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## (54) Title: METHOD OF CLEANING AND/OR SANITIZING A SEPARATION MATRIX

(57) **Abstract:** The present invention concerns a method of cleaning and/or sanitizing a separation matrix comprising multimers of immunoglobulin-binding alkali-stabilized Protein A domains covalently coupled to a porous support. The method comprises the steps of: a) optionally purifying a mixture comprising a first immunoglobulin using the separation matrix; b) providing a cleaning liquid comprising at least 50% by volume of an aqueous alkali metal hydroxide solution; and c) cleaning and/or sanitizing the separation matrix by contacting the cleaning liquid with the separation matrix for a predetermined contact time. The alkali-stabilized Protein A domains comprise mutants of a parental Fc-binding domain of Staphylococcus Protein A (SpA), as defined by SEQ ID NO 51 or SEQ ID NO 52, wherein the amino acid residues at positions 13 and 44 of SEQ ID NO 51 or 52 are asparagines and wherein at least the asparagine residue at position 3 of SEQ ID NO 51 or 52 has been mutated to an amino acid selected from the group consisting of glutamic acid, lysine, tyrosine, threonine, phenylalanine, leucine, isoleucine, tryptophan, methionine, valine, alanine, histidine and arginine.



Method of cleaning and/or sanitizing a separation matrix

## TECHNICAL FIELD

The present invention relates to a method of cleaning and/or sanitizing separation matrices  
5 that comprise multimers of immunoglobulin-binding alkali-stabilized Protein A domains  
covalently coupled to a porous support. The present invention further relates to a method of  
preventing carryover in the purification of immunoglobulins with a separation matrix  
comprising multimers of immunoglobulin-binding alkali-stabilized Protein A domains covalently  
coupled to a porous support, as well as to use of a cleaning liquid comprising at least 70% by  
10 volume of an aqueous alkali metal hydroxide solution for the sanitization of a separation  
matrix.

## BACKGROUND ART

Immunoglobulins represent the most prevalent biopharmaceutical products in either  
15 manufacture or development worldwide. The high commercial demand for and hence value of  
this particular therapeutic market has led to the emphasis being placed on pharmaceutical  
companies to maximize the productivity of their respective immunoglobulin manufacturing  
processes whilst controlling the associated costs.

Affinity chromatography is used in most cases, as one of the key steps in the purification of  
20 these immunoglobulin molecules, such as monoclonal antibodies (mAbs) or polyclonal  
antibodies (pAbs). A particularly interesting class of affinity reagents is proteins capable of  
specific binding to invariable parts of an immunoglobulin molecule, such interaction being  
independent on the antigen-binding specificity of the antibody. Such reagents can be widely  
used for affinity chromatography recovery of immunoglobulins from different samples such as  
25 but not limited to serum or plasma preparations or cell culture derived feed stocks. An example  
of such a protein is staphylococcal protein A, containing domains capable of binding to the Fc  
and Fab portions of IgG immunoglobulins from different species. These domains are commonly  
denoted as the E-, D-, A-, B- and C-domains.

Staphylococcal protein A (SpA) based reagents have due to their high affinity and selectivity found a widespread use in the field of biotechnology, e.g. in affinity chromatography for capture and purification of antibodies as well as for detection or quantification. At present, SpA-based affinity medium probably is the most widely used affinity medium for isolation of monoclonal antibodies and their fragments from different samples including industrial cell culture supernatants. Accordingly, various matrices comprising protein A-ligands are commercially available, for example, in the form of native protein A (e.g. Protein A SEPHAROSE™, GE Healthcare, Uppsala, Sweden) and also comprised of recombinant protein A (e.g. rProtein A SEPHAROSE™, GE Healthcare). More specifically, the genetic manipulation performed in the commercial recombinant protein A product is aimed at facilitating the attachment thereof to a support and at increasing the productivity of the ligand.

These applications, like other affinity chromatography applications, require comprehensive attention to definite removal of contaminants. Such contaminants can for example be non-eluted molecules adsorbed to the stationary phase or matrix in a chromatographic procedure, such as non-desired biomolecules or microorganisms, including for example proteins, carbohydrates, lipids, endotoxins, bacteria and viruses. The removal of such contaminants from the matrix is usually performed after a first elution of the desired product in order to regenerate the matrix before subsequent use. Cleaning the matrix of contaminants usually involves a procedure known as cleaning-in-place (CIP), wherein agents capable of eluting contaminants from the stationary phase are used. Inactivation of microorganisms usually involves a procedure known as sanitization-in-place (SIP) wherein agents capable of inactivating microorganisms are used. Some agents are capable of both cleaning and sanitizing the separation matrix, depending on the concentration of the agent and the contact time with the separation matrix. One such class of agents often used is alkaline solutions that are passed over said stationary phase. At present the most extensively used cleaning and sanitizing agent for most separation matrices is NaOH, and the concentration thereof can range from 0.1 up to e.g. 1 M, depending on the degree and nature of contamination. However, this strategy is associated with exposing the matrix to solutions with pH-values above 13. For many affinity chromatography matrices containing proteinaceous affinity ligands such alkaline environment is a very harsh condition and consequently results in decreased capacities owing to instability of the ligand to the high pH involved.

An extensive research has therefore been focused on the development of engineered protein ligands that exhibit an improved capacity to withstand alkaline pH-values. For example, Gülich et al. (Susanne Gülich, Martin Linhult, Per-Åke Nygren, Mathias Uhlén, Sophia Hober, Journal of Biotechnology 80 (2000), 169-178) suggested protein engineering to improve the stability  
5 properties of a Streptococcal albumin-binding domain (ABD) in alkaline environments. Gülich et al. created a mutant of ABD, wherein all the four asparagine residues have been replaced by leucine (one residue), aspartate (two residues) and lysine (one residue). Further, Gülich et al. report that their mutant exhibits a target protein binding behavior similar to that of the native protein, and that affinity columns containing the engineered ligand show higher binding  
10 capacities after repeated exposure to alkaline conditions than columns prepared using the parental non-engineered ligand. Thus, it is concluded therein that all four asparagine residues can be replaced without any significant effect on structure and function.

Recent work shows that changes can also be made to protein A (SpA) to effect similar properties. US patent application publication US 2005/0143566, which is hereby incorporated  
15 by reference in its entirety, discloses that when at least one asparagine residue is mutated to an amino acid other than glutamine or aspartic acid, the mutation confers an increased chemical stability at pH-values of up to about 13-14 compared to the parental SpA, such as the B-domain of SpA, or Protein Z, a synthetic construct derived from the B-domain of SpA (US 5,143,844, incorporated by reference in its entirety). The authors show that when these  
20 mutated proteins are used as affinity ligands, the separation media as expected can better withstand cleaning procedures using alkaline agents. Further mutations of protein A domains with the purpose of increasing the alkali stability have also been published in US 8,329,860, JP 2006304633A, US 8,674,073, US 2010/0221844, US 2012/0208234, US 9,051,375, US 2014/0031522, US 2013/0274451 and WO 2014/146350, all of which are hereby incorporated  
25 by reference in their entireties. However, the currently available mutants are still sensitive to alkaline pH and the NaOH concentration during cleaning is usually limited to 0.1 M, which means that complete cleaning is difficult to achieve. Moreover, such a concentration of NaOH is insufficient in itself for effective sanitization of the affinity separation matrix, and therefore sanitization is often performed using solutions comprising alcohols such as ethanol or  
30 isopropanol. Higher NaOH concentrations, which would improve the cleaning and sanitization, lead to unacceptable capacity losses of the affinity separation matrix.

There is thus still a need in this field to obtain a separation matrix containing protein ligands having a further improved stability towards alkaline cleaning and sanitization procedures. There is also a need for such separation matrices with an improved binding capacity to allow for economically efficient purification of therapeutic antibodies.

5

## SUMMARY OF THE INVENTION

The inventors of the present invention have recognised that alcohol solutions are suboptimal for the sanitization of affinity separation matrices. Alcohols are flammable, subject to regulation, and difficult to dispose of. Moreover, the inventors of the present invention have  
10 recognised that low concentration NaOH, such as 0.1 M NaOH, provides insufficient cleaning in order to allow the separation matrix to be used for the purification of a variety of different immunoglobulin products. Instead, the separation matrix is typically only used to purify separate batches of a single product in order to prevent carryover of impurities from one product to another. The inventors have recognised that concentrated aqueous alkali metal  
15 hydroxide solutions have a number of advantages as cleaning and storage solutions. Concentrated alkali metal hydroxide solutions are bacteriostatic and can inactivate most viruses, bacteria, yeasts, fungi and endotoxins. They are capable of effectively stripping the separation matrix of impurities to such a degree that the same separation matrix may be used to purify a variety of immunoglobulins. This facilitates the use of the separation matrix as a separation  
20 platform. Moreover, concentrated alkali metal hydroxide solutions are relatively cheap, easily disposed of, and removal from the separation matrix is simple to detect using pH and/or conductivity measurements.

It is therefore an object of the present invention to provide a method for cleaning and sanitizing an affinity separation matrix for the purification of immunoglobulins which permits the use of  
25 concentrated alkali metal hydroxide solution.

This object is achieved by the method according to the appended claims of cleaning and/or sanitizing a separation matrix comprising multimers of immunoglobulin-binding alkali-stabilized Protein A domains covalently coupled to a porous support. The alkali-stabilized Protein A domains comprise mutants of a parental Fc-binding domain of Staphylococcus Protein A (SpA),  
30 as defined by, or having at least 80% such as at least 90%, 95% or 98% identity to, SEQ ID NO 51

or SEQ ID NO 52, wherein the amino acid residues at positions 13 and 44 of SEQ ID NO 51 or 52 are asparagines and wherein at least the asparagine residue at position 3 of SEQ ID NO 51 or 52 has been mutated to an amino acid selected from the group consisting of glutamic acid, lysine, tyrosine, threonine, phenylalanine, leucine, isoleucine, tryptophan, methionine, valine, alanine, histidine and arginine. The method comprises the steps of:

- a) optionally purifying a mixture comprising a first immunoglobulin using the separation matrix;
- b) providing a cleaning liquid comprising at least 50% by volume of an aqueous alkali metal hydroxide solution; and
- c) cleaning and/or sanitizing the separation matrix by contacting the cleaning liquid with the separation matrix for a predetermined contact time.

By using a separation matrix comprising multimers of immunoglobulin-binding alkali-stabilized Protein A domains as defined above, a highly alkali-stable separation matrix having a high dynamic binding capacity is obtained. The inventors of the present invention have observed that such separation matrices are relatively stable upon impregnation with concentrated aqueous alkali metal hydroxide solution, and substantially retain dynamic binding capacity after contact with such concentrated alkali metal hydroxide solution. This means that such a separation matrices are suitable for cleaning and/or sanitization with concentrated alkali metal hydroxide solutions.

Further mutations to the immunoglobulin-binding alkali-stabilized Protein A domains may provide further enhancement of properties such as enhanced alkali stability. For example, the glutamine residue at position 1 of SEQ ID NO 51 or 52 may be mutated to an alanine; and/or the asparagine or glutamic acid residue at position 35 of SEQ ID NO 51 or 52 may be mutated to an alanine.

The multimers of immunoglobulin-binding alkali-stabilized Protein A domains may be homomultimers selected from the group consisting of dimers, trimers, tetramers, pentamers, hexamers, heptamers, octamers or nonamers. By using an appropriate multimer, the immunoglobulin binding capacity and alkali stability of the separation matrix may be increased.

The multimers of immunoglobulin-binding alkali-stabilized Protein A domains may each comprise a C-terminal cysteine residue for covalent coupling to the porous support. The multimers of immunoglobulin-binding alkali-stabilized Protein A domains may be coupled to the porous support via thioether links. This provides a robust, alkali-stable and well-proven  
5 method of attaching the ligands to the solid support.

The separation matrix may comprise at least 11 mg/ml, such as at least 15 mg/ml, of the multimers of immunoglobulin-binding alkali-stabilized Protein A domains covalently coupled to the porous support. This ensures a separation matrix with a good binding capacity.

10 The porous support may comprise cross-linked polymer particles having a volume-weighted median diameter ( $d_{50,v}$ ) of 56-70 micrometers and a dry solids weight of 55-80 mg/ml. The porous support may for example be highly cross-linked agarose beads.

The aqueous alkali metal hydroxide solution used in the cleaning liquid may be sodium hydroxide solution, potassium hydroxide solution or a mixture thereof, preferably sodium hydroxide solution. Sodium hydroxide solution is relatively cheap, readily available and widely  
15 accepted for use as a cleaning and sanitization solution. The aqueous alkali metal hydroxide solution may have a molarity of from 500 mM to 5 M, such as from 1 M to 2 M. This ensures a solution with good bactericidal properties.

The cleaning liquid may in some instances further comprise a C2 – C7 alcohol, such as ethanol, isopropanol or benzyl alcohol. A cleaning liquid combining an alcohol and an alkali metal  
20 hydroxide may be more effective in inactivating certain microorganisms, such as some spore-forming bacteria.

The cleaning liquid may comprise at least 70% by volume aqueous alkali metal hydroxide solution, such as at least 90% by volume aqueous alkali metal hydroxide solution, preferably at least 99% by volume aqueous alkali metal hydroxide solution.

25 In some instances the cleaning liquid may consist of, or consist essentially of, aqueous alkali metal hydroxide solution.

The predetermined contact time may be a time sufficient to provide a 6- $\log_{10}$  reduction in endotoxin concentration and/or microorganism concentration in the separation matrix. The predetermined contact time may be from 10 minutes to 50 hours, such as from 30 minutes to

24 hours, or such as from 1 hour to 12 hours. Such contact times are typically sufficient to inactivate a wide range of microorganisms.

Steps a) – c) of the method above may be repeated at least 10 times, such as at least 50 times or 50 – 200 times. Thus, the separation matrix may be reused many times while still retaining  
5 sufficient capacity to achieve an acceptable purification.

The object of the invention is further achieved by a method of preventing carryover in the purification of immunoglobulins with a separation matrix comprising multimers of immunoglobulin-binding alkali-stabilized Protein A domains covalently coupled to a porous support, as defined by the appended claims. The method comprises the steps of purifying a first  
10 immunoglobulin and cleaning and sanitizing the separation matrix by performing the steps a) – c) of the method of cleaning and/or sanitizing a separation matrix as described above. The method further comprises the step of

d) purifying a mixture comprising a second immunoglobulin using the separation matrix, wherein the second immunoglobulin is different from the first immunoglobulin.

15 The alkali-stabilized Protein A domains comprise mutants of a parental Fc-binding domain of Staphylococcus Protein A (SpA), as defined by, or having at least 80% such as at least 90%, 95% or 98% identity to, SEQ ID NO 51 or SEQ ID NO 52, wherein the amino acid residues at positions 13 and 44 of SEQ ID NO 51 or 52 are asparagines and wherein at least the asparagine residue at position 3 of SEQ ID NO 51 or 52 has been mutated to an amino acid selected from the group  
20 consisting of glutamic acid, lysine, tyrosine, threonine, phenylalanine, leucine, isoleucine, tryptophan, methionine, valine, alanine, histidine and arginine.

Further objects, advantages and novel features of the present invention will become apparent to one skilled in the art from the following detailed description.

## DEFINITIONS

The terms "antibody" and "immunoglobulin" are used interchangeably herein, and are understood to include also fragments of antibodies, fusion proteins comprising antibodies or antibody fragments and conjugates comprising antibodies or antibody fragments.

- 5 The terms an "Fc-binding polypeptide", "Fc-binding domain" and "Fc-binding protein" mean a polypeptide, domain or protein respectively, capable of binding to the crystallisable part (Fc) of an antibody and includes e.g. Protein A and Protein G, or any fragment or fusion protein thereof that has maintained said binding property.

10 The term "linker" herein means an element linking two polypeptide units, monomers or domains to each other in a multimer.

The term "spacer" herein means an element connecting a polypeptide or a polypeptide multimer to a support.

15 The term "% identity" with respect to comparisons of amino acid sequences is determined by standard alignment algorithms such as, for example, Basic Local Alignment Tool (BLASTTM) described in Altshul et al. (1990) J. Mol. Biol., 215: 403-410. A web-based software for this is freely available from the US National Library of Medicine at [http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastp&PAGE\\_TYPE=BlastSearch&LINK\\_LOC=blasthome](http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastp&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome) . Here, the algorithm "blastp (protein-protein BLAST)" is used for alignment of a query sequence with a subject sequence and determining i.a. the % identity.

20 As used herein, the terms "comprises," "comprising," "containing," "having" and the like can have the meaning ascribed to them in U.S. Patent law and can mean "includes," "including," and the like; "consisting essentially of" or "consists essentially" likewise has the meaning ascribed in U.S. Patent law and the term is open-ended, allowing for the presence of more than that which is recited so long as basic or novel characteristics of that which is recited is not  
25 changed by the presence of more than that which is recited, but excludes prior art embodiments.

## BRIEF DESCRIPTION OF THE DRAWINGS

For a fuller understanding of the present invention and further objects and advantages of it, the detailed description set out below should be read together with the accompanying figures, and in which:

- 5     Fig. 1        shows an alignment of the Fc-binding domains as defined by SEQ ID NO:1-7 and 51-52.
- Fig. 2        shows results from Example 2 for the alkali stability of parental and mutated tetrameric Zvar (SEQ ID NO 7) polypeptide variants coupled to an SPR biosensor chip.
- 10    Fig. 3        shows results from Example 4 for the alkali stability (0.5 M NaOH) of parental and mutated tetrameric Zvar (SEQ ID NO 7) polypeptide variants coupled to agarose beads.
- Fig. 4        shows results from Example 4 for the alkali stability (1.0 M NaOH) of parental and mutated tetrameric Zvar (SEQ ID NO 7) polypeptide variants coupled to agarose
- 15                beads.
- Fig. 5        shows results from Example 7 for the alkali stability (1.0 M NaOH) of agarose beads with different amounts of mutated multimer variants (SEQ ID NO. 20) coupled. The results are plotted as the relative remaining dynamic capacity (Qb10%, 6 min residence time) vs. incubation time in 1 M NaOH.
- 20    Fig. 6        shows results from Example 7 for the alkali stability (1.0 M NaOH) of agarose beads with different amounts of mutated multimer variants (SEQ ID NO. 20) coupled. The results are plotted as the relative remaining dynamic capacity (Qb10%, 6 min residence time) after 31 h incubation in 1 M NaOH vs. the ligand content of the prototypes.
- 25    Fig. 7        shows results from a pH gradient elution of polyclonal human IgG a) from the reference matrix MabSelect SuRe LX and b) a matrix according to the invention.
- Fig. 8        shows analyses of the IgG1, IgG2 and IgG4 components in fractions from the chromatograms of Fig. 7. a) reference matrix and b) matrix according to the

invention. For each fraction, the first bar (blue) represents IgG1, the second (red) IgG 4 and the third (green) IgG 2.

Fig. 9 shows results from accelerated alkali stability measurements with 1 M NaOH incubation for the reference matrix MabSelect SuRe LX (MSS LX) and a matrix according to the invention. The stability is expressed as the percentage of the 10% breakthrough capacity remaining after incubation.

Fig. 10 shows results from repeated CIP cycles using 2M NaOH as the cleaning liquid for the reference matrix MabSelect SuRe (MSS) and a separation matrix according to the invention (Inv. Ex). The capacity of each separation matrix relative to initial capacity (determined as load volume to 35% breakthrough DBC) is shown as a function of cycle number.

Fig. 11 shows results from incubation with 1 M NaOH the reference matrix MabSelect SuRe LX (MSS LX) and a separation matrix according to the invention (Inv. Ex). The relative fluorescence as a function of time due to binding of labelled hIgG is shown for each of the separation matrices incubated for set periods of 0, 16 and 32 hours.

Fig. 12 shows results from incubation with 1 M NaOH the reference matrix MabSelect SuRe LX (MSS LX) and a separation matrix according to the invention (Inv. Ex). The relative fluorescence as a function of time due to binding of labelled hIgG is shown for each of the separation matrices incubated for set periods of up to 32 hours.

Fig. 13 shows results from the inactivation of *B. subtilis* spores using cleaning fluids comprising 1 M NaOH.

## DETAILED DESCRIPTION

One aspect of the present invention concerns a method of cleaning and/or sanitizing a separation matrix comprising multimers of immunoglobulin-binding alkali-stabilized Protein A domains covalently coupled to a porous support.

Throughout this detailed description, two separate numbering conventions may be used.

Unless otherwise stated, the amino acid residue position numbering convention of Fig 1 is used, and the position numbers are designated as corresponding to those in SEQ ID NO 4-7. This applies also to multimers, where the position numbers designate the positions in the

5 polypeptide units or monomers according to the convention of Fig. 1, unless otherwise stated. However, throughout the claims, summary of invention and on occasion in the detailed description, the position numbers corresponding to those of SEQ ID NO 51 and 52 are used. Note that position 1 of SEQ ID NO 51 or SEQ ID NO 52 corresponds to position 9 of SEQ ID NO 4-7, and in this manner the different numbering conventions may be interconverted.

10 The immunoglobulin-binding alkali-stabilized Protein A domains of the invention, also termed herein as “the polypeptide”, comprise, consist essentially of, or consist of mutants of a parental Fc-binding domain of Staphylococcus Protein A (SpA), as defined by, or having at least 80% such as at least 90%, 95% or 98% identity to, SEQ ID NO 51 or SEQ ID NO 52, wherein the amino acid residues at positions 13 and 44 of SEQ ID NO 51 or 52 are asparagines and wherein at least the  
15 asparagine residue at position 3 of SEQ ID NO 51 or 52 has been mutated to an amino acid selected from the group consisting of glutamic acid, lysine, tyrosine, threonine, phenylalanine, leucine, isoleucine, tryptophan, methionine, valine, alanine, histidine and arginine.

SEQ ID NO 51 (truncated Zvar)

QQ NAFYEILHLP NLTEEQRNAF IQSLKDDPSQ SANLLAEAKK LNDAQ

20 SEQ ID NO 52 (truncated C domain)

QQ NAFYEILHLP NLTEEQRNGF IQSLKDDPSV SKEILAEAKK LNDAQ

Such immunoglobulin-binding alkali-stabilized Protein A domains may comprise, consist essentially of, or consist of mutants of a parental Fc-binding domain of Staphylococcus Protein A (SpA), as defined by, or having at least 90%, at least 95% or at least 98% identity to, SEQ ID  
25 NO: 1 (E-domain), SEQ ID NO: 2 (D-domain), SEQ ID NO:3 (A-domain), SEQ ID NO:22 (variant A-domain), SEQ ID NO: 4 (B-domain), SEQ ID NO: 5 (C-domain), SEQ ID NO:6 (Protein Z) , SEQ ID NO:7 (Zvar), SEQ ID NO 51 (Zvar without the linker region amino acids 1-8 and 56-58) or SEQ ID NO 52 (C-domain without the linker region amino acids 1-8 and 56-58) as illustrated in Fig. 1, wherein at least the asparagine (or serine, in the case of SEQ ID NO 2) residue at the position  
30 corresponding to position 11 in SEQ ID NO:4-7 has been mutated to an amino acid selected

from the group consisting of glutamic acid, lysine, tyrosine, threonine, phenylalanine, leucine, isoleucine, tryptophan, methionine, valine, alanine, histidine and arginine, and wherein the asparagine residues corresponding to positions 21 and 52 in SEQ ID NO:4-7 (positions 13 and 44 of SEQ ID NO 51 or 52) are conserved.

