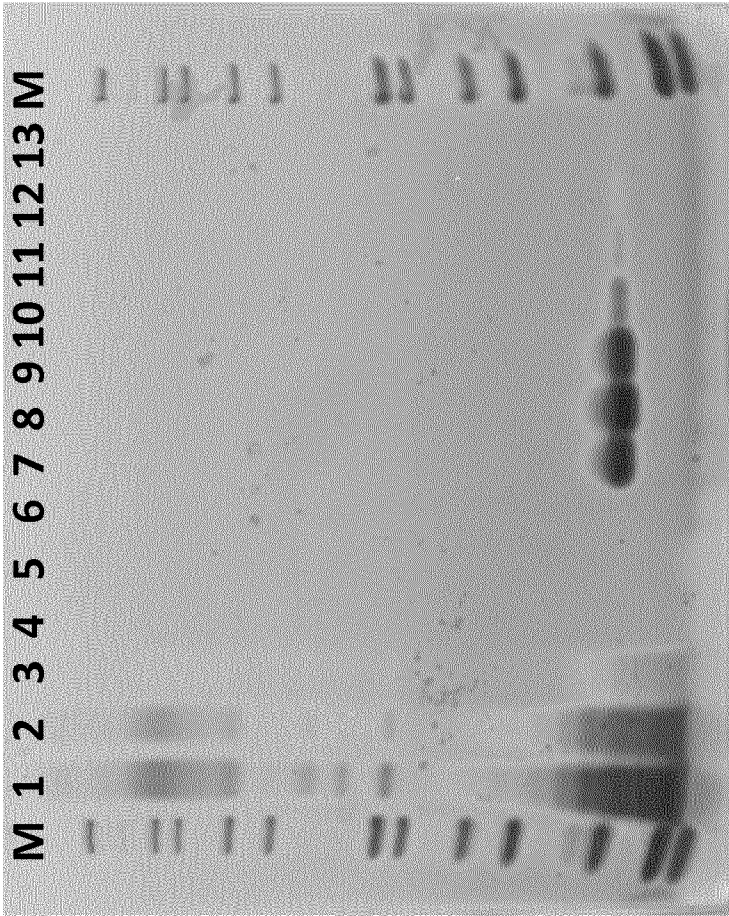


FIGURE 13B

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2013/072359

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07K14/00 C07K14/195 G01N33/68
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C07K G01N

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

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

EPO-Internal, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	EP 2 077 272 A1 (AFFIBODY AB [SE]) 8 July 2009 (2009-07-08) The query sequence SEQ ID NO:55 has over 70% identity over 49 positions in a common overlap (range (q:s): 1-49:5-53) with subject EPOP:HB435314 (length: 53) from EP2077272-A1/2 published on 2009-07-08. The query sequence SEQ ID NO:53 has 77.10 % identity (87.50 % similarity) over 48 positions in a common overlap (range (q:s): 2-49:1-48) with subject GSP:AXD39421 (length: 48) from W02009080811-A1 published on 2009-07-02. The affibody of SEQ ID N°2 his similar to the amino acid sequence of claim 7iii)iv).; the whole document ----- -/-	1-15



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

18 February 2014

Date of mailing of the international search report

26/02/2014

Name and mailing address of the ISA/

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040,
Fax: (+31-70) 340-3016

Authorized officer

Vix, Olivier

INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2013/072359

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 2009/016043 A2 (AFFIBODY AB [SE]; ABRAHMSEN LARS [SE]; JONSSON ANDREAS [SE]; DOGAN JAK) 5 February 2009 (2009-02-05) cited in the application the whole document	1-15
Y	----- E. WAHLBERG ET AL: "An affibody in complex with a target protein: Structure and coupled folding", PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES, vol. 100, no. 6, 18 March 2003 (2003-03-18), pages 3185-3190, XP055102112, ISSN: 0027-8424, DOI: 10.1073/pnas.0436086100 see abstract, page3186-87and page 3190	1-15
Y	----- HE YANAN ET AL: "An artificially evolved albumin binding module facilitates chemical shift epitope mapping of GA domain interactions with phylogenetically diverse albumins", PROTEIN SCIENCE, CAMBRIDGE UNIVERSITY PRESS, vol. 16, 13 June 2007 (2007-06-13), pages 1490-1494, XP002508987, ISSN: 0961-8368, DOI: 10.1110/PS.072799507 the whole document	1-9,11, 14,15
Y	----- DE-CHÂTEAU ET AL: "Protein PAB, a mosaic albumin-binding bacterial protein representing the first contemporary example of module shuffling.", JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 269, no. 16, 1 April 1994 (1994-04-01), pages 12147-12151, XP055047839, ISSN: 0021-9258 In protein PAB, the region is called the GA module (protein G-related albumin-binding module), see Fig. 2 the indicated GA sequence of pab gene from P. magnus; the whole document ----- -/--	1-9,11, 14,15

INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2013/072359

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	JOHAN NILVEBRANT ET AL: "Engineering Bispecificity into a Single Albumin-Binding Domain", PLOS ONE, vol. 6, no. 10, 1 January 2011 (2011-01-01), page e25791, XP055047771, ISSN: 1932-6203, DOI: 10.1371/journal.pone.0025791 in particular see abstract, pages 4-5 and Fig. 1-2 and page 8,-11 -----	1-15
Y	CRAMER ET AL: "Crystal structure of a bacterial albumin-binding domain at 1.4A resolution", FEBS LETTERS, ELSEVIER, AMSTERDAM, NL, vol. 581, no. 17, 28 June 2007 (2007-06-28), pages 3178-3182, XP022133711, ISSN: 0014-5793, DOI: 10.1016/J.FEBSLET.2007.06.003 in particular see pages 3180-3181 -----	1-9,11, 14,15
Y	S. LEJON: "Crystal Structure and Biological Implications of a Bacterial Albumin Binding Module in Complex with Human Serum Albumin", JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 279, no. 41, 16 July 2004 (2004-07-16), pages 42924-42928, XP055090932, ISSN: 0021-9258, DOI: 10.1074/jbc.M406957200 in particular see abstract, page 42926-42927 -----	1-9,11, 14,15
Y	WO 2009/019117 A1 (AFFIBODY AB [SE]; BERGMAN THOMAS [SE]; BLOMQVIST ANDERS [SE]; GRAESLUN) 12 February 2009 (2009-02-12) The query sequence SEQ ID NO:1 has 63.33 % identity (65.52 % over the entire subject, 65.52 % over the entire query) in a common overlap (range (q:s): 1-29:1-29) with subject GSP:AWH91233 (length: 29) from WO2009019117-A1 published on 2009-02-12. The query sequence SEQ ID NO:53 has 74.00 % identity (63.79 % over the entire subject, 75.51 % over the entire query) in a common overlap (range (q:s): 1-49:7-55) with subject GSP:AWH91430 (length: 58) from WO2009019117-A1 published on 2009-02-12.; the whole document ----- -/--	1-9,11, 14,15

INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2013/072359

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 2011/056124 A1 (AFFIBODY AB [SE]; FREJD FREDRIK [SE]; GUNNERIUSSON ELIN [SE]; KRONQVIS) 12 May 2011 (2011-05-12) D9 discloses insulin-like growth factor 1 receptor (IGF-1R) binding polypeptides.; the whole document	1-15
Y	----- TASHIRO M ET AL: "High-resolution solution NMR structure of the Z domain of staphylococcal protein A", JOURNAL OF MOLECULAR BIOLOGY, ACADEMIC PRESS, UNITED KINGDOM, vol. 272, no. 4, 3 October 1997 (1997-10-03), pages 573-590, XP004453779, ISSN: 0022-2836, DOI: 10.1006/JMBI.1997.1265 The amino acid sequence in Figure 2 discloses the amino acid sequence of the Z domain of Staphylococcal Protein A which is 58 residues long, comprises 3 helices and is highly similar to the sequence of the ABD binding polypeptide listed in the application claim 10.; the whole document	1-15
Y	----- JAWID N. AHMAD ET AL: "Novel high-affinity binders of human interferon gamma derived from albumin-binding domain of protein G", PROTEINS: STRUCTURE, FUNCTION, AND BIOINFORMATICS, vol. 80, no. 3, 24 March 2012 (2012-03-24), pages 774-789, XP055099184, ISSN: 0887-3585, DOI: 10.1002/prot.23234 Obtention of novel affinity starting from a 3 helix bundle scaffold: the authors mention on page 775 that the ABD scaffold has the potential to yield to binders of other targets than HSA. Rational selection of scaffold residues and randomization can achieve this result.; the whole document ----- -/--	1-15

INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2013/072359

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	TOVE ALM ET AL: "A small bispecific protein selected for orthogonal affinity purification", BIOTECHNOLOGY JOURNAL, vol. 5, no. 6, 1 June 2010 (2010-06-01), pages 605-617, XP055099189, ISSN: 1860-6768, DOI: 10.1002/biot.201000041 defines the dual binding of the ABD module towards HSA and the Z domain of protein A. Thus, the Z-domain was known to bind ABD. See oabstract, page 606, pages 610-611; the whole document	1-15
Y	----- NYGREN PER-AKE: "Alternative binding proteins: affibody binding proteins developed from a small three-helix bundle scaffold", FEBS JOURNAL, BLACKWELL PUBLISHING, LONDON, GB, vol. 275, no. 11, 1 June 2008 (2008-06-01), pages 2668-2676, XP002601623, ISSN: 1742-464X, DOI: 10.1111/J.1742-4658.2008.06438.X documents discloses a strategy for library design for target binding : selection of defined positions based on the 3D of the three-helix bundle affibody protein scaffold Z and randomization of those positions (library construction). See page 2669-2670 and page 2673 and figure 1.; the whole document	1-15
Y	----- Karin Nord; Gunneriusson E; Ringdahl J; Ståhl S; Uhlén M; Nygren P A: "Binding proteins selected from combinatorial libraries of an alpha-helical bacterial receptor domain.", Nature Biotech., vol. 15, no. 8 15 August 1997 (1997-08-15), pages 772-777, XP055099206, Retrieved from the Internet: URL:http://www.nature.com/nbt/journal/v15/n8/pdf/nbt0897-772.pdf [retrieved on 2014-01-29] cited in the application the whole document ----- -/--	1,2

INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2013/072359

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	J. LÖFBLÖM ET AL: "Affibody molecules: Engineered proteins for therapeutic, diagnostic and biotechnological applications", FEBS LETTERS, vol. 584, no. 12, 1 June 2010 (2010-06-01) , pages 2670-2680, XP055054512, ISSN: 0014-5793, DOI: 10.1016/j.febslet.2010.04.014 the whole document	1-15
X,P	----- WO 2013/126006 A1 (SWEDISH ORPHAN BIOVITRUM AB PUBL [SE]) 29 August 2013 (2013-08-29) The query sequence SEQ ID NO:55 has 75.50 % identity (87.80 % similarity) over 49 positions in a common overlap (range (q:s): 1-49:1-49) with subject GSP:BAS31543 (length: 49) from WO2013126006-A1 published on 2013-08-29.. The query sequence SEQ ID NO:54 has 79.59 % identity (91.84 % similarity) over 49 positions in a common overlap (range (q:s): 1-49:1-49) with subject GSP:BAS31548 (length: 49) from WO2013126006-A1 published on 2013-08-29.; the whole document	1-15
A	----- BRAISTED A C ET AL: "Minimizing a binding domain from protein A", PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES, NATIONAL ACADEMY OF SCIENCES, US, vol. 93, no. 12, 11 June 1996 (1996-06-11) , pages 5688-5692, XP002555464, ISSN: 0027-8424, DOI: 10.1073/PNAS.93.12.5688 the whole document	1-15

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/EP2013/072359

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 2077272	A1	08-07-2009	AT 551351 T 15-04-2012
		AU 2008339897 A1 02-07-2009	
		CA 2710140 A1 02-07-2009	
		CN 101983204 A 02-03-2011	
		DK 2231693 T3 29-05-2012	
		EP 2077272 A1 08-07-2009	
		EP 2231693 A1 29-09-2010	
		ES 2383574 T3 22-06-2012	
		JP 2011509073 A 24-03-2011	
		US 2010286366 A1 11-11-2010	
		WO 2009080811 A1 02-07-2009	
WO 2009016043	A2	05-02-2009	AU 2008281913 A1 05-02-2009
		CA 2694139 A1 05-02-2009	
		CN 101765608 A 30-06-2010	
		DE 08786222 T1 25-11-2010	
		EP 2190863 A2 02-06-2010	
		EP 2546261 A2 16-01-2013	
		ES 2346178 T1 13-10-2010	
		JP 2010534486 A 11-11-2010	
		US 2010273979 A1 28-10-2010	
		WO 2009016043 A2 05-02-2009	
WO 2009019117	A1	12-02-2009	EP 2183275 A1 12-05-2010
		US 2010292434 A1 18-11-2010	
		WO 2009019117 A1 12-02-2009	
WO 2011056124	A1	12-05-2011	EP 2496598 A1 12-09-2012
		JP 2013509861 A 21-03-2013	
		US 2012270801 A1 25-10-2012	
		WO 2011056124 A1 12-05-2011	
WO 2013126006	A1	29-08-2013	NONE