- 5 A number of the Fc-binding domains listed above are shown aligned in Fig. 1. The parental, i.e. non-engineered, Staphylococcus Protein A (SpA) comprises five Fc-binding domains termed domain E (SEQ ID NO 1), D (SEQ ID NO 2), A (SEQ ID NO 3), B (SEQ ID NO 4) and C (SEQ ID NO 5). Protein Z (SEQ ID NO:6) is a mutated B-domain as disclosed in US5143844, hereby incorporated by reference in its entirety. SEQ ID NO 7 denotes a further mutated variant of Protein Z, here  
 10 called Zvar, with the mutations N3A,N6D,N23T. SEQ ID NO:22 (not shown in Fig. 1) is a natural variant of the A-domain in Protein A from Staphylococcus aureus strain N315, having an A46S mutation, using the position terminology of Fig. 1. SEQ ID NO 51 is Zvar (SEQ ID NO 7) without the linker region amino acids at positions 1-8 and 56-58. SEQ ID NO 52 is the C-domain of protein A without the linker region amino acids 1-8 and 56-58) as illustrated in Fig. 1.
- 15 The mutation of N11 (N3 of SEQ ID NO 51:52) in these domains, together with the conservation of the asparagine residues N21 and N52 (N13 and N44 of SEQ ID NO 51:52) confers an improved alkali stability in comparison with the parental domain/polypeptide, without impairing the immunoglobulin-binding properties. Hence, the polypeptide can also be described as an Fc- or immunoglobulin-binding polypeptide, or alternatively as an Fc- or  
 20 immunoglobulin-binding polypeptide unit.

Described in alternative language, the immunoglobulin-binding alkali-stabilized Protein A domains may comprise, consist essentially of, or consist of a sequence as defined by, or having at least 90%, at least 95% or at least 98% identity to SEQ ID NO 53.

SEQ ID NO 53

- 25 X<sub>1</sub>Q X<sub>2</sub>AFYEILX<sub>3</sub>LP NLTEEQRX<sub>4</sub>X<sub>5</sub>F IX<sub>6</sub>X<sub>7</sub>LKDX<sub>8</sub>PSX<sub>9</sub> SX<sub>10</sub>X<sub>11</sub>X<sub>12</sub>LAEAKX<sub>13</sub> X<sub>14</sub>NX<sub>15</sub>AQ

wherein individually of each other:

X<sub>1</sub>=A, Q or is deleted

X<sub>2</sub>=E,K,Y,T,F,L,W,I,M,V,A,H or R

X<sub>3</sub>=H or K

X<sub>4</sub>=A or N

X<sub>5</sub>=A, G, S, Y, Q, T, N, F, L, W, I, M, V, D, E, H, R or K, such as S, Y, Q, T, N, F, L, W, I, M, V, D, E, H, R or K

X<sub>6</sub>=Q or E

X<sub>7</sub>=S or K

5 X<sub>8</sub>=E or D

X<sub>9</sub>=Q, V or is deleted

X<sub>10</sub>=K, R, A or is deleted

X<sub>11</sub>=A, E, N or is deleted

X<sub>12</sub>=I or L

10 X<sub>13</sub>=K or R

X<sub>14</sub>=L or Y

X<sub>15</sub>=D, F, Y, W, K or R

Specifically, the amino acid residues in SEQ ID NO 53 may individually of each other be:

X<sub>1</sub> = A or is deleted

15 X<sub>2</sub> = E

X<sub>3</sub> = H

X<sub>4</sub> = N

X<sub>6</sub> = Q

X<sub>7</sub> = S

20 X<sub>8</sub> = D

X<sub>9</sub> = V or is deleted

X<sub>10</sub> = K or is deleted

X<sub>11</sub> = A or is deleted

X<sub>12</sub> = I

25 X<sub>13</sub> = K

X<sub>14</sub> = L

In certain embodiments, the amino acid residues in SEQ ID NO 53 may be:

X<sub>1</sub>=A, X<sub>2</sub> = E, X<sub>3</sub> = H, X<sub>4</sub> = N, X<sub>6</sub> = Q, X<sub>7</sub> = S, X<sub>8</sub> = D, X<sub>9</sub> = V, X<sub>10</sub> = K, X<sub>11</sub> = A, X<sub>12</sub> = I, X<sub>13</sub> = K, X<sub>14</sub> = L. In some embodiments X<sub>2</sub> = E, X<sub>3</sub> = H, X<sub>4</sub> = N, X<sub>5</sub>=A, X<sub>6</sub> = Q, X<sub>7</sub> = S, X<sub>8</sub> = D, X<sub>12</sub> = I, X<sub>13</sub> = K, X<sub>14</sub> = L and

30 X<sub>15</sub>=D and one or more of X<sub>1</sub>, X<sub>9</sub>, X<sub>10</sub> and X<sub>11</sub> is deleted. In further embodiments, X<sub>1</sub>=A, X<sub>2</sub> = E, X<sub>3</sub>

= H, X<sub>4</sub> = N, X<sub>5</sub> = S, Y, Q, T, N, F, L, W, I, M, V, D, E, H, R or K, X<sub>6</sub> = Q, X<sub>7</sub> = S, X<sub>8</sub> = D, X<sub>9</sub> = V, X<sub>10</sub> = K, X<sub>11</sub> = A, X<sub>12</sub> = I, X<sub>13</sub> = K, X<sub>14</sub> = L and X<sub>15</sub> = D, or alternatively X<sub>1</sub> = A, X<sub>2</sub> = E, X<sub>3</sub> = H, X<sub>4</sub> = N, X<sub>5</sub> = A, X<sub>6</sub> = Q, X<sub>7</sub> = S, X<sub>8</sub> = D, X<sub>9</sub> = V, X<sub>10</sub> = K, X<sub>11</sub> = A, X<sub>12</sub> = I, X<sub>13</sub> = K, X<sub>14</sub> = L and X<sub>15</sub> = F, Y, W, K or R.

In some embodiments, the amino acid residues may individually of each other be:

- 5 a) X<sub>1</sub> = A or is deleted, X<sub>2</sub> = E, X<sub>3</sub> = H, X<sub>4</sub> = N, X<sub>6</sub> = Q, X<sub>7</sub> = S, X<sub>8</sub> = D, X<sub>9</sub> = V or is deleted, X<sub>10</sub> = K or is deleted, X<sub>11</sub> = A or is deleted, X<sub>12</sub> = I, X<sub>13</sub> = K, X<sub>14</sub> = L;
- b) X<sub>1</sub> = A, X<sub>2</sub> = E, X<sub>3</sub> = H, X<sub>4</sub> = N, X<sub>5</sub> = A, X<sub>6</sub> = Q, X<sub>7</sub> = S, X<sub>8</sub> = D, X<sub>9</sub> = V, X<sub>10</sub> = K, X<sub>11</sub> = A, X<sub>12</sub> = I, X<sub>13</sub> = K, X<sub>14</sub> = L and X<sub>15</sub> = D;
- c) X<sub>1</sub> is A, X<sub>2</sub> = E, X<sub>3</sub> = H, X<sub>4</sub> = N, X<sub>6</sub> = Q, X<sub>7</sub> = S, X<sub>8</sub> = D, X<sub>9</sub> = V, X<sub>10</sub> = K, X<sub>11</sub> = A, X<sub>12</sub> = I, X<sub>13</sub> = K, X<sub>14</sub> = L  
10 and X<sub>15</sub> = D; or
- d) X<sub>1</sub> is A, X<sub>3</sub> = H, X<sub>4</sub> = N, X<sub>5</sub> = A, X<sub>6</sub> = Q, X<sub>7</sub> = S, X<sub>8</sub> = D, X<sub>9</sub> = V, X<sub>10</sub> = K, X<sub>11</sub> = A, X<sub>12</sub> = I, X<sub>13</sub> = K, X<sub>14</sub> = L and X<sub>15</sub> = D.

The N11 (X<sub>2</sub>) mutation (e.g. a N11E or N11K mutation) may be the only mutation or the polypeptide may also comprise further mutations, such as substitutions in at least one of the  
15 positions corresponding to positions 3, 6, 9, 10, 15, 18, 23, 28, 29, 32, 33, 36, 37, 40, 42, 43, 44, 47, 50, 51, 55 and 57 in SEQ ID NO:4-7. In one or more of these positions, the original amino acid residue may e.g. be substituted with an amino acid which is not asparagine, proline or cysteine. The original amino acid residue may e.g. be substituted with an alanine, a valine, a threonine, a serine, a lysine, a glutamic acid or an aspartic acid. Further, one or more amino  
20 acid residues may be deleted, e.g. from positions 1-6 and/or from positions 56-58.

In some embodiments, the amino acid residue at the position corresponding to position 9 in SEQ ID NO:4-7 (X<sub>1</sub>) is an amino acid other than glutamine, asparagine, proline or cysteine, such as an alanine or it can be deleted. The combination of the mutations at positions 9 and 11 provides particularly good alkali stability, as shown by the examples. In specific embodiments,  
25 in SEQ ID NO: 7 the amino acid residue at position 9 is an alanine and the amino acid residue at position 11 is a lysine or glutamic acid, such as a lysine. Mutations at position 9 are also discussed in copending application PCT/SE2014/050872, which is hereby incorporated by reference in its entirety.

In some embodiments, the amino acid residue at the position corresponding to position 50 in SEQ ID NO:4-7 (X<sub>13</sub>) is an arginine or a glutamic acid.

5 In certain embodiments, the amino acid residue at the position corresponding to position 3 in SEQ ID NO:4-7 is an alanine and/or the amino acid residue at the position corresponding to position 6 in SEQ ID NO:4-7 is an aspartic acid. One of the amino acid residues at positions 3 and 6 may be an asparagine and in an alternative embodiment both amino acid residues at positions 3 and 6 may be asparagines.

10 In some embodiments the amino acid residue at the position corresponding to position 43 in SEQ ID NO:4-7 (X<sub>11</sub>) is an alanine or a glutamic acid, such as an alanine or it can be deleted. In specific embodiments, the amino acid residues at positions 9 and 11 in SEQ ID NO: 7 are alanine and lysine/glutamic acid respectively, while the amino acid residue at position 43 is alanine or glutamic acid.

In certain embodiments the amino acid residue at the position corresponding to position 28 in SEQ ID NO:4-7 (X<sub>5</sub>) is an alanine or an asparagine, such as an alanine.

15 In some embodiments the amino acid residue at the position corresponding to position 40 in SEQ ID NO:4-7 (X<sub>9</sub>) is selected from the group consisting of asparagine, alanine, glutamic acid and valine, or from the group consisting of glutamic acid and valine, or valine, or it can be deleted. In specific embodiments, the amino acid residues at positions 9 and 11 in SEQ ID NO: 7 are alanine and glutamic acid respectively, while the amino acid residue at position 40 is valine.  
20 Optionally, the amino acid residue at position 43 may then be alanine or glutamic acid.

In certain embodiments, the amino acid residue at the position corresponding to position 42 in SEQ ID NO:4-7 (X<sub>10</sub>) is an alanine, lysine or arginine or it can be deleted.

In some embodiments the amino acid residue at the position corresponding to position 18 in SEQ ID NO:4-7 (X<sub>3</sub>) is a lysine or a histidine, such as a lysine.

25 In certain embodiments the amino acid residue at the position corresponding to position 33 in SEQ ID NO:4-7 (X<sub>7</sub>) is a lysine or a serine, such as a lysine.

In some embodiments the amino acid residue at the position corresponding to position 37 in SEQ ID NO:4-7 (X<sub>8</sub>) is a glutamic acid or an aspartic acid, such as a glutamic acid.

In certain embodiments the amino acid residue at the position corresponding to position 51 in SEQ ID NO:4-7 (X<sub>14</sub>) is a tyrosine or a leucine, such as a tyrosine.

In some embodiments, the amino acid residue at the position corresponding to position 44 in SEQ ID NO:4-7 (X<sub>12</sub>) is a leucine or an isoleucine. In specific embodiments, the amino acid residues at positions 9 and 11 in SEQ ID NO: 7 are alanine and lysine/glutamic acid respectively, while the amino acid residue at position 44 is isoleucine. Optionally, the amino acid residue at position 43 may then be alanine or glutamic acid.

In some embodiments, the amino acid residues at the positions corresponding to positions 1, 2, 3 and 4 or to positions 3, 4, 5 and 6 in SEQ ID NO: 4-7 have been deleted. In specific variants of these embodiments, the parental polypeptide is the C domain of Protein A (SEQ ID NO: 5). The effects of these deletions on the native C domain are described in US9018305 and US8329860, which are hereby incorporated by reference in their entireties.

In certain embodiments, the mutation in SEQ ID NO 4-7, such as in SEQ ID NO 7, is selected from the group consisting of: N11K; N11E; N11Y; N11T; N11F; N11L; N11W; N11I; N11M; N11V; N11A; N11H; N11R; N11E,Q32A; N11E,Q32E,Q40E; N11E,Q32E,K50R; Q9A,N11E,N43A; Q9A,N11E,N28A,N43A; Q9A,N11E,Q40V,A42K,N43E,L44I; Q9A,N11E,Q40V,A42K,N43A,L44I; N11K,H18K,S33K,D37E,A42R,N43A,L44I,K50R,L51Y; Q9A,N11E,N28A,Q40V,A42K,N43A,L44I; Q9A,N11K,H18K,S33K,D37E,A42R,N43A,L44I,K50R,L51Y; N11K, H18K, D37E, A42R, N43A, L44I; Q9A, N11K, H18K, D37E, A42R, N43A, L44I; Q9A, N11K, H18K, D37E, A42R, N43A, L44I, K50R; Q9A,N11K,H18K,D37E,A42R; Q9A,N11E,D37E,Q40V,A42K,N43A,L44I and Q9A,N11E,D37E,Q40V,A42R,N43A,L44I. These mutations provide particularly high alkaline stabilities. The mutation in SEQ ID NO 4-7, such as in SEQ ID NO 7, can also be selected from the group consisting of N11K; N11Y; N11F; N11L; N11W; N11I; N11M; N11V; N11A; N11H; N11R; Q9A,N11E,N43A; Q9A,N11E,N28A,N43A; Q9A,N11E,Q40V,A42K,N43E,L44I; Q9A,N11E,Q40V,A42K,N43A,L44I; Q9A,N11E,N28A,Q40V,A42K,N43A,L44I; N11K,H18K,S33K,D37E,A42R,N43A,L44I,K50R,L51Y; Q9A,N11K,H18K,S33K,D37E,A42R,N43A,L44I,K50R,L51Y; N11K, H18K, D37E, A42R, N43A, L44I; Q9A, N11K, H18K, D37E, A42R, N43A, L44I and Q9A, N11K, H18K, D37E, A42R, N43A, L44I, K50R.

In some embodiments, the polypeptide comprises or consists essentially of a sequence defined by or having at least 90%, 95% or 98% identity to an amino acid sequence selected from the group consisting of: SEQ ID NO 8, SEQ ID NO 9, SEQ ID NO 10, SEQ ID NO 11, SEQ ID NO 12, SEQ ID NO 13, SEQ ID NO 14, SEQ ID NO 15, SEQ ID NO 16, SEQ ID NO 23, SEQ ID NO 24, SEQ ID NO 25, SEQ ID NO 26, SEQ ID NO 27, SEQ ID NO 28, SEQ ID NO 29, SEQ ID NO 36, SEQ ID NO 37, SEQ ID NO 38, SEQ ID NO 39, SEQ ID NO 40, SEQ ID NO 41, SEQ ID NO 42, SEQ ID NO 43, SEQ ID NO 44, SEQ ID NO 45, SEQ ID NO 46, SEQ ID NO 47, SEQ ID NO 48, SEQ ID NO 49 and SEQ ID NO 50. It may e.g. comprise or consist essentially of a sequence defined by or having at least 90%, 95% or 98% identity to an amino acid sequence selected from the group consisting of: SEQ ID NO 8, SEQ ID NO 9, SEQ ID NO 10, SEQ ID NO 11, SEQ ID NO 16, SEQ ID NO 23, SEQ ID NO 24, SEQ ID NO 25, SEQ ID NO 26, SEQ ID NO 27, SEQ ID NO 28 and SEQ ID NO 29. It can also comprise or consist essentially of a sequence defined by or having at least 90%, 95% or 98% identity to an amino acid sequence selected from the group consisting of: SEQ ID NO 8, SEQ ID NO 9, SEQ ID NO 10, SEQ ID NO 11, SEQ ID NO 16, SEQ ID NO 23, SEQ ID NO 24, SEQ ID NO 25, SEQ ID NO 27, SEQ ID NO 28, SEQ ID NO 38, SEQ ID NO 40; SEQ ID NO 41; SEQ ID NO 42; SEQ ID NO 43, SEQ ID NO 44, SEQ ID NO 45, SEQ ID NO 46, SEQ ID NO 47 and SEQ ID NO 48.

In certain embodiments, the polypeptide comprises or consists essentially of a sequence defined by or having at least 90%, 95% or 98% identity to an amino acid sequence selected from the group consisting of SEQ ID NO 54-70; comprises or consists essentially of a sequence defined by or having at least 90%, 95% or 98% identity to an amino acid sequence selected from the group consisting of SEQ ID NO 71-75; or it may comprise or consist essentially of a sequence defined by or having at least 90%, 95% or 98% identity to an amino acid sequence selected from the group consisting of SEQ ID NO 76-79. It may further comprise or consist essentially of a sequence defined by or having at least 90%, 95% or 98% identity to an amino acid sequence selected from the group consisting of SEQ ID NO 89-95.

The polypeptide may e.g. be defined by a sequence selected from the groups above or from subsets of these groups, but it may also comprise additional amino acid residues at the N- and/or C-terminal end, e.g. a leader sequence at the N-terminal end and/or a tail sequence at the C-terminal end.

SEQ ID NO 8 Zvar(Q9A,N11E,N43A)

VDAKFDKE**AQ** EAFYEILHLP NLTEEQRNAF IQSLKDDPSQ SA**ALL**AEAKK LND**AQ**APK

SEQ ID NO 9 Zvar(Q9A,N11E,N28A,N43A)

VDAKFDKE**AQ** EAFYEILHLP NLTEEQR**AAF** IQSLKDDPSQ SA**ALL**AEAKK LND**AQ**APK

5

SEQ ID NO 10 Zvar(Q9A,N11E,Q40V,A42K,N43E,L44I)

VDAKFDKE**AQ** EAFYEILHLP NLTEEQRNAF IQSLKDDPS**V** S**KEIL**AEAKK LND**AQ**APK

SEQ ID NO 11 Zvar(Q9A,N11E,Q40V,A42K,N43A,L44I)

10 VDAKFDKE**AQ** EAFYEILHLP NLTEEQRNAF IQSLKDDPS**V** S**KAIL**AEAKK LND**AQ**APK

SEQ ID NO 12 Zvar(N11E,Q32A)

VDAKFDKE**QQ** EAFYEILHLP NLTEEQRNAF I**ASL**KDDPSQ SAN**LLA**AEAKK LND**AQ**APK

15 SEQ ID NO 13 Zvar(N11E)

VDAKFDKE**QQ** EAFYEILHLP NLTEEQRNAF IQSLKDDPSQ SAN**LLA**AEAKK LND**AQ**APK

SEQ ID NO 14 Zvar(N11E,Q32E,Q40E)

VDAKFDKE**QQ** EAFYEILHLP NLTEEQRNAF I**ESL**KDDP**SE** SAN**LLA**AEAKK LND**AQ**APK

20

SEQ ID NO 15 Zvar(N11E,Q32E,K50R)

VDAKFDKE**QQ** EAFYEILHLP NLTEEQRNAF I**ESL**KDDPSQ SAN**LLA**EA**KR** LND**AQ**APK

SEQ ID NO 16 Zvar(N11K)

25 VDAKFDKE**QQ** **KAF**YEILHLP NLTEEQRNAF IQSLKDDPSQ SAN**LLA**AEAKK LND**AQ**APK

SEQ ID NO 23 Zvar(N11K,H18K,S33K,D37E,A42R,N43A,L44I,K50R,L51Y)

VDAKFDKE**QQ** **KAF**YEIL**KLP** NLTEEQRNAF IQ**KLK**DEPSQ S**RAIL**AEAK**R** YND**AQ**APK

30 SEQ ID NO 24 Zvar(Q9A,N11E,N28A,Q40V,A42K,N43A,L44I)

VDAKFDKE**AQ** EAFYEILHLP NLTEEQR**AAF** IQSLKDDPS**V** S**KAIL**AEAKK LND**AQ**APK

SEQ ID NO 25 Zvar(Q9A,N11K,H18K,S33K,D37E,A42R,N43A,L44I,K50R,L51Y)

VDAKFDKEAQ KAFYEILKLP NLTEEQRAAF IQKLKDEPSQ SRAILAEAKR YNDAQAPK

SEQ ID NO 26 Zvar(N11K, H18K, D37E, A42R, N43A, L44I)

5 VDAKFDKEQQ KAFYEILKLP NLTEEQRNAF IQSLKDEPSQ SRAILAEAKK LNDAAQAPK

SEQ ID NO 27 Zvar(Q9A, N11K, H18K, D37E, A42R, N43A, L44I)

VDAKFDKEAQ KAFYEILKLP NLTEEQRNAF IQSLKDEPSQ SRAILAEAKK LNDAAQAPK

10 SEQ ID NO 28 Zvar(Q9A, N11K, H18K, D37E, A42R, N43A, L44I, K50R)

VDAKFDKEAQ KAFYEILKLP NLTEEQRNAF IQSLKDEPSQ SRAILAEAKR LNDAAQAPK

SEQ ID NO 29 Zvar(Q9A,N11K,H18K,D37E,A42R)

VDAKFDKEAQ KAFYEILKLP NLTEEQRNAF IQSLKDEPSQ SRNLLAEAKK LNDAAQAPK

15

SEQ ID NO 36 B(Q9A,N11E,Q40V,A42K,N43A,L44I)

ADNKFNKEAQ EAFYEILHLP NLNEEQRNGF IQSLKDDPSV SKAILAEAKK LNDAAQAPK

SEQ ID NO 37 C(Q9A,N11E,E43A)

20 ADNKFNKEAQ EAFYEILHLP NLTEEQRNGF IQSLKDDPSV SKAILAEAKK LNDAAQAPK

SEQ ID NO 38 Zvar(N11Y)

VDAKFDKEQQ YAFYEILHLP NLTEEQRNAF IQSLKDDPSQ SANLLAEAKK LNDAAQAPK

25 SEQ ID NO 39 Zvar(N11T)

VDAKFDKEQQ TAFYEILHLP NLTEEQRNAF IQSLKDDPSQ SANLLAEAKK LNDAAQAPK

SEQ ID NO 40 Zvar(N11F)

VDAKFDKEQQ FAFYEILHLP NLTEEQRNAF IQSLKDDPSQ SANLLAEAKK LNDAAQAPK

30

SEQ ID NO 41 Zvar(N11L)

VDAKFDKEQQ LAFYEILHLP NLTEEQRNAF IQSLKDDPSQ SANLLAEAKK LNDAAQAPK

SEQ ID NO 42 Zvar(N11W)

VDAKFDKEQQ **W**AFYEILHLP NLTEEQRNAF IQSLKDDPSQ SANLLAEAKK LNDAAQAPK

5 SEQ ID NO 43 Zvar(N11I)

VDAKFDKEQQ **I**AFYEILHLP NLTEEQRNAF IQSLKDDPSQ SANLLAEAKK LNDAAQAPK

SEQ ID NO 44 Zvar(N11M)

VDAKFDKEQQ **M**AFYEILHLP NLTEEQRNAF IQSLKDDPSQ SANLLAEAKK LNDAAQAPK

10

SEQ ID NO 45 Zvar(N11V)

VDAKFDKEQQ **V**AFYEILHLP NLTEEQRNAF IQSLKDDPSQ SANLLAEAKK LNDAAQAPK

SEQ ID NO 46 Zvar(N11A)

15 VDAKFDKEQQ **A**AFYEILHLP NLTEEQRNAF IQSLKDDPSQ SANLLAEAKK LNDAAQAPK

SEQ ID NO 47 Zvar(N11H)

VDAKFDKEQQ **H**AFYEILHLP NLTEEQRNAF IQSLKDDPSQ SANLLAEAKK LNDAAQAPK

20 SEQ ID NO 48 Zvar(N11R)

VDAKFDKEQQ **R**AFYEILHLP NLTEEQRNAF IQSLKDDPSQ SANLLAEAKK LNDAAQAPK

SEQ ID NO 49 Zvar(Q9A,N11E,D37E,Q40V,A42K,N43A,L44I)

VDAKFDKE**A**Q **E**AFYEILHLP NLTEEQRNAF IQSLKDE**P**SV **S**KAILAEAKK LNDAAQAPK

25

SEQ ID NO 50 Zvar(Q9A,N11E,D37E,Q40V,A42R,N43A,L44I)

VDAKFDKE**A**Q **E**AFYEILHLP NLTEEQRNAF IQSLKDE**P**SV **S**RAILAEAKK LNDAAQAPK

SEQ ID NO 54 Zvar(Q9A,N11E, A29G,Q40V,A42K,N43A,L44I)

30 VDAKFDKE**A**Q **E**AFYEILHLP NLTEEQR**N**GF IQSLKDDPSV **S**KAILAEAKK LNDAAQAPK

SEQ ID NO 55 Zvar(Q9A,N11E, A29S,Q40V,A42K,N43A,L44I)

VDAKFDKE**AAQ** EAFYEILHLP NLTEEQRNS**F** IQSLKDDPS**V** **SKAILAEAKK** LNDAQAPK

SEQ ID NO 56 Zvar(Q9A,N11E, A29Y,Q40V,A42K,N43A,L44I)

VDAKFDKE**AAQ** EAFYEILHLP NLTEEQRNY**F** IQSLKDDPS**V** **SKAILAEAKK** LNDAQAPK

5

SEQ ID NO 57 Zvar(Q9A,N11E, A29Q,Q40V,A42K,N43A,L44I)

VDAKFDKE**AAQ** EAFYEILHLP NLTEEQRN**QF** IQSLKDDPS**V** **SKAILAEAKK** LNDAQAPK

SEQ ID NO 58 Zvar(Q9A,N11E, A29T,Q40V,A42K,N43A,L44I)

10 VDAKFDKE**AAQ** EAFYEILHLP NLTEEQRNT**F** IQSLKDDPS**V** **SKAILAEAKK** LNDAQAPK

SEQ ID NO 59 Zvar(Q9A,N11E, A29N,Q40V,A42K,N43A,L44I)

VDAKFDKE**AAQ** EAFYEILHLP NLTEEQRN**NF** IQSLKDDPS**V** **SKAILAEAKK** LNDAQAPK

15 SEQ ID NO 60 Zvar(Q9A,N11E, A29F,Q40V,A42K,N43A,L44I)

VDAKFDKE**AAQ** EAFYEILHLP NLTEEQRN**FF** IQSLKDDPS**V** **SKAILAEAKK** LNDAQAPK

SEQ ID NO 61 Zvar(Q9A,N11E, A29L,Q40V,A42K,N43A,L44I)

VDAKFDKE**AAQ** EAFYEILHLP NLTEEQRN**LF** IQSLKDDPS**V** **SKAILAEAKK** LNDAQAPK

20

SEQ ID NO 62 Zvar(Q9A,N11E, A29W,Q40V,A42K,N43A,L44I)

VDAKFDKE**AAQ** EAFYEILHLP NLTEEQRN**WF** IQSLKDDPS**V** **SKAILAEAKK** LNDAQAPK

SEQ ID NO 63 Zvar(Q9A,N11E, A29I,Q40V,A42K,N43A,L44I)

25 VDAKFDKE**AAQ** EAFYEILHLP NLTEEQRN**IF** IQSLKDDPS**V** **SKAILAEAKK** LNDAQAPK

SEQ ID NO 64 Zvar(Q9A,N11E, A29M,Q40V,A42K,N43A,L44I)

VDAKFDKE**AAQ** EAFYEILHLP NLTEEQRN**MF** IQSLKDDPS**V** **SKAILAEAKK** LNDAQAPK

30 SEQ ID NO 65 Zvar(Q9A,N11E, A29V,Q40V,A42K,N43A,L44I)

VDAKFDKE**AAQ** EAFYEILHLP NLTEEQRN**VF** IQSLKDDPS**V** **SKAILAEAKK** LNDAQAPK

SEQ ID NO 66 Zvar(Q9A,N11E, A29D,Q40V,A42K,N43A,L44I)

VDAKFDKEAQ EAFYEILHLP NLTEEQRNDF IQSLKDDPSV SKAILAEAKK LNDAQAPK

SEQ ID NO 67 Zvar(Q9A,N11E, A29E,Q40V,A42K,N43A,L44I)

5 VDAKFDKEAQ EAFYEILHLP NLTEEQRNEF IQSLKDDPSV SKAILAEAKK LNDAQAPK

SEQ ID NO 68 Zvar(Q9A,N11E, A29H,Q40V,A42K,N43A,L44I)

VDAKFDKEAQ EAFYEILHLP NLTEEQRNHF IQSLKDDPSV SKAILAEAKK LNDAQAPK

10 SEQ ID NO 69 Zvar(Q9A,N11E, A29R,Q40V,A42K,N43A,L44I)

VDAKFDKEAQ EAFYEILHLP NLTEEQRNR F IQSLKDDPSV SKAILAEAKK LNDAQAPK

SEQ ID NO 70 Zvar(Q9A,N11E, A29K,Q40V,A42K,N43A,L44I)

VDAKFDKEAQ EAFYEILHLP NLTEEQRNKF IQSLKDDPSV SKAILAEAKK LNDAQAPK

15

SEQ ID NO 71 Zvar(Q9A,N11E, Q40V,A42K,N43A,L44I,D53F)

VDAKFDKEAQ EAFYEILHLP NLTEEQRNAF IQSLKDDPSV SKAILAEAKK LNFAQAPK

SEQ ID NO 72 Zvar(Q9A,N11E, Q40V,A42K,N43A,L44I,D53Y)

20 VDAKFDKEAQ EAFYEILHLP NLTEEQRNAF IQSLKDDPSV SKAILAEAKK LNYAQAPK

SEQ ID NO 73 Zvar(Q9A,N11E, Q40V,A42K,N43A,L44I,D53W)

VDAKFDKEAQ EAFYEILHLP NLTEEQRNAF IQSLKDDPSV SKAILAEAKK LNWAQAPK

25 SEQ ID NO 74 Zvar(Q9A,N11E, Q40V,A42K,N43A,L44I,D53K)

VDAKFDKEAQ EAFYEILHLP NLTEEQRNAF IQSLKDDPSV SKAILAEAKK LNKAQAPK

SEQ ID NO 75 Zvar(Q9A,N11E, Q40V,A42K,N43A,L44I,D53R)

VDAKFDKEAQ EAFYEILHLP NLTEEQRNAF IQSLKDDPSV SKAILAEAKK LNRAQAPK

30

SEQ ID NO 76 Zvar(Q9del,N11E, Q40V,A42K,N43A,L44I)

VDAKFDKE\_Q EAFYEILHLP NLTEEQRNAF IQSLKDDPSV SKAILAEAKK LNDAQAPK

SEQ ID NO 77 Zvar(Q9A,N11E, Q40del,A42K,N43A,L44I)

VDAKFDKE**AQ** EAFYEILHLP NLTEEQRNAF IQSLKDDPS\_ **SK**ILAEAKK LNDQAQPK

SEQ ID NO 78 Zvar(Q9A,N11E, Q40V,A42del,N43A,L44I)

5 VDAKFDKE**AQ** EAFYEILHLP NLTEEQRNAF IQSLKDDPSV S\_**A**ILAEAKK LNDQAQPK

SEQ ID NO 79 Zvar(Q9A,N11E, Q40V,A42K,N43del,L44I)

VDAKFDKE**AQ** EAFYEILHLP NLTEEQRNAF IQSLKDDPSV **SK**\_ILAEAKK LNDQAQPK

10 SEQ ID NO 89 Zvar(D2del,A3del,K4del,Q9A,N11E,Q40V,A42K,N43A,L44I)

V\_\_\_FDKE**AQ** EAFYEILHLP NLTEEQRNAF IQSLKDDPSV **SK**ILAEAKK LNDQAQPK

SEQ ID NO 90 Zvar(V1del,D2del,Q9A,N11E,Q40V,A42K,N43A,L44I,K58del)

\_\_\_AKFDKE**AQ** EAFYEILHLP NLTEEQRNAF IQSLKDDPSV **SK**ILAEAKK LNDQAQPK\_

15

SEQ ID NO 91 Zvar(K4del,F5del,D6del,K7del,E8del,Q9A,N11E,Q40V,A42K,N43A,L44I)

VDA\_\_\_\_\_AQ EAFYEILHLP NLTEEQRNAF IQSLKDDPSV **SK**ILAEAKK LNDQAQPK

SEQ ID NO 92 Zvar(Q9A,N11E,Q40V,A42K,N43A,L44I,A56del,P57del,K58del)

20 VDAKFDKE**AQ** EAFYEILHLP NLTEEQRNAF IQSLKDDPSV **SK**ILAEAKK LNDQAQ\_\_\_

SEQ ID NO 93 Zvar(V1del,,D2del,A3del,Q9A,N11E,Q40V,A42K,N43A,L44I)

\_\_\_KFDKE**AQ** EAFYEILHLP NLTEEQRNAF IQSLKDDPSV **SK**ILAEAKK LNDQAQPK

25 SEQ ID NO 94

Zvar(V1del,D2del,A3del,K4del,F5del,D6del,K7del,E8del,Q9A,N11E,Q40V,A42K,N43A,L44I)

\_\_\_\_\_AQ EAFYEILHLP NLTEEQRNAF IQSLKDDPSV **SK**ILAEAKK LNDQAQPK

SEQ ID NO 95 Zvar(Q9A,N11E,Q40V,A42K,N43A,L44I,K58\_insYEDG)

30 VDAKFDKE**AQ** EAFYEILHLP NLTEEQRNAF IQSLKDDPSV **SK**ILAEAKK LNDQAQPKYE **DG**

The separation matrix comprises multimers of the immunoglobulin-binding alkali-stabilized Protein A domains. Such multimers comprise, consist essentially of, or consist of a plurality of immunoglobulin-binding alkali-stabilized Protein A domains (polypeptide units) as defined by any embodiment disclosed above. The use of multimers may increase the immunoglobulin binding capacity and multimers may also have a higher alkali stability than monomers. The multimer can e.g. be a dimer, a trimer, a tetramer, a pentamer, a hexamer, a heptamer, an octamer or a nonamer. The multimer may be a homomultimer, where all the units in the multimer are identical or it can be a heteromultimer, where at least one unit differs from the others. Advantageously, all the units in the multimer are alkali stable, such as by comprising the mutations/conservations disclosed above. The polypeptides can be linked to each other directly by peptide bonds between the C-terminal and N-terminal ends of the polypeptides.

Alternatively, two or more units in the multimer can be linked by linkers comprising oligomeric or polymeric species, such as linkers comprising peptides with up to 25 or 30 amino acids, such as 3-25 or 3-20 amino acids. The linkers may e.g. comprise or consist essentially of a peptide sequence defined by, or having at least 90% identity or at least 95% identity, with an amino acid sequence selected from the group consisting of APKVDAKFDKE, APKVDNKFNKE, APKADNKFNKE, APKVFDKE, APAKFDKE, AKFDKE, APKVDA, VDAKFDKE, APKKFDKE, APK, APKYEDGVDAKFDKE and YEDG or alternatively selected from the group consisting of APKADNKFNKE, APKVFDKE, APAKFDKE, AKFDKE, APKVDA, VDAKFDKE, APKKFDKE, APKYEDGVDAKFDKE and YEDG. They can also consist essentially of a peptide sequence defined by or having at least 90% identity or at least 95% identity with an amino acid sequence selected from the group consisting of APKADNKFNKE, APKVFDKE, APAKFDKE, AKFDKE, APKVDA, VDAKFDKE, APKKFDKE, APK and APKYEDGVDAKFDKE. In some embodiments the linkers do not consist of the peptides APKVDAKFDKE or APKVDNKFNKE, or alternatively do not consist of the peptides APKVDAKFDKE, APKVDNKFNKE, APKFNKE, APKFDKE, APKVDKE or APKADKE.

The nature of such a linker should preferably not destabilize the spatial conformation of the protein units. This can e.g. be achieved by avoiding the presence of proline in the linkers. Furthermore, said linker should preferably also be sufficiently stable in alkaline environments not to impair the properties of the mutated protein units. For this purpose, it is advantageous if the linkers do not contain asparagine. It can additionally be advantageous if the linkers do not contain glutamine. The multimer may further at the N-terminal end comprise a plurality of

amino acid residues e.g. originating from the cloning process or constituting a residue from a cleaved off signaling sequence. The number of additional amino acid residues may e.g. be 20 or less, such as 15 or less, such as 10 or less or 5 or less. As a specific example, the multimer may comprise an AQ, AQGT, VDAKFDKE, AQVDAKFDKE or AQGTVDAKFDKE sequence at the N-terminal end.

In certain embodiments, the multimer may comprise, or consist essentially, of a sequence selected from the group consisting of: SEQ ID NO 80-87. These and additional sequences are listed below and named as Parent(Mutations)<sub>n</sub>, where n is the number of monomer units in a multimer.

10 SEQ ID NO 17 Zvar(Q9A,N11E,N43A)<sub>4</sub>

AQGT VDAKFDKEAQ EAFYEILHLP NLTEEQRNAF IQSLKDDPSQ SAALLAEAKK LNDAQAPK  
VDAKFDKEAQ EAFYEILHLP NLTEEQRNAF IQSLKDDPSQ SAALLAEAKK LNDAQAPK VDAKFDKEAQ  
EAFYEILHLP NLTEEQRNAF IQSLKDDPSQ SAALLAEAKK LNDAQAPK VDAKFDKEAQ EAFYEILHLP  
NLTEEQRNAF IQSLKDDPSQ SAALLAEAKK LNDAQAPKC

15

SEQ ID NO 18 Zvar(Q9A,N11E,N28A,N43A)<sub>4</sub>

AQGT VDAKFDKEAQ EAFYEILHLP NLTEEQRAAF IQSLKDDPSQ SAALLAEAKK LNDAQAPK  
VDAKFDKEAQ EAFYEILHLP NLTEEQRAAF IQSLKDDPSQ SAALLAEAKK LNDAQAPK VDAKFDKEAQ  
EAFYEILHLP NLTEEQRAAF IQSLKDDPSQ SAALLAEAKK LNDAQAPK VDAKFDKEAQ EAFYEILHLP  
20 NLTEEQRAAF IQSLKDDPSQ SAALLAEAKK LNDAQAPKC

SEQ ID NO 19 Zvar(Q9A,N11E,Q40V,A42K,N43E,L44I)<sub>4</sub>

AQGT VDAKFDKEAQ EAFYEILHLP NLTEEQRNAF IQSLKDDPSV SKEILAEAKK LNDAQAPK  
VDAKFDKEAQ EAFYEILHLP NLTEEQRNAF IQSLKDDPSV SKEILAEAKK LNDAQAPK VDAKFDKEAQ  
25 EAFYEILHLP NLTEEQRNAF IQSLKDDPSV SKEILAEAKK LNDAQAPK VDAKFDKEAQ EAFYEILHLP  
NLTEEQRNAF IQSLKDDPSV SKEILAEAKK LNDAQAPKC

SEQ ID NO 20 Zvar(Q9A,N11E,Q40V,A42K,N43A,L44I)<sub>4</sub>

AQGT VDAKFDKEAQ EAFYEILHLP NLTEEQRNAF IQSLKDDPSV SKAILAEAKK LNDAQAPK  
30 VDAKFDKEAQ EAFYEILHLP NLTEEQRNAF IQSLKDDPSV SKAILAEAKK LNDAQAPK VDAKFDKEAQ  
EAFYEILHLP NLTEEQRNAF IQSLKDDPSV SKAILAEAKK LNDAQAPK VDAKFDKEAQ EAFYEILHLP

NLTEEQRNAF IQSLKDDPSV SKAILAEAKK LNDAAQAPKC

SEQ ID NO 30 Zvar(N11K,H18K,S33K,D37E,A42R,N43A,L44I,K50R,L51Y)4

AQGT VDAKFDKEQQ KAFYEILKLP NLTEEQRNAF IQKLKDEPSQ SRAILAEAKR YNDAQAPK

- 5 VDAKFDKEQQ KAFYEILKLP NLTEEQRNAF IQKLKDEPSQ SRAILAEAKR YNDAQAPK VDAKFDKEQQ  
KAFYEILKLP NLTEEQRNAF IQKLKDEPSQ SRAILAEAKR YNDAQAPK VDAKFDKEQQ KAFYEILKLP  
NLTEEQRNAF IQKLKDEPSQ SRAILAEAKR YNDAQAPKC

SEQ ID NO 31 Zvar(Q9A,N11K,H18K,D37E,A42R)4

- 10 AQGT VDAKFDKEAQ KAFYEILKLP NLTEEQRNAF IQSLKDEPSQ SRNLLAEAKK LNDAAQAPK  
VDAKFDKEAQ KAFYEILKLP NLTEEQRNAF IQSLKDEPSQ SRNLLAEAKK LNDAAQAPK VDAKFDKEAQ  
KAFYEILKLP NLTEEQRNAF IQSLKDEPSQ SRNLLAEAKK LNDAAQAPK VDAKFDKEAQ KAFYEILKLP  
NLTEEQRNAF IQSLKDEPSQ SRNLLAEAKK LNDAAQAPKC

- 15 SEQ ID NO 32 Zvar(Q9A,N11E,N28A,Q40V,A42K,N43A,L44I)4

AQGT VDAKFDKEAQ EAFYEILHLP NLTEEQRAAF IQSLKDDPSV SKAILAEAKK LNDAAQAPK  
VDAKFDKEAQ EAFYEILHLP NLTEEQRAAF IQSLKDDPSV SKAILAEAKK LNDAAQAPK VDAKFDKEAQ  
EAFYEILHLP NLTEEQRAAF IQSLKDDPSV SKAILAEAKK LNDAAQAPK VDAKFDKEAQ EAFYEILHLP  
NLTEEQRAAF IQSLKDDPSV SKAILAEAKK LNDAAQAPKC

20

SEQ ID NO 33 Zvar(Q9A,N11E,Q40V,A42K,N43A,L44I)6

AQGT VDAKFDKEAQ EAFYEILHLP NLTEEQRNAF IQSLKDDPSV SKAILAEAKK LNDAAQAPK  
VDAKFDKEAQ EAFYEILHLP NLTEEQRNAF IQSLKDDPSV SKAILAEAKK LNDAAQAPK VDAKFDKEAQ  
EAFYEILHLP NLTEEQRNAF IQSLKDDPSV SKAILAEAKK LNDAAQAPK VDAKFDKEAQ EAFYEILHLP  
25 NLTEEQRNAF IQSLKDDPSV SKAILAEAKK LNDAAQAPK VDAKFDKEAQ EAFYEILHLP NLTEEQRNAF  
IQSLKDDPSV SKAILAEAKK LNDAAQAPK VDAKFDKEAQ EAFYEILHLP NLTEEQRNAF IQSLKDDPSV  
SKAILAEAKK LNDAAQAPKC

SEQ ID NO 34 Zvar(Q9A,N11E,D37E,Q40V,A42K,N43A,L44I)4

- 30 AQGT VDAKFDKEAQ EAFYEILHLP NLTEEQRNAF IQSLKDEPSV SKAILAEAKK LNDAAQAPK  
VDAKFDKEAQ EAFYEILHLP NLTEEQRNAF IQSLKDEPSV SKAILAEAKK LNDAAQAPK VDAKFDKEAQ

EA FYEILHLP NLTEEQRNAF IQSLKDEPSV **SKAILAEAKK** LND AQAPK VDAKFDKEAQ EAFYEILHLP  
NLTEEQRNAF IQSLKDEPSV **SKAILAEAKK** LND AQAPKC

SEQ ID NO 35 Zvar(Q9A,N11E,D37E,Q40V,A42R,N43A,L44I)4

- 5 AQGT VDAKFDKEAQ EAFYEILHLP NLTEEQRNAF IQSLKDEPSV **SRAILAEAKK** LND AQAPK  
VDAKFDKEAQ EAFYEILHLP NLTEEQRNAF IQSLKDEPSV **SRAILAEAKK** LND AQAPK VDAKFDKEAQ  
EAFYEILHLP NLTEEQRNAF IQSLKDEPSV **SRAILAEAKK** LND AQAPK VDAKFDKEAQ EAFYEILHLP  
NLTEEQRNAF IQSLKDEPSV **SRAILAEAKK** LND AQAPKC

- 10 SEQ ID NO 80 Zvar(Q9A,N11E,Q40V,A42K,N43A,L44I)2 with D2, A3 and K4 in linker deleted  
VDAKFDKEAQ EAFYEILHLP NLTEEQRNAF IQSLKDDPSV **SKAILAEAKK** LND **AQAPK VFDKEAQ**  
EAFYEILHLP NLTEEQRNAF IQSLKDDPSV **SKAILAEAKK** LND AQAPKC

SEQ ID NO 81 Zvar(Q9A,N11E,Q40V,A42K,N43A,L44I)2 with K58, V1 and D2 in linker deleted

- 15 VDAKFDKEAQ EAFYEILHLP NLTEEQRNAF IQSLKDDPSV **SKAILAEAKK** LND **AQAP AKFDKEAQ**  
EAFYEILHLP NLTEEQRNAF IQSLKDDPSV **SKAILAEAKK** LND AQAPKC

SEQ ID NO 82 Zvar(Q9A,N11E,Q40V,A42K,N43A,L44I)2 with P57, K58, V1, D2 and A3 in linker  
deleted

- 20 VDAKFDKEAQ EAFYEILHLP NLTEEQRNAF IQSLKDDPSV **SKAILAEAKK** LND **AQAP AKFDKEAQ**  
EAFYEILHLP NLTEEQRNAF IQSLKDDPSV **SKAILAEAKK** LND AQAPKC

SEQ ID NO 83 Zvar(Q9A,N11E,Q40V,A42K,N43A,L44I)2 with K4, F5, D6, K7 and E8 in linker  
deleted

- 25 VDAKFDKEAQ EAFYEILHLP NLTEEQRNAF IQSLKDDPSV **SKAILAEAKK** LND **AQAPK VDAAQ**  
EAFYEILHLP NLTEEQRNAF IQSLKDDPSV **SKAILAEAKK** LND AQAPKC

SEQ ID NO 84 Zvar(Q9A,N11E,Q40V,A42K,N43A,L44I)2 with A56, P57 and K58 in linker deleted

- 30 VDAKFDKEAQ EAFYEILHLP NLTEEQRNAF IQSLKDDPSV **SKAILAEAKK** LND **AQ VDAKFDKEAQ**  
EAFYEILHLP NLTEEQRNAF IQSLKDDPSV **SKAILAEAKK** LND AQAPKC

SEQ ID NO 85 Zvar(Q9A,N11E,Q40V,A42K,N43A,L44I)2 with V1, D2 and A3 in linker deleted

VDAKFDKEAQ EAFYEILHLP NLTEEQRNAF IQSLKDDPSV SKAILAEAKK LND**AQAPK** KFDKEAQ  
EAFYEILHLP NLTEEQRNAF IQSLKDDPSV SKAILAEAKK LND**AQAPK**C

SEQ ID NO 86 Zvar(Q9A,N11E,Q40V,A42K,N43A,L44I)<sub>2</sub> with V1, D2, A3, K4, F5, D6, K7 and E8 in  
5 linker deleted

VDAKFDKEAQ EAFYEILHLP NLTEEQRNAF IQSLKDDPSV SKAILAEAKK LND**AQAPK** AQ  
EAFYEILHLP NLTEEQRNAF IQSLKDDPSV SKAILAEAKK LND**AQAPK**C

SEQ ID NO 87 Zvar(Q9A,N11E,Q40V,A42K,N43A,L44I)<sub>2</sub> with YEDG inserted in linker between  
10 K58 and V1

VDAKFDKEAQ EAFYEILHLP NLTEEQRNAF IQSLKDDPSV SKAILAEAKK LND**AQAPK** YEDG  
**VDAKFDKEAQ** EAFYEILHLP NLTEEQRNAF IQSLKDDPSV SKAILAEAKK LND**AQAPK**C

SEQ ID NO 88 Zvar<sub>2</sub>

15 VDAKFDKEAQ EAFYEILHLP NLTEEQRNAF IQSLKDDPSV SKAILAEAKK LND**AQAPK** VDAKFDKEAQ  
EAFYEILHLP NLTEEQRNAF IQSLKDDPSV SKAILAEAKK LND**AQAPK**C

In some embodiments, the polypeptide and/or multimer, as disclosed above, further comprises  
at the C-terminal or N-terminal end one or more coupling elements, selected from the group  
20 consisting of one or more cysteine residues, a plurality of lysine residues and a plurality of  
histidine residues. The coupling element(s) may also be located within 1-5 amino acid residues,  
such as within 1-3 or 1-2 amino acid residues from the C-terminal or N-terminal end. The  
coupling element may e.g. be a single cysteine at the C-terminal end. The coupling element(s)  
may be directly linked to the C- or N-terminal end, or it/they may be linked via a stretch  
25 comprising up to 15 amino acids, such as 1-5, 1-10 or 5-10 amino acids. This stretch should  
preferably also be sufficiently stable in alkaline environments not to impair the properties of  
the mutated protein. For this purpose, it is advantageous if the stretch does not contain  
asparagine. It can additionally be advantageous if the stretch does not contain glutamine. An  
advantage of having a C-terminal cysteine is that endpoint coupling of the protein can be  
30 achieved through reaction of the cysteine thiol with an electrophilic group on a support. This  
provides excellent mobility of the coupled protein which is important for the binding capacity.

The alkali stability of the polypeptide or multimer can be assessed by coupling it to a surface plasmon resonance (SPR) chip, e.g. to Biacore CM5 sensor chips as described in the examples, using e.g. NHS- or maleimide coupling chemistries, and measuring the immunoglobulin-binding capacity of the chip, typically using polyclonal human IgG, before and after incubation in  
5 alkaline solutions at a specified temperature, e.g. 22 +/- 2 °C. The incubation can e.g. be performed in 0.5 M NaOH for a number of 10 min cycles, such as 100, 200 or 300 cycles. The IgG capacity of the matrix after 100 10 min incubation cycles in 0.5 M NaOH at 22 +/- 2 °C can be at least 55, such as at least 60, at least 80 or at least 90% of the IgG capacity before the incubation. Alternatively, the remaining IgG capacity after 100 cycles for a particular mutant  
10 measured as above can be compared with the remaining IgG capacity for the parental polypeptide/multimer. In this case, the remaining IgG capacity for the mutant may be at least 105%, such as at least 110%, at least 125%, at least 150% or at least 200% of the parental polypeptide/multimer.

The immunoglobulin-binding alkali-stabilized Protein A domains and/or multimers thereof may  
15 be encoded by a nucleic acid sequence, such as an RNA sequence or a DNA sequence encoding the polypeptide or multimer. A vector, such as a plasmid, which in addition to the coding sequence comprises the required signal sequences, may be used for expression of the polypeptide or multimer. The vector may comprise nucleic acid encoding a multimer as described above, wherein the separate nucleic acids encoding each unit may have homologous  
20 or heterologous DNA sequences.

An expression system, which comprises a nucleic acid or a vector as disclosed above, may be used for expression of the polypeptide or multimer. The expression system may e.g. be a gram-positive or gram-negative prokaryotic host cell system, e.g. *E.coli* or *Bacillus sp.* which has been modified to express the present polypeptide or multimer. Alternatively, the expression system  
25 may be a eukaryotic host cell system, such as a yeast, e.g. *Pichia pastoris* or *Saccharomyces cerevisiae*, or mammalian cells, e.g. CHO cells.

The separation matrix comprises, consists essentially of, or consists of multimers of immunoglobulin-binding alkali-stabilized Protein A domains as described above, covalently coupled to a porous support.

The separation matrix may comprise at least 11, such as 11-21, 15-21 or 15-18 mg/ml Fc-binding ligands covalently coupled to a porous support, wherein:

a) the ligands comprise multimers of alkali-stabilized Protein A domains,

b) the porous support comprises cross-linked polymer particles having a volume-weighted

5 median diameter ( $d_{50,v}$ ) of 56-70, such as 56-66, micrometers and a dry solids weight of 55-80, such as 60-78 or 65-78, mg/ml. The cross-linked polymer particles may further have a pore size corresponding to an inverse gel filtration chromatography  $K_d$  value of 0.69-0.85, such as 0.70-0.85 or 0.69-0.80, for dextran of Mw 110 kDa. The multimers may e.g. comprise tetramers, pentamers, hexamers or heptamers of alkali-stabilized Protein A domains, such as hexamers of  
10 alkali-stabilized Protein A domains. The combination of the high ligand contents with the particle size range, the dry solids weight range and the optional  $K_d$  range provides for a high binding capacity, e.g. such that the 10% breakthrough dynamic binding capacity for IgG is at least 45 mg/ml, such as at least 50 or at least 55 mg/ml at 2.4 min residence time. Alternatively, or additionally, the 10% breakthrough dynamic binding capacity for IgG may be at least 60  
15 mg/ml, such as at least 65, at least 70 or at least 75 mg/ml at 6 min residence time.

The alkali-stabilized Protein A domain multimers are highly selective for IgG and the separation matrix can suitably have a dissociation constant for human IgG2 of below 0.2 mg/ml, such as below 0.1 mg/ml, in 20 mM phosphate buffer, 180 mM NaCl, pH 7.5. This can be determined according to the adsorption isotherm method described in N Pakiman et al: J Appl Sci 12, 1136-  
20 1141 (2012).

In certain embodiments the separation matrix comprises at least 15, such as 15-21 or 15-18 mg/ml Fc-binding ligands covalently coupled to a porous support, wherein the ligands comprise multimers of alkali-stabilized Protein A domains. These multimers can suitably be as disclosed in any of the embodiments described above or as specified below.

25 In some embodiments the separation matrix comprises 5 – 25, such as 5-20 mg/ml, 5 – 15 mg/ml, 5 – 11 mg/ml or 6 – 11 mg/ml of the polypeptide or multimer coupled to the support. The amount of coupled polypeptide/multimer can be controlled by the concentration of polypeptide/multimer used in the coupling process, by the activation and coupling conditions used and/or by the pore structure of the support used. As a general rule the absolute binding  
30 capacity of the matrix increases with the amount of coupled polypeptide/multimer, at least up

to a point where the pores become significantly constricted by the coupled polypeptide/multimer. Without being bound by theory, it appears though that for the  $K_d$  values recited for the support, the constriction of the pores by coupled ligand is of lower significance. The relative binding capacity per mg coupled polypeptide/multimer will decrease at high  
5 coupling levels, resulting in a cost-benefit optimum within the ranges specified above.

Such a separation matrix is useful for separation of immunoglobulins or other Fc-containing proteins and, due to the improved alkali stability of the polypeptides/multimers, the matrix will withstand highly alkaline conditions during cleaning, which is essential for long-term repeated use in a bioprocess separation setting. The alkali stability of the matrix can be assessed by  
10 measuring the immunoglobulin-binding capacity, typically using polyclonal human IgG, before and after incubation in alkaline solutions at a specified temperature, e.g.  $22 \pm 2^\circ\text{C}$ . The incubation can e.g. be performed in 0.5 M or 1.0 M NaOH for a number of 15 min cycles, such as 100, 200 or 300 cycles, corresponding to a total incubation time of 25, 50 or 75 h. The IgG capacity of the matrix after 96-100 15 min incubation cycles or a total incubation time of 24 or  
15 25 h in 0.5 M NaOH at  $22 \pm 2^\circ\text{C}$  can be at least 80, such as at least 85, at least 90 or at least 95% of the IgG capacity before the incubation. The capacity of the matrix after a total incubation time of 24 h in 1.0 M NaOH at  $22 \pm 2^\circ\text{C}$  can be at least 70, such as at least 80 or at least 90% of the IgG capacity before the incubation. The the 10% breakthrough dynamic binding capacity ( $Q_{b10\%}$ ) for IgG at 2.4 min or 6 min residence time may e.g. be reduced by less than  
20 20 % after incubation 31 h in 1.0 M aqueous NaOH at  $22 \pm 2^\circ\text{C}$ .

As the skilled person will understand, the expressed polypeptide or multimer should be purified to an appropriate extent before being immobilized to a support. Such purification methods are well known in the field, and the immobilization of protein-based ligands to supports is easily carried out using standard methods. Suitable methods and supports will be discussed below in  
25 more detail.

The porous support of the separation matrix may be of any suitable well-known kind. A conventional affinity separation matrix is often of organic nature and based on polymers that expose a hydrophilic surface to the aqueous media used, i.e. expose hydroxy (-OH), carboxy (-COOH), carboxamido (-CONH<sub>2</sub>, possibly in N- substituted forms), amino (-NH<sub>2</sub>, possibly in  
30 substituted form), oligo- or polyethylenoxy groups on their external and, if present, also on

internal surfaces. The porosity of the support can be expressed as a  $K_{av}$  or  $K_d$  value (the fraction of the pore volume available to a probe molecule of a particular size) measured by inverse size exclusion chromatography, e.g. according to the methods described in Gel Filtration Principles and Methods, Pharmacia LKB Biotechnology 1991, pp 6-13.  $K_{av}$  is  
5 determined as the ratio  $(V_e - V_0)/(V_t - V_0)$ , where  $V_e$  is the elution volume of a probe molecule (e.g. Dextran 110 kD),  $V_0$  is the void volume of the column (e.g. the elution volume of a high Mw void marker, such as raw dextran) and  $V_t$  is the total volume of the column.  $K_d$  can be determined as  $(V_e - V_0)/V_i$ , where  $V_i$  is the elution volume of a salt (e.g. NaCl) able to access all the volume except the matrix volume (the volume occupied by the matrix polymer molecules).  
10 By definition, both  $K_d$  and  $K_{av}$  values always lie within the range 0 – 1. The  $K_{av}$  value can advantageously be 0.6 – 0.95, e.g. 0.7 – 0.90 or 0.6 – 0.8, as measured with dextran of Mw 110 kDa as a probe molecule. The  $K_d$  value as measured with dextran of Mw 110 kDa can suitably be 0.68-0.90, such as 0.68-0.85 or 0.70-0.85. An advantage of this is that the support has a large fraction of pores able to accommodate both the polypeptides/multimers of the invention and  
15 immunoglobulins binding to the polypeptides/multimers and to provide mass transport of the immunoglobulins to and from the binding sites.

The polypeptides or multimers may be attached to the porous support via conventional coupling techniques utilising e.g. thiol, amino and/or carboxy groups present in the ligand. Bisepoxides, epichlorohydrin, CNBr, N-hydroxysuccinimide (NHS) etc are well-known coupling  
20 reagents. Between the support and the polypeptide/multimer, a molecule known as a spacer can be introduced, which improves the availability of the polypeptide/multimer and facilitates the chemical coupling of the polypeptide/multimer to the support. Depending on the nature of the polypeptide/multimer and the coupling conditions, the coupling may be a multipoint coupling (e.g. via a plurality of lysines) or a single point coupling (e.g. via a single cysteine).

25 In certain embodiments the polypeptides or multimers are coupled to the support via thioether bonds. Methods for performing such coupling are well-known in this field and easily performed by the skilled person in this field using standard techniques and equipment. Thioether bonds are flexible and stable and generally suited for use in affinity chromatography. In particular when the thioether bond is via a terminal or near-terminal cysteine residue on the polypeptide  
30 or multimer, the mobility of the coupled polypeptide/multimer is enhanced which provides improved binding capacity and binding kinetics. In some embodiments the

polypeptide/multimer is coupled via a C-terminal cysteine provided on the protein as described above. This allows for efficient coupling of the cysteine thiol to electrophilic groups, e.g. epoxide groups, halohydrin groups etc. on a support, resulting in a thioether bridge coupling.

In certain embodiments the support comprises a polyhydroxy polymer, such as a

5 polysaccharide. Examples of polysaccharides include e.g. dextran, starch, cellulose, pullulan, agar, agarose etc. Polysaccharides are inherently hydrophilic with low degrees of nonspecific interactions, they provide a high content of reactive (activatable) hydroxyl groups and they are generally stable towards alkaline cleaning solutions used in bioprocessing.

10 In some embodiments the support comprises agar or agarose. The supports used in the present invention can easily be prepared according to standard methods, such as inverse suspension gelation (S Hjertén: Biochim Biophys Acta 79(2), 393-398 (1964). Alternatively, the base matrices are commercially available products, such as crosslinked agarose beads sold under the name of SEPHAROSE™ FF (GE Healthcare). In an embodiment, which is especially advantageous for large-scale separations, the support has been adapted to increase its rigidity using the

15 methods described in US6602990 or US7396467, which are hereby incorporated by reference in their entireties, and hence renders the matrix more suitable for high flow rates.

In certain embodiments the support, such as a polymer, polysaccharide or agarose support, is crosslinked, such as with hydroxyalkyl ether crosslinks. Crosslinker reagents producing such crosslinks can be e.g. epihalohydrins like epichlorohydrin, diepoxides like butanediol diglycidyl

20 ether, allylating reagents like allyl halides or allyl glycidyl ether. Crosslinking is beneficial for the rigidity of the support and improves the chemical stability. Hydroxyalkyl ether crosslinks are alkali stable and do not cause significant nonspecific adsorption.

Alternatively, the porous support is based on synthetic polymers, such as polyvinyl alcohol, polyhydroxyalkyl acrylates, polyhydroxyalkyl methacrylates, polyacrylamides,

25 polymethacrylamides etc. In case of hydrophobic polymers, such as matrices based on divinyl and monovinyl-substituted benzenes, the surface of the matrix is often hydrophilised to expose hydrophilic groups as defined above to a surrounding aqueous liquid. Such polymers are easily produced according to standard methods, see e.g. "Styrene based polymer supports developed by suspension polymerization" (R Arshady: Chimica e L'Industria 70(9), 70-75 (1988)).

30 Alternatively, a commercially available product, such as SOURCE™ (GE Healthcare) is used. In

another alternative, the porous support according to the invention comprises a support of inorganic nature, e.g. silica, zirconium oxide etc.

In yet another embodiment, the solid support is in another form such as a surface, a chip, capillaries, or a filter (e.g. a membrane or a depth filter matrix).

- 5 As regards the shape of the matrix according to the invention, in one embodiment the matrix is in the form of a porous monolith. In an alternative embodiment, the matrix is in beaded or particle form that can be porous or non-porous. Matrices in beaded or particle form can be used as a packed bed or in a suspended form. Suspended forms include those known as expanded beds and pure suspensions, in which the particles or beads are free to move. In case  
10 of monoliths, packed bed and expanded beds, the separation procedure commonly follows conventional chromatography with a concentration gradient. In case of pure suspension, batch-wise mode will be used.

The separation matrix as disclosed above has excellent alkali stability and may be cleaned and/or sanitized using an alkaline cleaning liquid. The method of cleaning and/or sanitizing the  
15 separation matrix comprises the following steps:

- a) optionally purifying a mixture comprising a first immunoglobulin using the separation matrix;
- b) providing a cleaning liquid comprising at least 50% by volume of an aqueous alkali metal hydroxide solution; and
- 20 c) cleaning and/or sanitizing the separation matrix by contacting the cleaning liquid with the separation matrix for a predetermined contact time.

Cleaning and/or sanitization may be performed on separation matrix not previously used in order to sanitize the matrix prior to use, for example after packing a column comprising the separation matrix for the first time. Cleaning and/or sanitization may also be performed after  
25 using the separation matrix to purify an immunoglobulin, i.e. step a) of the method above. The steps of using the separation matrix to purify an immunoglobulin and cleaning and/or sanitizing the separation matrix afterwards may be repeated in order to maximize use of the separation matrix. The steps may be repeated at least 10 times, such as at least 50 times or 50 – 200 times. The step c) of cleaning/sanitizing may be performed after each step a) of purifying an

immunoglobulin, or may be performed less frequently, such as after every second purification, or after every tenth purification. The step c) of cleaning and/or sanitizing the separation matrix need not be performed using the same conditions each time. For example, the separation matrix may be cleaned after each purification step using a first set of conditions, and after  
5 every  $n^{\text{th}}$  purification be sanitized using more stringent conditions.

Because aqueous alkali metal hydroxide solutions are effective cleaning agents and bactericidal in themselves, by cleaning and sanitizing using a cleaning liquid comprising such aqueous alkali metal hydroxide solutions there is a lesser need for using alcohols as cleaning and sanitizing agents. Alkali metal hydroxides are relatively cheap compared to alcohols and are subject to  
10 less regulatory burden. Moreover, they are non-flammable and easier to dispose of.

The separation matrix is a separation matrix as disclosed above, comprising multimers of immunoglobulin-binding alkali-stabilized Protein A domains covalently coupled to a porous support, wherein the alkali-stabilized Protein A domains comprise mutants of a parental Fc-binding domain of Staphylococcus Protein A (SpA), as defined by, or having at least 80% such as  
15 at least 90%, 95% or 98% identity to, SEQ ID NO 51 or SEQ ID NO 52, wherein the amino acid residues at positions 13 and 44 of SEQ ID NO 51 or 52 are asparagines, and wherein at least the asparagine residue at position 3 of SEQ ID NO 51 or 52 has been mutated to an amino acid selected from the group consisting of glutamic acid, lysine, tyrosine, threonine, phenylalanine, leucine, isoleucine, tryptophan, methionine, valine, alanine, histidine and arginine. The  
20 immunoglobulin-binding alkali-stabilized Protein A domains may comprise further mutations. For example, the glutamine residue at position 1 of SEQ ID NO 51 or 52 may be mutated to an alanine; and/or the asparagine or glutamic acid residue at position 35 of SEQ ID NO 51 or 52 may be mutated to an alanine.

Due to the high alkaline stability of the alkali-stabilized Protein A domains, the separation  
25 matrix can tolerate extensive cleaning and sanitization procedures, even using concentrated alkali metal hydroxide solutions, without excessive loss of binding capacity. For example, the separation matrix may retain at least 80% of its original dynamic binding capacity after cleaning and/or sanitization.

The cleaning liquid comprises at least 50% by volume of an aqueous alkali metal hydroxide  
30 solution. The aqueous alkali metal hydroxide solution may comprise a single alkali metal

hydroxide or a mixture of alkali metal hydroxides, such as sodium hydroxide, potassium hydroxide, or a mixture of sodium hydroxide and potassium hydroxide. The aqueous alkali metal hydroxide solution may have a molarity of from 500 mM to 5 M, such as from 1 M to 2 M, expressed as the total combined concentration of alkali metal hydroxides if a mixture of  
5 alkali metal hydroxides is used. The cleaning liquid may essentially consist of, or consist of, the aqueous alkali metal hydroxide solution. However, the cleaning liquid may in some embodiments also comprise further components. Such further components may include alcohols, such as a C<sub>2</sub> – C<sub>7</sub> alcohol, such as ethanol, isopropanol or benzoic alcohol. Such further components may include salts, such as sodium chloride. The use of alcohols and/or salts in the  
10 cleaning liquid may increase the efficacy of the cleaning liquid in inhibiting or inactivating certain microorganisms, such as spore-forming bacteria.

Non-limiting examples of cleaning liquids include:

Sodium hydroxide solution (0.5 M, 1 M, 2 M or 5 M);

Potassium hydroxide solution (0.5 M, 1 M, 2 M or 5 M);

15 Sodium hydroxide solution (0.5 M, 1 M, 2 M or 5 M) with 10-20% by volume ethanol;

Sodium hydroxide solution (0.5 M, 1 M, 2 M or 5 M) with 10-50% by volume isopropanol;

Sodium hydroxide solution (0.5 M, 1 M, 2 M or 5 M) with 1-5% by volume benzyl alcohol;

Potassium hydroxide solution (0.5 M, 1 M, 2 M or 5 M) with 10-20% by volume ethanol;

Potassium hydroxide solution (0.5 M, 1 M, 2 M or 5 M) with 10-50% by volume isopropanol; or

20 Potassium hydroxide solution (0.5 M, 1 M, 2 M or 5 M) with 1-5% by volume benzyl alcohol.

The use of relatively concentrated alkali metal hydroxide solution, such as 0.5 M – 5 M solution, provides exceptional removal of matrix contaminants such as proteins and nucleic acids, as well as quickly and effectively inactivating viruses, bacteria, yeast and fungi. A commonly applied measure of sanitization efficacy is a 6-log<sub>10</sub> reduction in the microorganism or contaminant  
25 being measured. Sanitizing with concentrated alkali metal hydroxide solution is capable of providing a 6-log<sub>10</sub> reduction in most microorganisms and endotoxins.

During cleaning of the separation matrix, the cleaning liquid should be passed through the separation matrix with a suitable flow rate, in order to flush contaminants from the matrix. During sanitization, the cleaning liquid is initially passed through the separation matrix with a suitable flow rate until the separation matrix is fully permeated with the cleaning liquid. The cleaning liquid is preferably passed through the separation matrix with a suitable flow rate for the entire duration of the sanitization step. However, if desired the flow of cleaning liquid through the separation matrix may be stopped once the separation matrix is fully permeated with the cleaning liquid. This reduces the volume of cleaning fluid required to perform a sanitization step, but provides a poorer cleaning of the separation matrix as compared to a procedure where a constant flow of cleaning fluid is applied.

Because aqueous alkali metal hydroxide solutions are so effective in removing contaminants from the separation matrix, the separation matrix may be used as a purification platform for the purification of a variety of immunoglobulins, with a lower risk of host cell protein contamination or carryover. After purification of a first immunoglobulin product, the separation matrix is first cleaned and sanitized thoroughly using a cleaning liquid comprising aqueous alkali metal hydroxide solution prior to use in purification of the second immunoglobulin product.

### Examples

#### 20 Mutagenesis of protein

Site-directed mutagenesis was performed by a two-step PCR using oligonucleotides coding for the mutations. As template a plasmid containing a single domain of either Z, B or C was used. The PCR fragments were ligated into an E. coli expression vector. DNA sequencing was used to verify the correct sequence of inserted fragments.

25 To form multimers of mutants an Acc I site located in the starting codons (GTA GAC) of the B, C or Z domain was used, corresponding to amino acids VD. The vector for the monomeric domain was digested with Acc I and phosphatase treated. Acc I sticky-ends primers were designed, specific for each variant, and two overlapping PCR products were generated from each template. The PCR products were purified and the concentration was estimated by comparing

the PCR products on a 2% agarose gel. Equal amounts of the pair wise PCR products were hybridized (90°C -> 25°C in 45min) in ligation buffer. The resulting product consists approximately to ¼ of fragments likely to be ligated into an Acc I site (correct PCR fragments and/or the digested vector). After ligation and transformation colonies were PCR screened to  
5 identify constructs containing the desired mutant. Positive clones were verified by DNA sequencing.

#### Construct expression and purification

The constructs were expressed in the bacterial periplasm by fermentation of *E. coli* K12 in standard media. After fermentation the cells were heat-treated to release the periplasm  
10 content into the media. The constructs released into the medium were recovered by microfiltration with a membrane having a 0.2 µm pore size.

Each construct, now in the permeate from the filtration step, was purified by affinity. The permeate was loaded onto a chromatography medium containing immobilized IgG (IgG Sepharose 6FF, GE Healthcare). The loaded product was washed with phosphate buffered  
15 saline and eluted by lowering the pH.

The elution pool was adjusted to a neutral pH (pH 8) and reduced by addition of dithiothreitol. The sample was then loaded onto an anion exchanger. After a wash step the construct was eluted in a NaCl gradient to separate it from any contaminants. The elution pool was  
20 concentrated by ultrafiltration to 40-50 mg/ml. It should be noted that the successful affinity purification of a construct on an immobilized IgG medium indicates that the construct in question has a high affinity to IgG.

The purified ligands were analyzed with RPC LC-MS to determine the purity and to ascertain that the molecular weight corresponded to the expected (based on the amino acid sequence).

#### Example 1

The purified monomeric ligands listed in Table 1, further comprising for SEQ ID NO 8-16, 23-28 and 36-48 an AQGT leader sequence at the N-terminus and a cysteine at the C terminus, were immobilized on Biacore CM5 sensor chips (GE Healthcare, Sweden), using the amine coupling

kit of GE Healthcare (for carbodiimide coupling of amines on the carboxymethyl groups on the chip) in an amount sufficient to give a signal strength of about 200-1500 RU in a Biacore surface plasmon resonance (SPR) instrument (GE Healthcare, Sweden) . To follow the IgG binding capacity of the immobilized surface 1mg/ml human polyclonal IgG (Gammanorm) was flowed

5 over the chip and the signal strength (proportional to the amount of binding) was noted. The surface was then cleaned-in-place (CIP), i.e. flushed with 500mM NaOH for 10 minutes at room temperature (22 +/- 2°C). This was repeated for 96-100 cycles and the immobilized ligand alkaline stability was followed as the remaining IgG binding capacity (signal strength) after each cycle. The results are shown in Table 1 and indicate that at least the ligands Zvar(N11K)1,

10 Zvar(N11E)1, Zvar(N11Y)1, Zvar(N11T)1, Zvar(N11F)1, Zvar(N11L)1, Zvar(N11W)1, ZN11I)1, Zvar(N11M)1, Zvar(N11V)1, Zvar(N11A)1, Zvar(N11H1), Zvar(N11R)1, Zvar(N11E,Q32A)1, Zvar(N11E,Q32E,Q40E)1 and Zvar(N11E,Q32E,K50R)1, Zvar(Q9A,N11E,N43A)1, Zvar(Q9A,N11E,N28A,N43A)1, Zvar(Q9A,N11E,Q40V,A42K,N43E,L44I)1, Zvar(Q9A,N11E,Q40V,A42K,N43A,L44I)1, Zvar(Q9A,N11E,N28A,Q40V,A42K,N43A,L44I)1,

15 Zvar(N11K,H18K,S33K,D37E,A42R,N43A,L44I,K50R,L51Y)1, Zvar(Q9A,N11K,H18K,S33K,D37E,A42R,N43A,L44I,K50R,L51Y)1, Zvar(N11K, H18K, D37E, A42R, N43A, L44I)1, Zvar(Q9A, N11K, H18K, D37E, A42R, N43A, L44I)1 and Zvar(Q9A, N11K, H18K, D37E, A42R, N43A, L44I, K50R)1, as well as the varieties of Zvar(Q9A,N11E,Q40V,A42K,N43A,L44I)1 having G,S,Y,Q,T,N,F,L,W,I,M,V,D,E,H,R or K in position

20 29, the varieties of Zvar(Q9A,N11E,Q40V,A42K,N43A,L44I)1 having F,Y,W,K or R in position 53 and the varieties of Zvar(Q9A,N11E,Q40V,A42K,N43A,L44I)1 where Q9, Q40, A42 or N43 has been deleted, have an improved alkali stability compared to the parental structure Zvar1, used as the reference. Further, the ligands B(Q9A,N11E,Q40V,A42K,N43A,L44I)1 and C(Q9A,N11E,E43A)1 have an improved stability compared to the parental B and C domains,

25 used as references.

Table 1. Monomeric ligands, evaluated by Biacore (0.5 M NaOH).

| Ligand                | Sequence        | Capacity<br>after 96-<br>100<br>cycles | Reference<br>capacity<br>after 96-<br>100 cycles | Capacity<br>relative<br>to<br>reference |
|-----------------------|-----------------|----------------------------------------|--------------------------------------------------|-----------------------------------------|
| Zvar(N11E,Q32A)1      | SEQ ID<br>NO 12 | 57%                                    | 55%                                              | 1.036                                   |
| Zvar(N11E)1           | SEQ ID<br>NO 13 | 59%                                    | 55%                                              | 1.073                                   |
| Zvar(N11E,Q32E,Q40E)1 | SEQ ID<br>NO 14 | 52%                                    | 51%                                              | 1.020                                   |
| Zvar(N11E,Q32E,K50R)1 | SEQ ID<br>NO 15 | 53%                                    | 51%                                              | 1.039                                   |
| Zvar(N11K)1           | SEQ ID<br>NO 16 | 62%                                    | 49%                                              | 1.270                                   |
| Zvar(N11Y)1           | SEQ ID<br>NO 38 | 55%                                    | 46%                                              | 1.20                                    |
| Zvar(N11T)1           | SEQ ID<br>NO 39 | 50%                                    | 46%                                              | 1.09                                    |
| Zvar(N11F)1           | SEQ ID<br>NO 40 | 55%                                    | 46%                                              | 1.20                                    |
| Zvar(N11L)1           | SEQ ID<br>NO 41 | 57%                                    | 47%                                              | 1.21                                    |
| Zvar(N11W)1           | SEQ ID<br>NO 42 | 57%                                    | 47%                                              | 1.21                                    |
| Zvar(N11I)1           | SEQ ID<br>NO 43 | 57%                                    | 47%                                              | 1.21                                    |
| Zvar(N11M)1           | SEQ ID<br>NO 44 | 58%                                    | 46%                                              | 1.26                                    |
| Zvar(N11V)1           | SEQ ID<br>NO 45 | 56%                                    | 46%                                              | 1.22                                    |
| Zvar(N11A)1           | SEQ ID<br>NO 46 | 58%                                    | 46%                                              | 1.26                                    |
| Zvar(N11H)1           | SEQ ID<br>NO 47 | 57%                                    | 46%                                              | 1.24                                    |
| Zvar(N11R)1           | SEQ ID<br>NO 48 | 59%                                    | 46%                                              | 1.28                                    |
| Zvar(Q9A,N11E,N43A)1  | SEQ ID<br>NO 8  | 70%                                    | 47%                                              | 1.49                                    |

|                                                         |                 |     |     |      |
|---------------------------------------------------------|-----------------|-----|-----|------|
| Zvar(Q9A,N11E,N28A,N43A)1                               | SEQ ID<br>NO 9  | 68% | 47% | 1.45 |
| Zvar(Q9A,N11E,Q40V,A42K,N43E,L44I)1                     | SEQ ID<br>NO 10 | 67% | 47% | 1.43 |
| Zvar(Q9A,N11E,Q40V,A42K,N43A,L44I)1                     | SEQ ID<br>NO 11 | 66% | 47% | 1.40 |
| Zvar(Q9A,N11E,N28A,Q40V,A42K,N43A,L44I)1                | SEQ ID<br>NO 24 | 65% | 48% | 1.35 |
| Zvar(N11K,H18K,S33K,D37E,A42R,N43A,L44I,K50R,L51Y)1     | SEQ ID<br>NO 23 | 67% | 46% | 1.46 |
| Zvar(Q9A,N11K,H18K,S33K,D37E,A42R,N43A,L44I,K50R,L51Y)1 | SEQ ID<br>NO 25 | 59% | 46% | 1.28 |
| Zvar(N11K, H18K, D37E, A42R, N43A, L44I)1               | SEQ ID<br>NO 26 | 59% | 45% | 1.31 |
| Zvar(Q9A, N11K, H18K, D37E, A42R, N43A, L44I)1          | SEQ ID<br>NO 27 | 63% | 45% | 1.40 |
| Zvar(Q9A, N11K, H18K, D37E, A42R, N43A, L44I, K50R)1    | SEQ ID<br>NO 28 | 67% | 45% | 1.49 |
| B(Q9A,N11E,Q40V,A42K,N43A,L44I)1                        | SEQ ID<br>NO 36 | 39% | 35% | 1.11 |
| C(Q9A,N11E,E43A)1                                       | SEQ ID<br>NO 37 | 60% | 49% | 1.22 |
| Zvar(Q9A,N11E, <b>A29G</b> ,Q40V,A42K,N43A,L44I)1       | SEQ ID<br>NO 54 | 69% | 48% | 1.44 |
| Zvar(Q9A,N11E, <b>A29S</b> ,Q40V,A42K,N43A,L44I)1       | SEQ ID<br>NO 55 | 66% | 48% | 1.38 |
| Zvar(Q9A,N11E, <b>A29Y</b> ,Q40V,A42K,N43A,L44I)1       | SEQ ID<br>NO 56 | 61% | 48% | 1.27 |
| Zvar(Q9A,N11E, <b>A29Q</b> ,Q40V,A42K,N43A,L44I)1       | SEQ ID<br>NO 57 | 60% | 47% | 1.28 |
| Zvar(Q9A,N11E, <b>A29T</b> ,Q40V,A42K,N43A,L44I)1       | SEQ ID<br>NO 58 | 60% | 47% | 1.28 |
| Zvar(Q9A,N11E, <b>A29N</b> ,Q40V,A42K,N43A,L44I)1       | SEQ ID<br>NO 59 | 61% | 47% | 1.30 |
| Zvar(Q9A,N11E, <b>A29F</b> ,Q40V,A42K,N43A,L44I)1       | SEQ ID<br>NO 60 | 62% | 46% | 1.35 |
| Zvar(Q9A,N11E, <b>A29L</b> ,Q40V,A42K,N43A,L44I)1       | SEQ ID<br>NO 61 | 61% | 46% | 1.33 |
| Zvar(Q9A,N11E, <b>A29W</b> ,Q40V,A42K,N43A,L44I)1       | SEQ ID<br>NO 62 | 60% | 46% | 1.30 |

|                                                   |                 |     |     |      |
|---------------------------------------------------|-----------------|-----|-----|------|
| Zvar(Q9A,N11E, <b>A29I</b> ,Q40V,A42K,N43A,L44I)1 | SEQ ID<br>NO 63 | 58% | 47% | 1.23 |
| Zvar(Q9A,N11E, <b>A29M</b> ,Q40V,A42K,N43A,L44I)1 | SEQ ID<br>NO 64 | 62% | 47% | 1.32 |
| Zvar(Q9A,N11E, <b>A29V</b> ,Q40V,A42K,N43A,L44I)1 | SEQ ID<br>NO 65 | 62% | 47% | 1.32 |
| Zvar(Q9A,N11E, <b>A29D</b> ,Q40V,A42K,N43A,L44I)1 | SEQ ID<br>NO 66 | 56% | 47% | 1.19 |
| Zvar(Q9A,N11E, <b>A29E</b> ,Q40V,A42K,N43A,L44I)1 | SEQ ID<br>NO 67 | 57% | 47% | 1.21 |
| Zvar(Q9A,N11E, <b>A29H</b> ,Q40V,A42K,N43A,L44I)1 | SEQ ID<br>NO 68 | 57% | 47% | 1.21 |
| Zvar(Q9A,N11E, <b>A29R</b> ,Q40V,A42K,N43A,L44I)1 | SEQ ID<br>NO 69 | 58% | 46% | 1.26 |
| Zvar(Q9A,N11E, <b>A29K</b> ,Q40V,A42K,N43A,L44I)1 | SEQ ID<br>NO 70 | 59% | 46% | 1.28 |
| Zvar(Q9A,N11E,Q40V,A42K,N43A,L44I, <b>D53F</b> )1 | SEQ ID<br>NO 71 | 58% | 46% | 1.26 |
| Zvar(Q9A,N11E,Q40V,A42K,N43A,L44I, <b>D53Y</b> )1 | SEQ ID<br>NO 72 | 59% | 46% | 1.28 |
| Zvar(Q9A,N11E,Q40V,A42K,N43A,L44I, <b>D53W</b> )1 | SEQ ID<br>NO 73 | 62% | 46% | 1.35 |
| Zvar(Q9A,N11E,Q40V,A42K,N43A,L44I, <b>D53K</b> )1 | SEQ ID<br>NO 74 | 65% | 46% | 1.41 |
| Zvar(Q9A,N11E,Q40V,A42K,N43A,L44I, <b>D53R</b> )1 | SEQ ID<br>NO 75 | 60% | 46% | 1.30 |
| Zvar( <b>Q9del</b> ,N11E,Q40V,A42K,N43A,L44I)1    | SEQ ID<br>NO 76 | 60% | 46% | 1.30 |
| Zvar(Q9A,N11E, <b>Q40del</b> ,A42K,N43A,L44I)1    | SEQ ID<br>NO 77 | 59% | 46% | 1.28 |
| Zvar(Q9A,N11E,Q40V, <b>A42del</b> ,N43A,L44I)1    | SEQ ID<br>NO 78 | 57% | 46% | 1.24 |
| Zvar(Q9A,N11E,Q40V,A42K, <b>N43del</b> ,L44I)1    | SEQ ID<br>NO 79 | 55% | 46% | 1.20 |

The Biacore experiment can also be used to determine the binding and dissociation rates between the ligand and IgG. This was used with the set-up as described above and with an IgG1 monoclonal antibody as probe molecule. For the reference Zvar1, the on-rate ( $10^5 \text{ M}^{-1}\text{s}^{-1}$ ) was

3.1 and the off-rate ( $10^5 \text{ s}^{-1}$ ) was 22.1, giving an affinity (off-rate/on-rate) of 713 pM. For Zvar(Q9A,N11E,Q40V,A42K,N43A,L44I)1 (SEQ ID NO. 11), the on-rate was 4.1 and the off-rate 43.7, with affinity 1070 pM. The IgG affinity was thus somewhat higher for the mutated variant.

## 5 Example 2

The purified dimeric, tetrameric and hexameric ligands listed in Table 2 were immobilized on Biacore CM5 sensor chips (GE Healthcare, Sweden), using the amine coupling kit of GE Healthcare (for carbodiimide coupling of amines on the carboxymethyl groups on the chip) in an amount sufficient to give a signal strength of about 200-1500 RU in a Biacore instrument (GE Healthcare, Sweden). To follow the IgG binding capacity of the immobilized surface 1mg/ml human polyclonal IgG (Gammanorm) was flowed over the chip and the signal strength (proportional to the amount of binding) was noted. The surface was then cleaned-in-place (CIP), i.e. flushed with 500mM NaOH for 10 minutes at room temperature ( $22 \pm 2^\circ\text{C}$ ). This was repeated for 300 cycles and the immobilized ligand alkaline stability was followed as the remaining IgG binding capacity (signal strength) after each cycle. The results are shown in Table 2 and in Fig. 2 and indicate that at least the ligands Zvar(Q9A,N11E,N43A)4, Zvar(Q9A,N11E,N28A,N43A)4, Zvar(Q9A,N11E,Q40V,A42K,N43E,L44I)4 and Zvar(Q9A,N11E,Q40V,A42K,N43A,L44I)4, Zvar(Q9A,N11E,D37E,Q40V,A42K,N43A,L44I)4 and Zvar(Q9A,N11E,D37E,Q40V,A42R,N43A,L44I)4 have an improved alkali stability compared to the parental structure Zvar4, which was used as a reference. The hexameric ligand Zvar(Q9A,N11E,Q40V,A42K,N43A,L44I)6 also has improved alkali stability compared to the parental structure Zvar6, used as a reference. Further, Zvar(Q9A,N11E,Q40V,A42K,N43A,L44I) dimers with deletions of a) D2,A3,K4; b) K58,V1,D2; c) P57,K58,V1,D2,A3; d) K4,F5,D6,K7,E8; e) A56,P57,K58; V1,D2,A3 or f) V1,D2,A3,K4,F5,D6,K7,E8 from the linker region between the two monomer units have improved alkali stability compared to the parental structure Zvar2, used as a reference. Also Zvar(Q9A,N11E,Q40V,A42K,N43A,L44I) dimers with an insertion of YEDG between K58 and V1 in the linker region have improved alkali stability compared to Zvar2.

Table 2. Dimeric, tetrameric and hexameric ligands, evaluated by Biacore (0.5M NaOH).

| Ligand                                                                             | SEQ ID NO: | Remaining capacity 100 cycles (%) | Capacity relative to ref. 100 cycles | Remaining capacity 200 cycles (%) | Capacity relative to ref. 200 cycles | Remaining capacity 300 cycles (%) | Capacity relative to ref. 300 cycles |
|------------------------------------------------------------------------------------|------------|-----------------------------------|--------------------------------------|-----------------------------------|--------------------------------------|-----------------------------------|--------------------------------------|
| Zvar4                                                                              | 21         | 67                                | 1                                    | 36                                | 1                                    | 16                                | 1                                    |
| Zvar(Q9A,N11E,N43A)4                                                               | 17         | 81                                | 1.21                                 | 62                                | 1.72                                 | 41                                | 2.56                                 |
| Zvar(Q9A,N11E,N28A,N43A)4                                                          | 18         | 80                                | 1.19                                 | 62                                | 1.72                                 | 42                                | 2.62                                 |
| Zvar(Q9A,N11E,Q40V,A42K,N43E,L44I)4                                                | 19         | 84                                | 1.25                                 | 65                                | 1.81                                 | 48                                | 3.00                                 |
| Zvar(Q9A,N11E,Q40V,A42K,N43A,L44I)4                                                | 20         | 90                                | 1.34                                 | 74                                | 2.06                                 | 57                                | 3.56                                 |
| Zvar(Q9A,N11E,N28A,Q40V,A42K,N43A,L44I)4                                           | 32         | 84                                | 1.24                                 | Not tested                        | Not tested                           | Not tested                        | Not tested                           |
| Zvar(Q9A,N11E,Q40V,A42K,N43A,L44I)6                                                | 33         | 87                                | 1.30                                 | Not tested                        | Not tested                           | Not tested                        | Not tested                           |
| Zvar(Q9A,N11E,D37E,Q40V,A42K,N43A,L44I)4                                           | 34         | 81                                | 1.13                                 | Not tested                        | Not tested                           | Not tested                        | Not tested                           |
| Zvar(Q9A,N11E,D37E,Q40V,A42R,N43A,L44I)4                                           | 35         | 84                                | 1.17                                 | Not tested                        | Not tested                           | Not tested                        | Not tested                           |
| Zvar(Q9A,N11E,Q40V,A42K,N43A,L44I)2 with D2, A3 and K4 in linker deleted           | 80         | 70                                | 1.27                                 | Not tested                        | Not tested                           | Not tested                        | Not tested                           |
| Zvar(Q9A,N11E,Q40V,A42K,N43A,L44I)2 with K58, V1 and D2 in linker deleted          | 81         | 76                                | 1.38                                 | Not tested                        | Not tested                           | Not tested                        | Not tested                           |
| Zvar(Q9A,N11E,Q40V,A42K,N43A,L44I)2 with P57, K58, V1, D2 and A3 in linker deleted | 82         | 74                                | 1.35                                 | Not tested                        | Not tested                           | Not tested                        | Not tested                           |
| Zvar(Q9A,N11E,Q40V,A42K,N43A,L44I)2 with K4, F5, D6, K7 and E8 in linker deleted   | 83         | 70                                | 1.30                                 | Not tested                        | Not tested                           | Not tested                        | Not tested                           |

|                                                                                              |    |    |      |            |            |            |            |
|----------------------------------------------------------------------------------------------|----|----|------|------------|------------|------------|------------|
| Zvar(Q9A,N11E,Q40V,A42K,N43A,L44I)2 with A56, P57 and K58 in linker deleted                  | 84 | 68 | 1.26 | Not tested | Not tested | Not tested | Not tested |
| Zvar(Q9A,N11E,Q40V,A42K,N43A,L44I)2 with V1, D2 and A3 in linker deleted                     | 85 | 75 | 1.39 | Not tested | Not tested | Not tested | Not tested |
| Zvar(Q9A,N11E,Q40V,A42K,N43A,L44I)2 with V1, D2, A3, K4, F5, D6, K7 and E8 in linker deleted | 86 | 62 | 1.13 | Not tested | Not tested | Not tested | Not tested |
| Zvar(Q9A,N11E,Q40V,A42K,N43A,L44I)2 with YEDG inserted in linker between K58 and V1          | 87 | 72 | 1.31 | Not tested | Not tested | Not tested | Not tested |
| Zvar2                                                                                        | 88 | 55 | 1    | Not tested | Not tested | Not tested | Not tested |

### Example 3

Example 2 was repeated with 100 CIP cycles of three ligands using 1 M NaOH instead of 500 mM as in Example 2. The results are shown in Table 3 and show that all three ligands have an improved alkali stability also in 1M NaOH, compared to the parental structure Zvar4 which was used as a reference.

Table 3. Tetrameric ligands, evaluated by Biacore (1M NaOH).

| Ligand                              | Sequence     | Remaining capacity 100 cycles (%) | Capacity relative to ref. 100 cycles |
|-------------------------------------|--------------|-----------------------------------|--------------------------------------|
| Zvar4                               | SEQ ID NO 21 | 27                                | 1                                    |
| Zvar(Q9A,N11E,N28A,N43A)4           | SEQ ID NO 18 | 55                                | 2.04                                 |
| Zvar(Q9A,N11E,Q40V,A42K,N43E,L44I)4 | SEQ ID NO 19 | 54                                | 2.00                                 |
| Zvar(Q9A,N11E,Q40V,A42K,N43A,L44I)4 | SEQ ID NO 20 | 56                                | 2.07                                 |

Example 4

The purified tetrameric ligands of Table 2 (all with an additional N-terminal cysteine) were immobilized on agarose beads using the methods described below and assessed for capacity and stability. The results are shown in Table 4 and Fig. 3.

5 Table 4. Matrices with tetrametric ligands, evaluated in columns (0.5 M NaOH).

| <b>Ligand</b>                                       | <b>SEQ ID NO.</b> | <b>Ligand content (mg/ml)</b> | <b>Initial IgG capacity Qb10 (mg/ml)</b> | <b>Remainin g IgG capacity Qb10 after six 4 h cycles (mg/ml)</b> | <b>Remainin g IgG capacity after six 4 h cycles (%)</b> | <b>Capaci ty retenti on relative to ref. after six 4 h cycles</b> |
|-----------------------------------------------------|-------------------|-------------------------------|------------------------------------------|------------------------------------------------------------------|---------------------------------------------------------|-------------------------------------------------------------------|
| Zvar4                                               | 21                | 7                             | 52.5                                     | 36.5                                                             | 60                                                      | 1                                                                 |
| Zvar4                                               | 21                | 12                            | 61.1                                     | 43.4                                                             | 71                                                      | 1                                                                 |
| Zvar(Q9A,N11E,N28A,N43A)4                           | 18                | 7.0                           | 49.1                                     | 44.1                                                             | 90                                                      | 1.50                                                              |
| Zvar(Q9A,N11E,N28A,N43A)4                           | 18                | 12.1                          | 50.0                                     | 46.2                                                             | 93                                                      | 1.31                                                              |
| Zvar(Q9A,N11E,Q40V,A42K,N43A,L44I)4                 | 20                | 7.2                           | 49.0                                     | 44.2                                                             | 90                                                      | 1.50                                                              |
| Zvar(Q9A,N11E,Q40V,A42K,N43A,L44I)4                 | 20                | 12.8                          | 56.3                                     | 53.6                                                             | 95                                                      | 1.34                                                              |
| Zvar(N11K,H18K,S33K,D37E,A42R,N43A,L44I,K50R,L51Y)4 | 30                | 9.7                           | 56.3                                     | 52.0                                                             | 92                                                      | 1.53                                                              |
| Zvar(Q9A,N11K,H18K,D37E,A42R)4                      | 31                | 10.8                          | 56.9                                     | 52.5                                                             | 92                                                      | 1.30                                                              |

**Activation**

The base matrix used was rigid cross-linked agarose beads of 85 micrometers (volume-weighted, d50V) median diameter, prepared according to the methods of US6602990, hereby  
 10 incorporated by reference in its entirety, and with a pore size corresponding to an inverse gel filtration chromatography Kav value of 0.70 for dextran of Mw 110 kDa, according to the methods described in Gel Filtration Principles and Methods, Pharmacia LKB Biotechnology 1991, pp 6-13.

25 mL (g) of drained base matrix, 10.0 mL distilled water and 2.02 g NaOH (s) was mixed in a 100 mL flask with mechanical stirring for 10 min at 25°C. 4.0 mL of epichlorohydrin was added and the reaction progressed for 2 hours. The activated gel was washed with 10 gel sediment volumes (GV) of water.

## 5 Coupling

To 20 mL of ligand solution (50 mg/mL) in a 50 ml Falcon tube, 169 mg NaHCO<sub>3</sub>, 21 mg Na<sub>2</sub>CO<sub>3</sub>, 175 mg NaCl and 7 mg EDTA, was added. The Falcon tube was placed on a roller table for 5-10 min, and then 77 mg of DTE was added. Reduction proceeded for >45 min. The ligand solution was then desalted on a PD10 column packed with Sephadex G-25. The ligand content in the  
10 desalted solution was determined by measuring the 276 nm UV absorption.

The activated gel was washed with 3-5 GV {0.1 M phosphate/1 mM EDTA pH 8.6} and the ligand was then coupled according to the method described in US6399750, hereby incorporated by reference in its entirety. All buffers used in the experiments had been degassed by nitrogen gas for at least 5-10 min. The ligand content of the gels could be controlled by varying the amount  
15 and concentration of the ligand solution.

After immobilization the gels were washed 3xGV with distilled water. The gels + 1 GV {0.1 M phosphate/1 mM EDTA/10% thioglycerol pH 8.6} was mixed and the tubes were left in a shaking table at room temperature overnight. The gels were then washed alternately with 3xGV {0.1 M TRIS/0.15 M NaCl pH 8.6} and 0.5 M HAc and then 8-10xGV with distilled water. Gel  
20 samples were sent to an external laboratory for amino acid analysis and the ligand content (mg/ml gel) was calculated from the total amino acid content.

## Protein

Gammanorm 165 mg/ml (Octapharma), diluted to 2mg/ml in Equilibration buffer.

## 25 Equilibration buffer

PBS Phosphate buffer 10 mM + 0.14 M NaCl + 0.0027 M KCl, pH 7,4 (Medicago)

## Adsorption buffer

PBS Phosphate buffer 10 mM + 0.14 M NaCl + 0.0027 M KCl, pH 7,4 (Medicago)

Elution buffers

100 mM acetate pH 2.9

Dynamic binding capacity

2 ml of resin was packed in TRICORN™ 5 100 columns. The breakthrough capacity was

5 determined with an ÄKTAExplorer 10 system at a residence time of 6 minutes (0.33 ml/min flow rate). Equilibration buffer was run through the bypass column until a stable baseline was obtained. This was done prior to auto zeroing. Sample was applied to the column until a 100% UV signal was obtained. Then, equilibration buffer was applied again until a stable baseline was obtained.

10 Sample was loaded onto the column until a UV signal of 85% of maximum absorbance was reached. The column was then washed with 5 column volumes (CV) equilibration buffer at flow rate 0.5ml/min. The protein was eluted with 5 CV elution buffer at a flow rate of 0.5 ml/min. Then the column was cleaned with 0.5M NaOH at flow rate 0.2 ml/min and re-equilibrated with equilibration buffer.

15 For calculation of breakthrough capacity at 10%, the equation below was used. That is the amount of IgG that is loaded onto the column until the concentration of IgG in the column effluent is 10% of the IgG concentration in the feed.

$$q_{10\%} = \frac{C_0}{V_c} \left[ V_{app} - V_{sys} - \int_{V_{sys}}^{V_{app}} \frac{A(V) - A_{sub}}{A_{100\%} - A_{sub}} * dv \right]$$

$A_{100\%}$  = 100% UV signal;

20  $A_{sub}$  = absorbance contribution from non-binding IgG subclass;

$A(V)$  = absorbance at a given applied volume;

$V_c$  = column volume;

$V_{app}$  = volume applied until 10% breakthrough;

$V_{sys}$  = system dead volume;

25  $C_0$  = feed concentration.

The dynamic binding capacity (DBC) at 10% breakthrough was calculated. The dynamic binding capacity (DBC) was calculated for 10 and 80% breakthrough.

CIP - 0.5 M NaOH

The 10% breakthrough DBC (Qb10) was determined both before and after repeated exposures to alkaline cleaning solutions. Each cycle included a CIP step with 0.5 M NaOH pumped through the column at a rate of 0.5/min for 20 min, after which the column was left standing for 4 h.

- 5 The exposure took place at room temperature (22 +/- 2°C). After this incubation, the column was washed with equilibration buffer for 20 min at a flow rate of 0.5 ml/min. Table 4 shows the remaining capacity after six 4 h cycles (i.e. 24 h cumulative exposure time to 0.5 M NaOH), both in absolute numbers and relative to the initial capacity.

## 10 Example 5

Example 4 was repeated with the tetrameric ligands shown in Table 5, but with 1.0 M NaOH used in the CIP steps instead of 0.5 M. The results are shown in Table 5 and in Fig. 4.

Table 5. Matrices with tetrametric ligands, evaluated in columns – 1.0 M NaOH.

| <b>Ligand</b>                                       | <b>SEQ ID NO.</b> | <b>Ligand content (mg/ml)</b> | <b>Initial IgG capacity Qb10 (mg/ml)</b> | <b>Remainin g IgG capacity Qb10 after six 4 h cycles (mg/ml)</b> | <b>Remainin g IgG capacity after six 4 h cycles (%)</b> | <b>Capaci ty retenti on relative to ref. after six 4 h cycles</b> |
|-----------------------------------------------------|-------------------|-------------------------------|------------------------------------------|------------------------------------------------------------------|---------------------------------------------------------|-------------------------------------------------------------------|
| Zvar4                                               | 21                | 12                            | 60.1                                     | 33.5                                                             | 56                                                      | 1                                                                 |
| Zvar(Q9A,N11E,Q40V,A42K,N43A,L44I)4                 | 20                | 12.8                          | 60.3                                     | 56.0                                                             | 93                                                      | 1.67                                                              |
| Zvar(N11K,H18K,S33K,D37E,A42R,N43A,L44I,K50R,L51Y)4 | 30                | 9.7                           | 62.1                                     | 48.1                                                             | 77                                                      | 1.44                                                              |

## Example 6

Base matrices

The base matrices used were a set of rigid cross-linked agarose bead samples of 59-93 micrometers (volume-weighted, d50V) median diameter (determined on a Malvern Mastersizer 2000 laser diffraction instrument), prepared according to the methods of US6602990 and with a pore size corresponding to an inverse gel filtration chromatography Kd value of 0.62-0.82 for dextran of Mw 110 kDa, according to the methods described above, using HR10/30 columns (GE Healthcare) packed with the prototypes in 0.2 M NaCl and with a range of dextran fractions as probe molecules (flow rate 0.2 ml/min). The dry weight of the bead samples ranged from 53 to 86 mg/ml, as determined by drying 1.0 ml drained filter cake samples at 105 °C overnight and weighing.

Table 6. Base matrix samples

| Base matrix | Kd    | d50v ( $\mu\text{m}$ ) | Dry weight (mg/ml) |
|-------------|-------|------------------------|--------------------|
| A18         | 0.704 | 59.0                   | 56.0               |
| A20         | 0.70  | 69.2                   | 55.8               |
| A27         | 0.633 | 87.2                   | 74.2               |
| A28         | 0.638 | 67.4                   | 70.2               |
| A29         | 0.655 | 92.6                   | 57.5               |
| A32         | 0.654 | 73.0                   | 70.5               |
| A33         | 0.760 | 73.1                   | 55.5               |
| A38         | 0.657 | 70.9                   | 56.2               |
| A39         | 0.654 | 66.0                   | 79.1               |
| A40         | 0.687 | 64.9                   | 74.9               |
| A41         | 0.708 | 81.7                   | 67.0               |
| A42         | 0.638 | 88.0                   | 59.4               |
| A43         | 0.689 | 87.5                   | 77.0               |
| A45         | 0.670 | 56.6                   | 66.0               |
| A52         | 0.620 | 53.10                  | 63.70              |
| A53         | 0.630 | 52.6                   | 86.0               |
| A54         | 0.670 | 61.3                   | 75.3               |
| A55         | 0.640 | 62.0                   | 69.6               |
| A56         | 0.740 | 61.0                   | 56.0               |
| A56-2       | 0.740 | 51.0                   | 56.0               |
| A62a        | 0.788 | 48.8                   | 70.1               |
| A62b        | 0.823 | 50.0                   | 46.9               |
| A63a        | 0.790 | 66.8                   | 59.6               |
| A63b        | 0.765 | 54.0                   | 79.0               |
| A65a        | 0.796 | 58.0                   | 60.0               |
| A65b        | 0.805 | 57.3                   | 46.0               |
| B5          | 0.793 | 69.0                   | 84.4               |
| C1          | 0.699 | 71.0                   | 73.4               |
| C2          | 0.642 | 66.5                   | 81.1               |
| C3          | 0.711 | 62.0                   | 82.0               |

|     |       |      |      |
|-----|-------|------|------|
| C4  | 0.760 | 62.0 | 82.0 |
| H31 | 0.717 | 82.0 | 59.0 |
| H35 | 0.710 | 81.1 | 61.0 |
| H40 | 0.650 | 52.8 | 65.0 |
| I1  | 0.640 | 50.0 | 67.0 |
| 41  | 0.702 | 81.6 | 60.6 |

### Coupling

100 ml base matrix was washed with 10 gel volumes distilled water on a glass filter. The gel was weighed (1 g = 1 ml) and mixed with 30 ml distilled water and 8.08 g NaOH (0.202 mol) in a 250 ml flask with an agitator. The temperature was adjusted to 27 +/- 2 °C in a water bath. 16 ml epichlorohydrin (0.202 mol) was added under vigorous agitation (about 250 rpm) during 90 +/- 10 minutes. The reaction was allowed to continue for another 80 +/- 10 minutes and the gel was then washed with >10 gel volumes distilled water on a glass filter until neutral pH was reached. This activated gel was used directly for coupling as below.

- 10 To 16.4 mL of ligand solution (50 mg/mL) in a 50 ml Falcon tube, 139 mg NaHCO<sub>3</sub>, 17.4 mg Na<sub>2</sub>CO<sub>3</sub>, 143.8 mg NaCl and 141 mg EDTA, was added. The Falcon tube was placed on a roller table for 5-10 min, and then 63 mg of DTE was added. Reduction proceeded for >45 min. The ligand solution was then desalted on a PD10 column packed with Sephadex G-25. The ligand content in the desalted solution was determined by measuring the 276 nm UV absorption.
- 15 The activated gel was washed with 3-5 GV {0.1 M phosphate/1 mM EDTA pH 8.6} and the ligand was then coupled according to the method described in US6399750 5.2.2, although with considerably higher ligand amounts (see below). All buffers used in the experiments had been degassed by nitrogen gas for at least 5-10 min. The ligand content of the gels was controlled by varying the amount and concentration of the ligand solution, adding 5-20 mg ligand per ml gel.
- 20 The ligand was either a tetramer (SEQ ID NO. 20) or a hexamer (SEQ ID NO. 33) of an alkali-stabilized mutant.

After immobilization the gels were washed 3xGV with distilled water. The gels + 1 GV {0.1 M phosphate/1 mM EDTA/10% thioglycerol pH 8.6} was mixed and the tubes were left in a shaking table at room temperature overnight. The gels were then washed alternately with 3xGV

{0.1 M TRIS/0.15 M NaCl pH 8.6} and 0.5 M HAc and then 8-10xGV with distilled water. Gel samples were sent to an external laboratory for amino acid analysis and the ligand content (mg/ml gel) was calculated from the total amino acid content.

#### Evaluation

- 5 The Qb10 % dynamic capacity for polyclonal human IgG at 2.4 and 6 min residence time was determined as outlined in Example 4.

Table 7. Prototype results

| Prototype | Base matrix | Ligand content (mg/ml) | Multimer | Qb10% 2.4 min (mg/ml) | Qb10% 6 min (mg/ml) |
|-----------|-------------|------------------------|----------|-----------------------|---------------------|
| N1        | A38         | 7.45                   | tetramer | 44.4                  | 58.25               |
| N2        | A20         | 7.3                    | tetramer | 45.12                 | 57.21               |
| N3        | A42         | 6.72                   | tetramer | 33.56                 | 50.02               |
| N4        | A29         | 7.3                    | tetramer | 36.34                 | 51.8                |
| N5        | A28         | 7.9                    | tetramer | 42.38                 | 58.25               |
| N6        | A39         | 6.96                   | tetramer | 41.88                 | 54.67               |
| N7        | A27         | 7.5                    | tetramer | 29.19                 | 48.73               |
| N8        | A43         | 6.99                   | tetramer | 33.43                 | 49.79               |
| N9        | A38         | 11.34                  | tetramer | 48.1                  | 72.78               |
| N10       | A20         | 10.6                   | tetramer | 50.66                 | 70.07               |
| N11       | A42         | 11.1                   | tetramer | 32.25                 | 57.78               |
| N12       | A29         | 11                     | tetramer | 34.85                 | 64.68               |
| N13       | A28         | 11.9                   | tetramer | 39.92                 | 63.75               |
| N14       | A39         | 10.48                  | tetramer | 44.37                 | 64.79               |
| N15       | A27         | 12.1                   | tetramer | 24.8                  | 55.56               |
| N16       | A43         | 10.51                  | tetramer | 31.82                 | 58.04               |
| N17       | A41         | 8.83                   | tetramer | 38.5                  | 56.8                |
| N18       | A41         | 8.83                   | tetramer | 37.84                 | 58.6                |
| N19       | A41         | 8.83                   | tetramer | 35.06                 | 57.23               |
| N20       | A41         | 5.0                    | tetramer | 35.64                 | 46.04               |
| N21       | A41         | 13.0                   | tetramer | 34.95                 | 62.23               |
| N22       | A40         | 13.15                  | tetramer | 56.85                 | 71.09               |
| N23       | A33         | 7.33                   | tetramer | 48.69                 | 55.76               |
| N24       | A40         | 11.03                  | tetramer | 54.96                 | 73.8                |
| 033A      | A38         | 7.5                    | tetramer | 44                    | 58                  |
| 033B      | A38         | 11.3                   | tetramer | 48                    | 73                  |
| 097A      | A20         | 7.3                    | tetramer | 45                    | 57                  |
| 097B      | A20         | 10.6                   | tetramer | 51                    | 70                  |

|      |      |      |          |     |     |
|------|------|------|----------|-----|-----|
| 003A | A28  | 7.9  | tetramer | 42  | 58  |
| 003B | A28  | 11.9 | tetramer | 40  | 64  |
| 003C | A28  | 15.8 | tetramer | 37  | 67  |
| 038A | A39  | 7.0  | tetramer | 42  | 55  |
| 038B | A39  | 10.5 | tetramer | 44  | 65  |
| 074  | A40  | 13.2 | tetramer | 57  | 71  |
| 093  | A33  | 7.3  | tetramer | 49  | 56  |
| 058A | A40  | 11.0 | tetramer | 55  | 74  |
| 077  | A18  | 8.2  | tetramer | 52  | 59  |
| 010  | A32  | 10.7 | tetramer | 40  | 57  |
| 099  | A32  | 13.3 | tetramer | 37  | 66  |
| 030A | B5   | 6.3  | tetramer | 32  | 38  |
| 030B | B5   | 9.6  | tetramer | 45  | 47  |
| 293A | C1   | 5.4  | tetramer | 38  | 47  |
| 293B | C1   | 10.8 | tetramer | 43  | 60  |
| 294A | C2   | 5.1  | tetramer | 39  | 46  |
| 294B | C2   | 10.5 | tetramer | 42  | 57  |
| 336A | H40  | 5.6  | tetramer | 47  | 52  |
| 336B | H40  | 9.1  | tetramer | 52  | 67  |
| 091  | A18  | 13.4 | tetramer | N/A | 63  |
| 092  | A20  | 12.8 | tetramer | 49  | 67  |
| 080  | A33  | 9.4  | tetramer | 51  | 58  |
| 089  | A40  | 6.1  | tetramer | 49  | 59  |
| 688A | A62a | 6.6  | tetramer | 41  | 46  |
| 688B | A62a | 14.8 | tetramer | 55  | 62  |
| 871  | A62a | 9.7  | tetramer | 48  | 60  |
| 934A | A63a | 6.6  | tetramer | 40  | 44  |
| 934B | A63a | 14.0 | tetramer | 48  | 56  |
| 017B | A65a | 13.1 | tetramer | 56  | 64  |
| 041A | A62b | 5.2  | tetramer | 40  | N/A |
| 041B | A62b | 11.1 | tetramer | 52  | N/A |
| 116A | A65b | 5.8  | tetramer | 42  | 46  |
| 116B | A65b | 8.8  | tetramer | 49  | 56  |

|      |      |      |          |    |    |
|------|------|------|----------|----|----|
| 017A | A65a | 6.1  | tetramer | 40 | 44 |
| 387A | A62a | 6.4  | tetramer | 43 | 45 |
| 387B | A62a | 7.5  | tetramer | 47 | 56 |
| 432  | A63a | 6.1  | tetramer | 39 | 44 |
| 433A | A65a | 6.6  | tetramer | 42 | 47 |
| 433B | A65a | 13.6 | tetramer | 52 | 61 |
| 579A | I1   | 6.1  | tetramer | 45 | 51 |
| 579B | I1   | 11.2 | tetramer | 57 | 68 |
| 064A | C3   | 5.9  | tetramer | 44 | 52 |
| 064B | C3   | 9.0  | tetramer | 49 | 62 |
| 064C | C3   | 14.3 | tetramer | 51 | 70 |
| 352A | C4   | 10.1 | tetramer | 55 | 63 |
| 352B | C4   | 14.4 | tetramer | 59 | 67 |
| 066A | C3   | 6.8  | hexamer  | 48 | 59 |
| 066B | C3   | 11.9 | hexamer  | 51 | 73 |
| 066C | C3   | 15.1 | hexamer  | 43 | 61 |
| 353A | C4   | 11.2 | hexamer  | 62 | 74 |
| 353B | C4   | 15.2 | hexamer  | 57 | 82 |
| 872A | A62a | 9.6  | hexamer  | 56 | 72 |
| 872B | A62a | 14.5 | hexamer  | 62 | 84 |
| 869A | H40  | 6.9  | hexamer  | 50 | 56 |
| 869B | H40  | 14.3 | hexamer  | 56 | 75 |
| 869C | H40  | 23.0 | hexamer  | 41 | 65 |
| 962A | H35  | 6.8  | hexamer  | 36 | 49 |
| 962B | H35  | 12.3 | hexamer  | 31 | 54 |
| 962C | H35  | 20.3 | hexamer  | 20 | 43 |
| 112A | A56  | 7.9  | hexamer  | 47 | 55 |
| 112B | A56  | 12.4 | hexamer  | 57 | 73 |
| 112C | A56  | 19.2 | hexamer  | 55 | 80 |
| 113A | A56  | 7.1  | hexamer  | 48 | 57 |
| 113B | A56  | 12.4 | hexamer  | 53 | 73 |
| 113C | A56  | 15.2 | hexamer  | 48 | 76 |
| 212A | H31  | 6.5  | hexamer  | 37 | 38 |

|      |       |      |         |    |    |
|------|-------|------|---------|----|----|
| 212B | H31   | 10.4 | hexamer | 50 | 61 |
| 212C | H31   | 20.0 | hexamer | 31 | 52 |
| 213A | A33   | 6.5  | hexamer | 44 | 53 |
| 213B | A33   | 10.9 | hexamer | 50 | 65 |
| 213C | A33   | 11.1 | hexamer | 50 | 68 |
| 432A | A20   | 6.4  | hexamer | 41 | 56 |
| 432B | A20   | 12.4 | hexamer | 38 | 64 |
| 432C | A20   | 21.1 | hexamer | 44 | 43 |
| 433A | A38   | 5.9  | hexamer | 47 | 57 |
| 433B | A38   | 11.6 | hexamer | 48 | 72 |
| 433C | A38   | 15.8 | hexamer | 36 | 62 |
| 742A | A54   | 6.7  | hexamer | 38 | 46 |
| 742B | A54   | 12.6 | hexamer | 45 | 52 |
| 742C | A54   | 21.1 | hexamer | 38 | 65 |
| 726A | A63b  | 6.4  | hexamer | 42 | 46 |
| 726B | A63b  | 10.6 | hexamer | 49 | 60 |
| 726C | A63b  | 16.7 | hexamer | 53 | 69 |
| 793A | A56-2 | 6.8  | hexamer | 50 | 58 |
| 793B | A56-2 | 12.5 | hexamer | 59 | 72 |
| 793C | A56-2 | 19.2 | hexamer | 61 | 82 |

### Example 7

A series of prototypes, prepared as above, with different ligand content (tetramer, SEQ ID

NO:20) were incubated in 1 M NaOH for 4, 8 and 31 hours at 22 +/- 2 °C and the dynamic IgG

5 capacity (Qb10%, 6 min residence time) was measured before and after incubation. The prototypes are shown in Table 8 and the results in Figs. 5 and 6. It can be seen that the stability towards this harsh alkali treatment increases with increasing ligand content.

Table 8. Samples for incubation in 1 M NaOH

| Prototype | Ligand content (mg/ml) | Qb10%, 6 min, before incubation (mg/ml) |
|-----------|------------------------|-----------------------------------------|
| N1        | 12                     | 78                                      |
| LE28      | 13                     | 79                                      |
| N17       | 16                     | 73                                      |
| N16       | 20                     | 73                                      |

Example 8

Two crosslinked agarose bead prototypes, prepared as above, with different ligand content (hexamer, SEQ ID NO:33), median bead diameter (d<sub>50,v</sub>) 62 µm and K<sub>d</sub> 0.70 for dextran of Mw 110 kD, were evaluated with a real mAb feed. The ligand content of prototype A was 14.3 mg/ml and of prototype B 18.9 mg/ml. For comparison, the commercial product MabSelect SuRe® LX (GE Healthcare Life Sciences) was used. The resins were packed in Tricorn columns (GE Healthcare Life Sciences) to bed heights of 10 cm, giving bed volumes of 2 ml and the columns were shown to have peak asymmetry within the 0.8-1.5 interval. The sample loaded was a clarified CHO cell supernatant with 4.9 mg/ml monoclonal IgG1 antibody at physiological pH and the experimental conditions were as listed below in Table 9 (CV = column volumes, RT = residence time).

Table 9. Conditions for evaluation with real feed.

|                   |                                                                                      |
|-------------------|--------------------------------------------------------------------------------------|
| Equilibration:    | 3 CV 20 mM phosphate, 150 mM NaCl pH 7.4, RT=3.4 min                                 |
| Sample loading:   | 43 mg mAb/ml resin, RT=6 min                                                         |
| Wash 1:           | 5 CV 20 mM phosphate, 500 mM NaCl pH 7.4, 1.5 CV at RT=6min and 3.5 CV at RT=3.4 min |
| Wash 2:           | 1 CV 50 mM acetate pH 6.0, RT=3.4 min                                                |
| Elution:          | 3 CV 50 mM acetate pH 3.5, RT=6 min, peak collected between 150 mAU-150 mAU          |
| Strip:            | 2 CV 100 mM acetate, RT=3.4 min                                                      |
| CIP:              | 3 CV 0.1 M NaOH, RT=6 min                                                            |
| Re-equilibration: | 5 CV 20 mM phosphate, 150 mM NaCl pH 7.4, RT=3.4 min                                 |

The mAb peak was collected using a UV watch function and the concentration of the mAb was determined by UV measurement at 280 nm (extinction coefficient 1.5). All absorbance detections were performed using a spectrophotometer, including the measurements for the yield calculations.

- 5 Samples for HCP (host cell protein) analyses were prepared by adding 10% Preservation buffer (0.2 M  $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$  (5.3%), 0.2 M  $\text{Na}_2\text{HPO}_4 \cdot 12 \text{H}_2\text{O}$  (94.7%), 0.5% Tween 20, 1% BSA pH 8) to the samples directly after each run made (e.g. 50  $\mu\text{l}$  preservation buffer to 450  $\mu\text{l}$  sample). The HCP content was measured using commercial anti-CHO antibodies (Cygnus Technologies) and a Gyrolab (Gyros AB, Sweden) work station.
- 10 The results are presented in Table 10 below and show that the performance of the prototypes is in the same range as for the commercial product. The HCP content in the feed was 331 000 ppm.

Table 10. Results from real feed evaluation

| Resin             | Yield (%) | Elution pool (CV) | HCP in pool (ppm) |
|-------------------|-----------|-------------------|-------------------|
| MabSelect SuRe LX | 90        | 1.5               | 914               |
| MabSelect SuRe LX | 95        | 1.6               | 1021              |
| Prototype A       | 96        | 1.3               | 1076              |
| Prototype A       | 95        | 1.3               | 1105              |
| Prototype B       | 96        | 1.3               | 1040              |
| Prototype B       | 93        | 1.3               | 1104              |

#### 15 Example 9

- A crosslinked agarose bead matrix prototype, prepared as above, with 14.5 mg/ml ligand (hexamer, SEQ ID NO:33), median bead diameter ( $d_{50,v}$ ) 57.4  $\mu\text{m}$ ,  $K_d$  0.72 for dextran of Mw 110 kD and dry weight 70.3 mg/ml, was evaluated for elution pH with two real mAb feeds (mAb1 2.4 g/l and mAb2 4.9 g/l) IgG1, physiological pH, and a sample of polyclonal human IgG
- 20 (Gammanorm, Octapharma). For comparison, the commercial product MabSelect SuRe<sup>®</sup> LX (GE Healthcare Life Sciences) was used. The resins were packed in Tricorn columns (GE Healthcare Life Sciences) to bed heights of 10 cm, giving bed volumes of 2 ml and the columns were shown

to have peak asymmetry within the 0.8-1.5 interval. The samples loaded were clarified CHO cell supernatants with IgG1 mAbs at physiological pH and the experimental conditions were as listed below in Table 11 (CV = column volumes, RT = residence time).

Table 11. Conditions for elution pH evaluation.

|                   |                                                      |
|-------------------|------------------------------------------------------|
| Equilibration:    | 5 CV 20 mM phosphate, 150 mM NaCl pH 7.4, RT=3.4 min |
| Sample loading:   | 10 mg mAb/ml resin, RT=6 min                         |
| Wash:             | 6 CV 20 mM phosphate, 150 mM NaCl pH 7.4, RT=3.4 min |
| Elution:          | 30 CV 100 mM citrate pH 6-3 gradient, RT=6 min       |
| CIP:              | 3 CV 0.1 M NaOH, RT=6 min                            |
| Re-equilibration: | 8 CV 20 mM phosphate, 150 mM NaCl pH 7.4, RT=3.4 min |

5

The results are shown below in Table 12 and indicate that the antibodies elute at similar pH levels as on the reference, although with some individual variation depending on the particular antibody-resin combination.

Table 12. Results from elution pH evaluation

| Sample         | Elution pH MabSelect SuRe LX   | Elution pH prototype           |
|----------------|--------------------------------|--------------------------------|
| mAb 1          | 3.67                           | 3.53                           |
| mAb 2          | 3.68                           | 3.80                           |
| Polyclonal IgG | 4.01 (peak 1)<br>3.70 (peak 2) | 4.24 (peak 1)<br>3.81 (peak 2) |

10

Fractions from the pH-gradient elution of polyclonal IgG were also analysed with respect to content of IgG1, IgG2 and IgG4, using a Biacore SPR instrument (GE Healthcare Life Sciences) with antibodies against the four different IgG classes immobilized on a CM5 Biacore chip.

The chromatograms for polyclonal IgG on the reference and the prototype are shown in Fig 7 and the IgG class analyses are shown in Fig. 8. The data show that all three classes bind to both resins in a similar way and that the first peak predominantly contains IgG2, while IgG1 and IgG4 elute mainly in the second peak. The anti-IgG3 antibodies cross-reacted with IgG4, so no

15

reliable results for IgG3 were obtained. IgG3 is generally known to show no or only weak binding to Protein A.

#### Example 10

5 A crosslinked agarose bead matrix prototype, prepared as above, with 12.6 mg/ml ligand (tetramer, SEQ ID NO:20), 84.9  $\mu\text{m}$  median bead diameter ( $d_{50,v}$ ),  $K_d$  0.71 for dextran  $M_w$  110 kD and 62.2 mg/ml dry weight, was evaluated with respect to alkali stability, using the commercial product MabSelect SuRe LX as a reference. Tricorn 5 columns packed with the resins to 10 cm bed height were flushed with 3 column volumes of 1 M NaOH. The flow was  
10 then stopped for 240 minutes (corresponding to 16 normal CIP cycles of 15 min/cycle) before washing out the NaOH solution by 3 column volumes of PBS buffer. The dynamic binding capacity for polyclonal IgG (Gammanorm, Octapharma) was then measured and the process was repeated with another injection of 1 M NaOH. The dynamic capacity was measured after each 240 min incubation cycle with 1 M NaOH. In the capacity measurements, the columns  
15 were equilibrated with PBS buffer before the 2 mg/ml sample was loaded (residence time 6 min) until a UV signal of 85% of maximum absorbance was reached. Then the column was washed with PBS buffer, eluted with 500 mM acetic acid pH 3.0 and re-equilibrated. The dynamic binding capacity at 10% and 80% breakthrough was calculated as described above. The results are shown in Fig. 9 and they show that the prototype was significantly more stable than  
20 the commercial product.

#### Example 11

A separation matrix according to the invention was tested for tolerance to repeated CIP cycles using 2M NaOH. The inventive example (Inv. Ex.) was compared to the commercial product  
25 MabSelect SuRe (MSS) as a reference. The study was performed for 50 cycles using an ÄKTA 4-column periodic counter current (PCC) chromatography setup from GE Healthcare.

Each column was packed using a solution of 20% ethanol + 0.2 M NaCl, with a flow of 3.5 ml/min for 10 min. The packing tube and top filter were removed and the adaptor was placed on the top of column. Following a further packing flow of 3.5 ml/min for 10 min, the adaptor was adjusted

against the bed surface. The packed column volume  $V_c$  for the inventive example was 1.04 ml, as compared to 1.02 ml for MabSelect SuRe.

The PCC setup was then run using the following conditions:

|                   |                                                              |
|-------------------|--------------------------------------------------------------|
| Equilibration:    | 3 column volumes (CV) 20 mM phosphate, 150 mM NaCl pH 7.4    |
| Sample loading:   | mAb 4.28 mg/ml; load until 35% breakthrough DBC; RT=2.4 min  |
| Wash 1:           | 1.5 CV 20 mM phosphate, 500 mM NaCl pH 7.0 200 cm/h + 3.5 CV |
| Wash 2:           | 1 CV 50 mM acetate pH 6.0                                    |
| Elution:          | 3 CV 50 mM acetate pH 3.5                                    |
| Strip:            | 2 CV 100 mM acetic acid pH 2.9                               |
| CIP:              | 3 CV 2 M NaOH (contact time 15 min)                          |
| Re-equilibration: | 10 CV 20 mM phosphate, 150 mM NaCl pH 7.4                    |

- 5 The results are shown in Figure 10, where the load volume to 35% breakthrough dynamic binding capacity of each matrix is normalized relative to its load volume to 35% DBC. It can be seen that the commercial reference MabSelect SuRe (MSS) loses a proportion of its immunoglobulin binding capacity each cycle and after 16 cycles has less than 80% of its original capacity remaining. The inventive example (Inv. Ex.) on the other hand shows little loss in binding capacity
- 10 and has over 95% of its original binding capacity remaining after 50 CIP cycles in 2 M NaOH, equivalent to a contact time of 750 minutes.

### Example 12

The ligand stability towards 1 M and 2 M NaOH was studied for a separation matrix according to the invention, using confocal microscopy. The inventive example (Inv. Ex.) was compared to the commercial product MabSelect SuRe LX (MSS LX) as a reference. The study was performed as follows:

#### Separation matrix preparation

Separation matrix gels were washed from their storage solution to water through centrifugation, and a 1:1 gel slurry was obtained. For each gel, 2 ml of the 1:1 slurry in water was added to a 50

mL Falcon tube, centrifuged and decanted. To each tube was added 19 ml of 1 M or 2 M NaOH (2 separation matrices \* 2 [NaOH] concentrations → 4 tubes in total). The gels were incubated at room temperature with shaking on Heidolph shaker (1300 rpm). A sample of 1500 µl of gel slurry was taken after 2, 4, 6, 8, 16, 24 and 32 h of incubation and washed in 2 ml Eppendorf tubes through centrifugation (13000 rpm, Eppendorf centrifuge). The washing steps were as follows:

2x1.8 mL MQ water

1x1.8 mL HAc buffer

2x1.8 mL Tris buffer

10 2x1.8 mL PBS buffer

After final decantation, the gels were resuspended with 75 µl PBS buffer to obtain approximately 1:1 PBS gel slurries.

#### Preincubation

A sample of each gel that had not been incubated in NaOH, i.e. the original 1:1 gel water slurry, was exchanged to give a 1:1 PBS slurry. A 16 µl sample of each gel slurry was added to 500 µl of Cy5-hlgG solution (Cy5-labelled human immunoglobulin G) and mixed end-over-end over the weekend, at room temperature, covered in tin foil.

#### Microscope settings

A Leica SP8 Confocal Microscope was used for the study. Microscope settings were determined using the inventive example and checked using MSS LX in order to make sure saturation was not obtained with either of the gels.

Objective: 63x/130 Glyc 21C (Leica)

Laser: 638 nm

Detector PTM gain: 629.6 V

25 Detector offset: -0.8%

Scanning speed: 400 Hz

Frame size: 512x512 pixels

Frame average: 2

Kinetics experiment (Adsorption of hlgG in gels as a function of time)

A sample of 15 µl of each NaOH-incubated and washed 1:1 gel slurry was added to 500 µl of Cy5-hlgG, giving a mixture of >300 mg Ab/ml gel. These mixtures were Incubated at room temperature on Heidolph shaker at 1300 rpm. At predetermined times (5, 10, 15, 30, 60, 90, 120, 180 and 240 minutes), 15 µl samples were taken out and imaged using the microscope. The confocal images were integrated and adsorption curves were obtained by calculating the relative fluorescence,  $Q_{rel}$ , of each bead, see eq 1.

$$Q_{rel} = \frac{F_{ring/volume} \cdot (r_{out}^3 - r_{in}^3)}{r_{out}^3} \quad (\text{eq. 1})$$

where  $r_{out}$  and  $r_{in}$  denote the outer and inner boundary radius of the fluorescent region of the bead (determined using the line tool in the microscope software, LAS-X) and

$$F_{ring/volume} = \frac{F_{ring}}{\frac{4}{3}\pi r_{out}^3} \quad (\text{eq. 2})$$

where  $F_{ring}$  is the total fluorescence of the fluorescent region of the bead, determined using the circle tool in LAS-X.

Combining eq 1 and 2 yields eq 3:

$$Q_{rel} = \frac{F_{ring} \cdot (r_{out}^3 - r_{in}^3)}{\frac{4}{3}\pi r_{out}^6} \quad (\text{eq. 3}).$$

This procedure follows from the work presented in A. Ljunglöf, J. Thömmes J. Chromatogr. A 813 (1998) 387-395.

Results, analysis and conclusions

The determined relative fluorescence  $Q_{rel}$  of the separation matrices as a function of time after incubation in 1M NaOH are shown as Figures 11a (MabSelect SuRe LX) and 11b (Inventive example). It can be seen that MSS LX (Fig. 11a) lost approximately 50% of its original binding capacity and reaches saturation faster following incubation for 16 hours in 1 M NaOH, as compared to the reference (no alkali treatment, i.e. 0 hours). Following incubation for 32 hours in 1M NaOH it has lost approximately all Ig-binding capacity. In contrast, the separation matrix of the inventive example (Fig. 11b) is stable to incubation in 1 M NaOH and maintains its original binding capacity after 32 hours incubation in 1 M NaOH, within experimental variation.

The determined relative fluorescence  $Q_{rel}$  of the separation matrices as a function of time after incubation in 2M NaOH are shown as Figures 12a (MabSelect SuRe LX) and 12b (Inventive example). It can be seen that the rate of capacity loss for MSS LX (Fig. 12a) is accelerated with incubation in 2M NaOH. After incubation for 8 hours approximately 50% of Ig-binding capacity is lost, and following incubation for 16 hours in 2M NaOH, more or less all Ig-binding capacity is lost. In contrast, the inventive example (Fig. 12b) is relatively stable to alkali and maintains, at least within experimental variation, its original binding capacity after 16 h or less of incubation in 2 M NaOH. However, it can be seen that after 24 h or more of incubation in 2 M NaOH the inventive example has lost binding capacity, and that approximately 50% of the binding capacity has been lost after 32 h of incubation. Also, saturation is reached faster as the binding capacity is lost, most clearly seen with the 32 h incubated samples.

These results demonstrate that the separation matrix of the inventive example is significantly more alkali stable than MabSelect SuRe LX and appears to withstand up to 16 h of 2 M NaOH incubation without significantly affecting mass transport and binding capacity.

### Example 13

The inactivation of *Bacillus subtilis* spores was investigated using various cleaning liquids according to the invention. Spore-forming *Bacillus subtilis* (ATCC No. 6633) is known to be among the more resistant microorganisms to NaOH sanitization. Colonies of *B. subtilis* were incubated with various cleaning liquids (1M NaOH, 1M NaOH with 2% BnOH, and 1M NaOH with 40% IPA), and the number of colony forming units (CFU/ml) was determined at various predetermined incubation times. Figure 13 shows the results of the study as CFU/ml as a function of incubation time and cleaning liquid. It can be seen that using 1M NaOH by itself is insufficient to fully inactivate *B. subtilis* even after 24 hours of sanitization. Adding 2% benzyl alcohol to the cleaning liquid does not provide any significant improvement. However, the use of 1M NaOH with 40% isopropyl alcohol as cleaning liquid allows the reduction of *B. subtilis* to below detectable levels after 4 hours of treatment.

## CLAIMS

1. A method of cleaning and/or sanitizing a separation matrix comprising multimers of immunoglobulin-binding alkali-stabilized Protein A domains covalently coupled to a porous support, wherein the method comprises the steps of:
    - 5 a) optionally purifying a mixture comprising a first immunoglobulin using the separation matrix;
    - b) providing a cleaning liquid comprising at least 50% by volume of an aqueous alkali metal hydroxide solution; and
    - 10 c) cleaning and/or sanitizing the separation matrix by contacting the cleaning liquid with the separation matrix for a predetermined contact time;

wherein the alkali-stabilized Protein A domains comprise mutants of a parental Fc-binding domain of Staphylococcus Protein A (SpA), as defined by SEQ ID NO 51 or SEQ ID NO 52, wherein the amino acid residues at positions 13 and 44 of SEQ ID NO 51 or 52 are asparagines and wherein at least the asparagine residue at position 3 of SEQ ID NO 51 or

  - 15 52 has been mutated to an amino acid selected from the group consisting of glutamic acid, lysine, tyrosine, threonine, phenylalanine, leucine, isoleucine, tryptophan, methionine, valine, alanine, histidine and arginine, such as to glutamic acid.
2. The method of claim 1, wherein the mutants comprise further mutations in one or more of positions 1, 2, 7, 10, 15, 20, 21, 24, 25, 28, 29, 32, 34, 35, 36, 39, 42 and 43 in SEQ ID
  - 20 NO 51 or 52.
3. A method according to claim 1 or 2, wherein the glutamine residue at position 1 of SEQ ID NO 51 or 52 has been mutated to an alanine.
  4. A method according to any one of claims 1-3, wherein the asparagine or glutamic acid residue at position 35 of SEQ ID NO 51 or 52 has been mutated to an alanine.
  - 25 5. A method according to any one of the preceding claims, wherein the multimers of immunoglobulin-binding alkali-stabilized Protein A domains are homomultimers selected

from the group consisting of dimers, trimers, tetramers, pentamers, hexamers, heptamers, octamers or nonamers.

- 5 6. A method according to any one of the preceding claims, wherein the multimers of immunoglobulin-binding alkali-stabilized Protein A domains each comprise a C-terminal cysteine residue for covalent coupling to the porous support.
7. A method according to any one of the preceding claims, wherein the multimers of immunoglobulin-binding alkali-stabilized Protein A domains are coupled to the porous support via thioether links.
- 10 8. A method according to any one of the preceding claims, wherein the separation matrix comprises at least 11 mg/ml, such as at least 15 mg/ml, 15-21, 17-21 or 18-20 mg/ml of the multimers of immunoglobulin-binding alkali-stabilized Protein A domains covalently coupled to the porous support.
9. A method according to any one of the preceding claims, wherein the porous support is highly cross-linked agarose beads.
- 15 10. A method according to any one of the preceding claims, wherein the aqueous alkali metal hydroxide solution is sodium hydroxide solution, potassium hydroxide solution or a mixture thereof, preferably sodium hydroxide solution.
11. A method according to any one of the preceding claims, wherein the aqueous alkali metal hydroxide solution has a molarity of from 500 mM to 5 M, such as from 1 M to 2 M.
- 20 12. A method according to any one of the preceding claims, wherein the cleaning liquid further comprises a C<sub>2</sub> – C<sub>7</sub> alcohol, such as ethanol, isopropanol or benzyl alcohol.
13. A method according to any one of the preceding claims, wherein the cleaning liquid comprises at least 70% by volume aqueous alkali metal hydroxide solution, such as at least 90% by volume aqueous alkali metal hydroxide solution, preferably at least 99% by volume aqueous alkali metal hydroxide solution.
- 25 14. A method according to any one of the preceding claims, wherein the predetermined contact time is a time sufficient to provide a 6-log<sub>10</sub> reduction in endotoxin concentration and/or microorganism concentration in the separation matrix.

15. A method according to any one of the preceding claims, wherein the predetermined contact time is from 10 minutes to 50 hours, such as from 30 minutes to 24 hours, or such as from 1 hour to 12 hours.
16. A method according to any one of the preceding claims, wherein the steps a) – c) are repeated at least 10 times, such as at least 50 times or 50 – 200 times.
17. A method of preventing carryover in the purification of immunoglobulins with a separation matrix comprising multimers of immunoglobulin-binding alkali-stabilized Protein A domains covalently coupled to a porous support, wherein the alkali-stabilized Protein A domains comprise mutants of a parental Fc-binding domain of Staphylococcus Protein A (SpA), as defined by SEQ ID NO 51 or SEQ ID NO 52, wherein the amino acid residues at positions 13 and 44 of SEQ ID NO 51 or 52 are asparagines and wherein at least the asparagine residue at position 3 of SEQ ID NO 51 or 52 has been mutated to an amino acid selected from the group consisting of glutamic acid, lysine, tyrosine, threonine, phenylalanine, leucine, isoleucine, tryptophan, methionine, valine, alanine, histidine and arginine; and wherein the method comprises:
- purifying a first immunoglobulin and cleaning and sanitizing the separation matrix by performing the steps a) – c) of the method of cleaning and/or sanitizing a separation matrix according to any one of claims 1-15; and further comprises the step of
- d) purifying a mixture comprising a second immunoglobulin using the separation matrix, wherein the second immunoglobulin is different from the first immunoglobulin.
18. The method according to any one of the preceding claims, wherein the separation matrix retains at least 80% of its original dynamic binding capacity after step c), such as at least 90% of its original dynamic binding capacity.
19. Use of a cleaning liquid comprising at least 70% by volume of an aqueous alkali metal hydroxide solution for the sanitization of a separation matrix comprising multimers of immunoglobulin-binding alkali-stabilized Protein A domains covalently coupled to a porous support.

| Alignment of Fc-binding domains |     |            |            |             |            |            |           |    |                 |
|---------------------------------|-----|------------|------------|-------------|------------|------------|-----------|----|-----------------|
| E                               | --- | -----AQQ   | NAFYQVLNMP | NLNADQRNGF  | IQSLKDDPSQ | SANVLGEAQK | LNDSQAPK  | 51 | (SEQ ID NO: 1)  |
| D                               | ADA | QQNKENKDQQ | SAFYEILNMP | NLNEEQRNGF  | IQSLKDDPSQ | STNVLGEAKK | LNESQAPK  | 61 | (SEQ ID NO: 2)  |
| A                               | --A | DNN-FNKEQQ | NAFYEILNMP | NLNEEQRNGF  | IQSLKDDPSQ | SANLLAEAKK | LNESQAPK  | 58 | (SEQ ID NO: 3)  |
| B                               | --- | ADNKENKEQQ | NAFYEILHLP | NLNEEQRNGF  | IQSLKDDPSQ | SANLLAEAKK | LNDAAQPK  | 58 | (SEQ ID NO: 4)  |
| C                               | --- | ADNKENKEQQ | NAFYEILHLP | NLTTEEQRNGF | IQSLKDDPSV | SKEILAEAKK | LNDAAQPK  | 58 | (SEQ ID NO: 5)  |
| Z                               | --- | VDNKENKEQQ | NAFYEILHLP | NLNEEQRNAF  | IQSLKDDPSQ | SANLLAEAKK | LNDAAQPK  | 58 | (SEQ ID NO: 6)  |
| Zvar                            | --- | VDAKFDKEQQ | NAFYEILHLP | NLTTEEQRNAF | IQSLKDDPSQ | SANLLAEAKK | LNDAAQPK  | 58 | (SEQ ID NO: 7)  |
|                                 |     |            |            |             |            |            |           |    |                 |
|                                 | --- | -----QQ    | NAFYEILHLP | NLTTEEQRNAF | IQSLKDDPSQ | SANLLAEAKK | LNDAAQ--- | 47 | (SEQ ID NO: 51) |
|                                 | --- | -----QQ    | NAFYEILHLP | NLTTEEQRNGF | IQSLKDDPSV | SKEILAEAKK | LNDAAQ--- | 47 | (SEQ ID NO: 52) |
| Pos                             | 1   | 10         | 20         | 30          | 40         | 50         | 58        |    |                 |

Fig. 1

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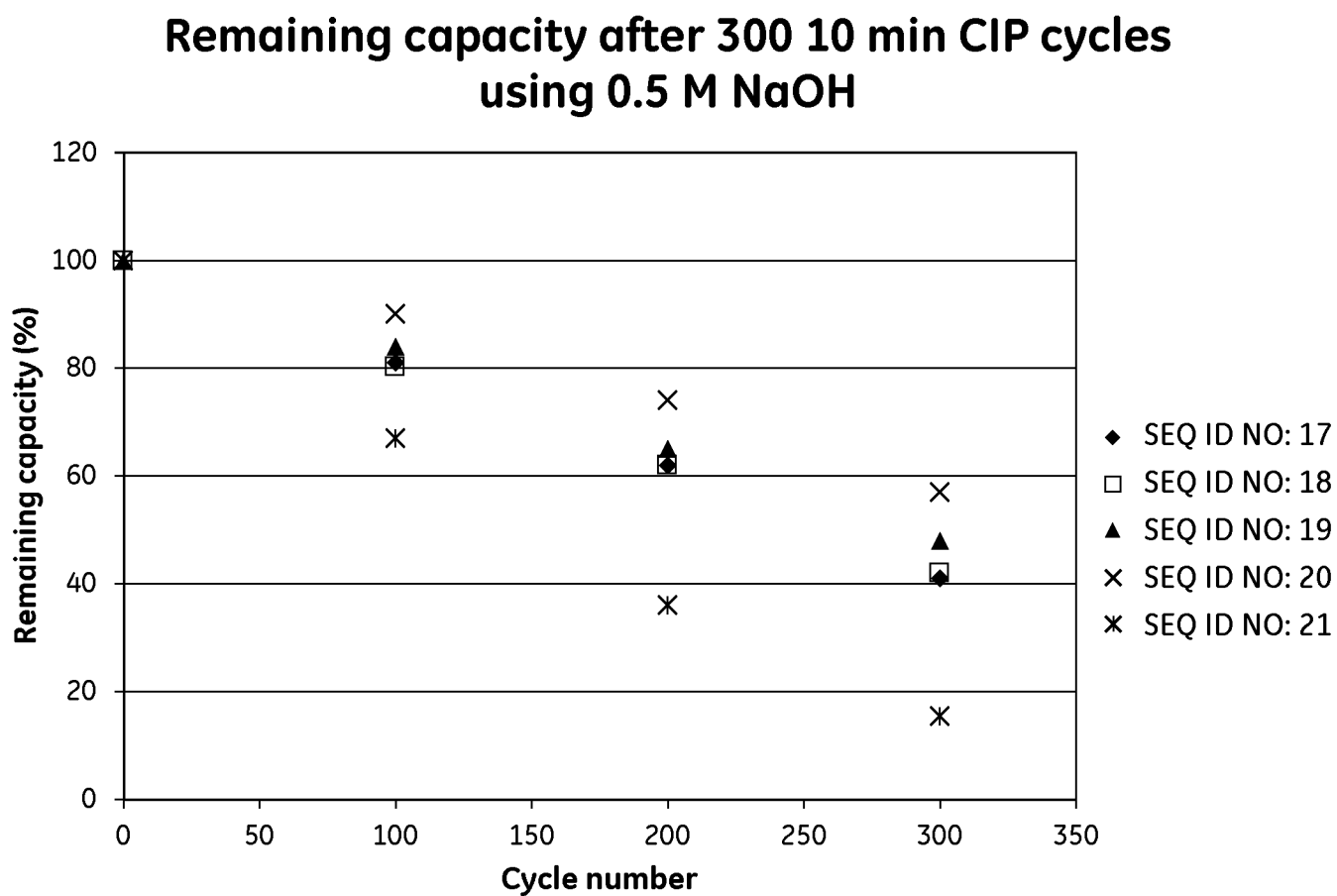


Fig. 2.

3/13

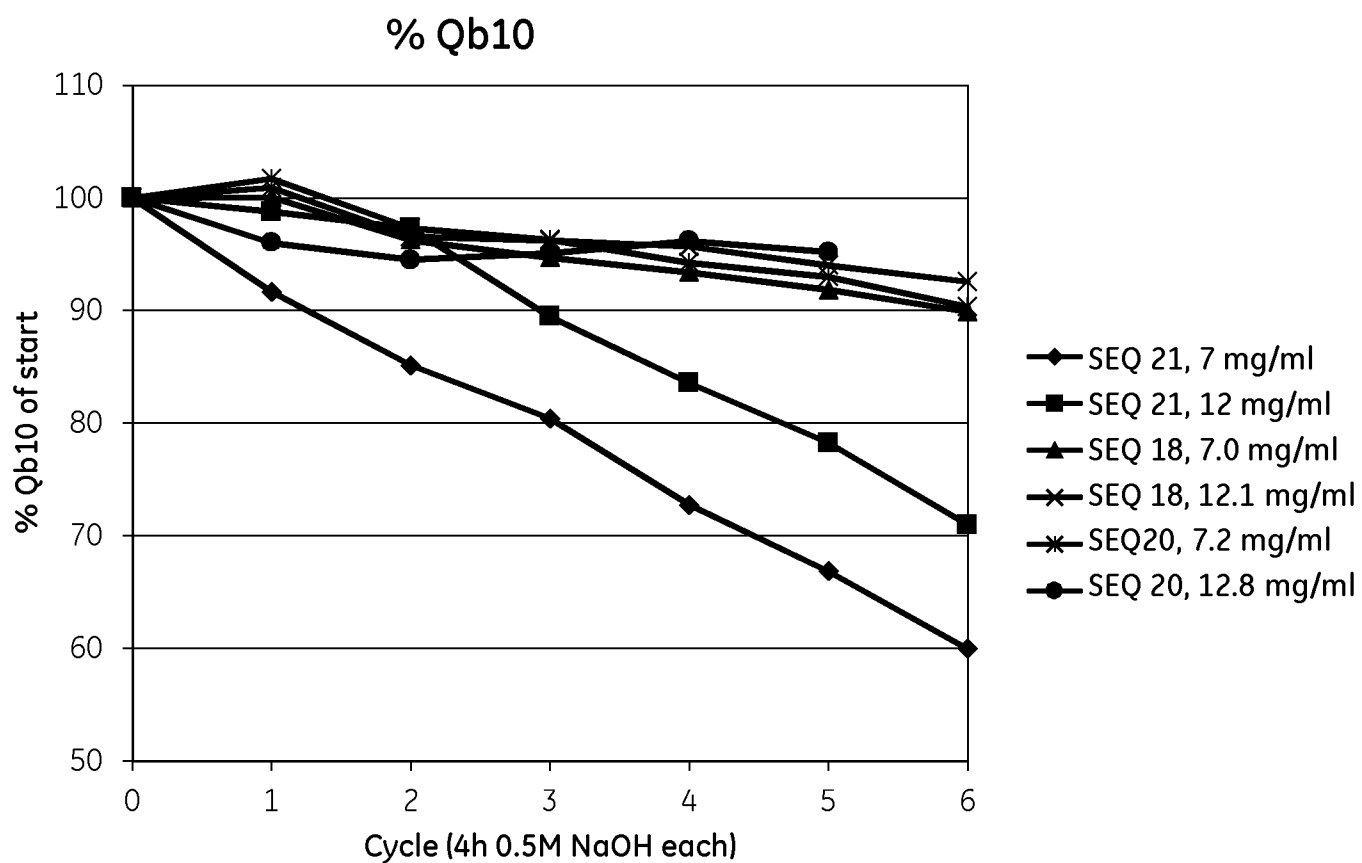


Fig. 3.

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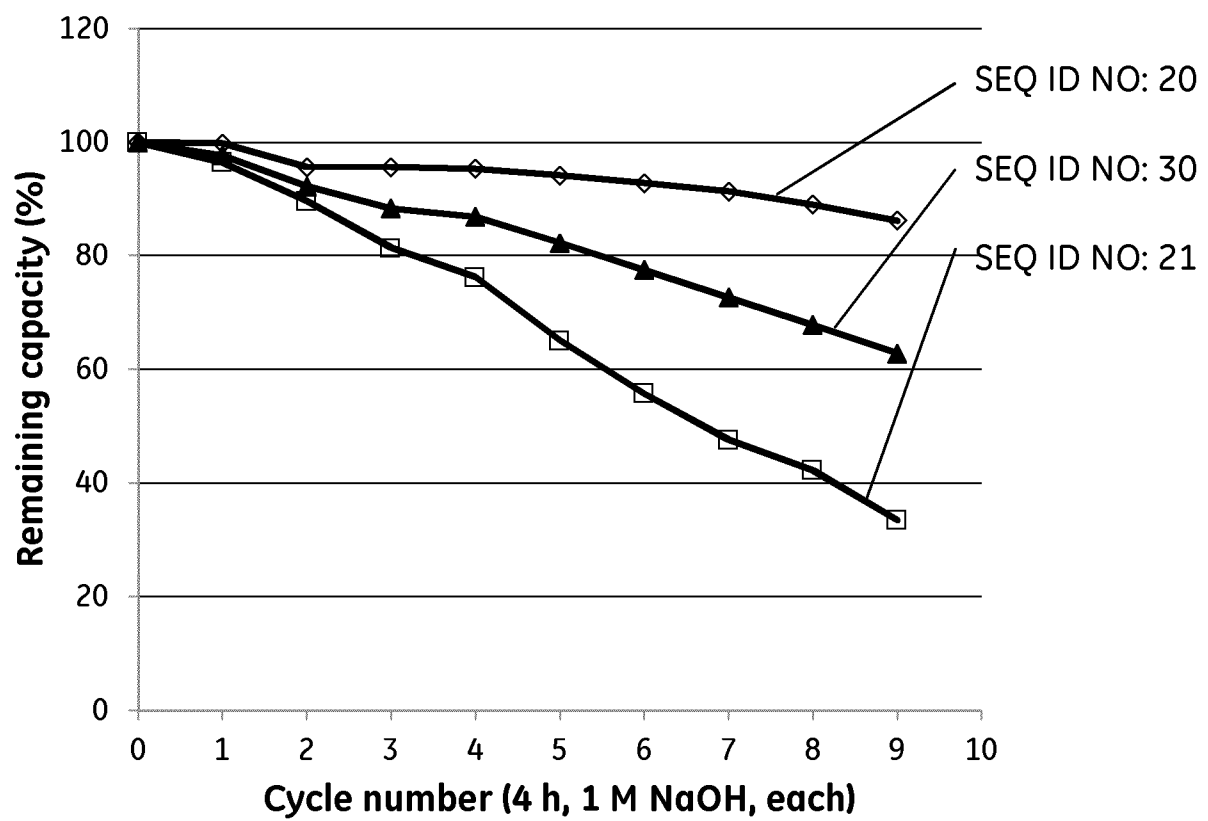


Fig. 4.

5/13

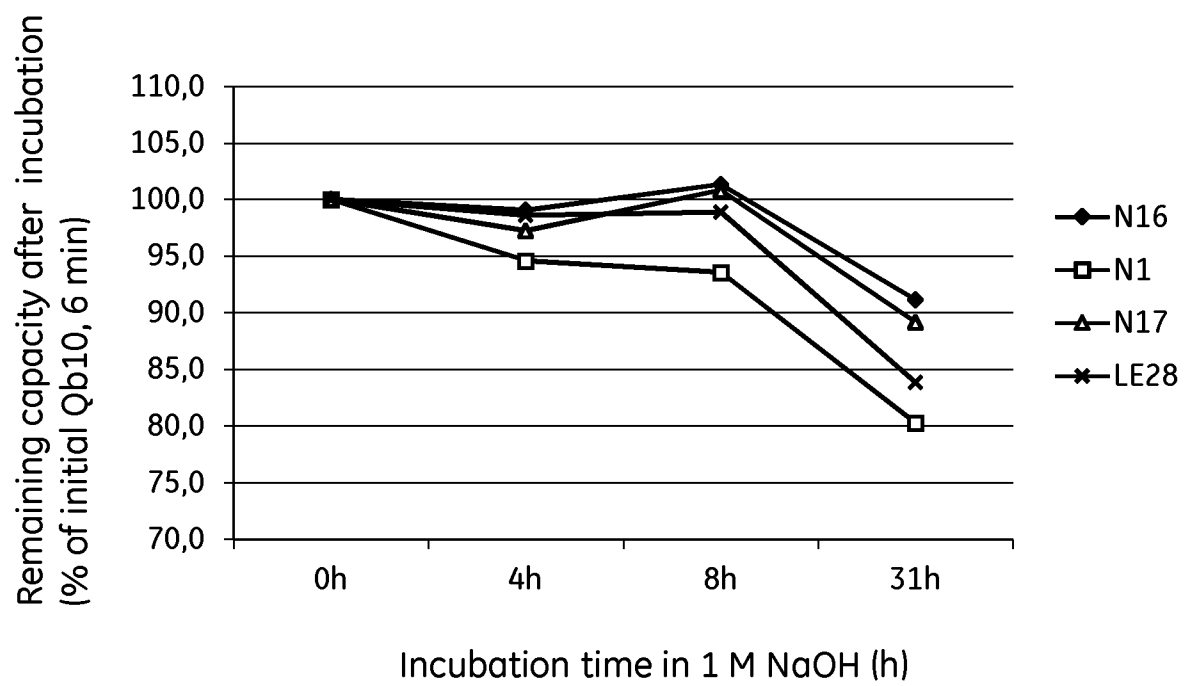


Fig. 5.

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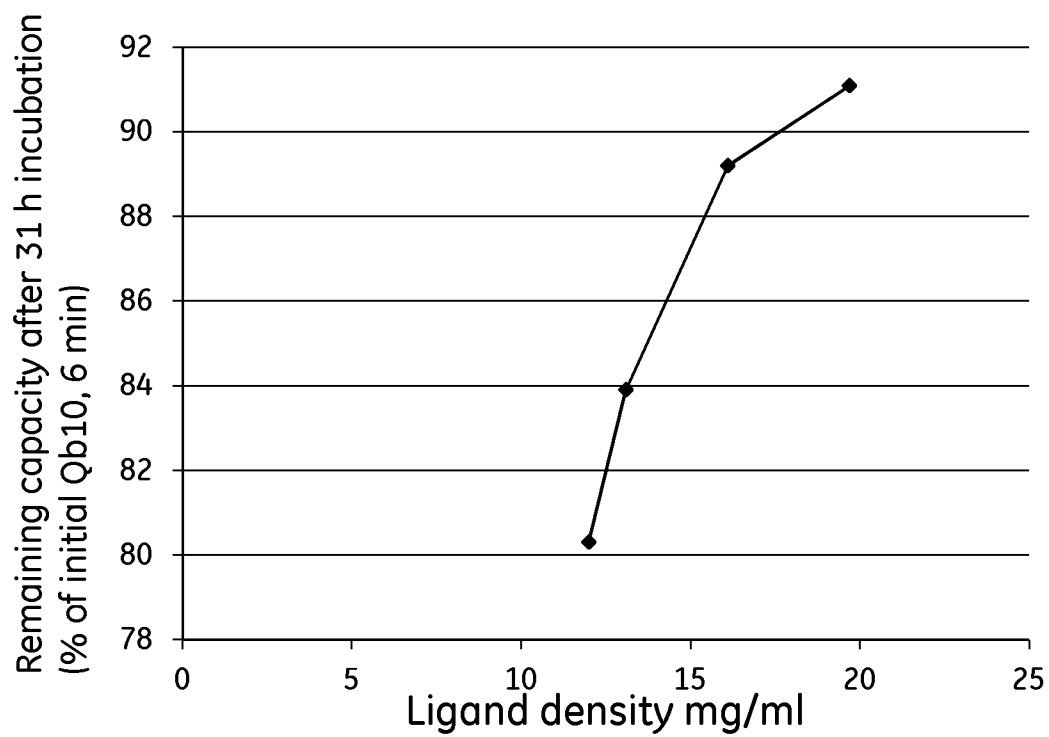


Fig. 6.

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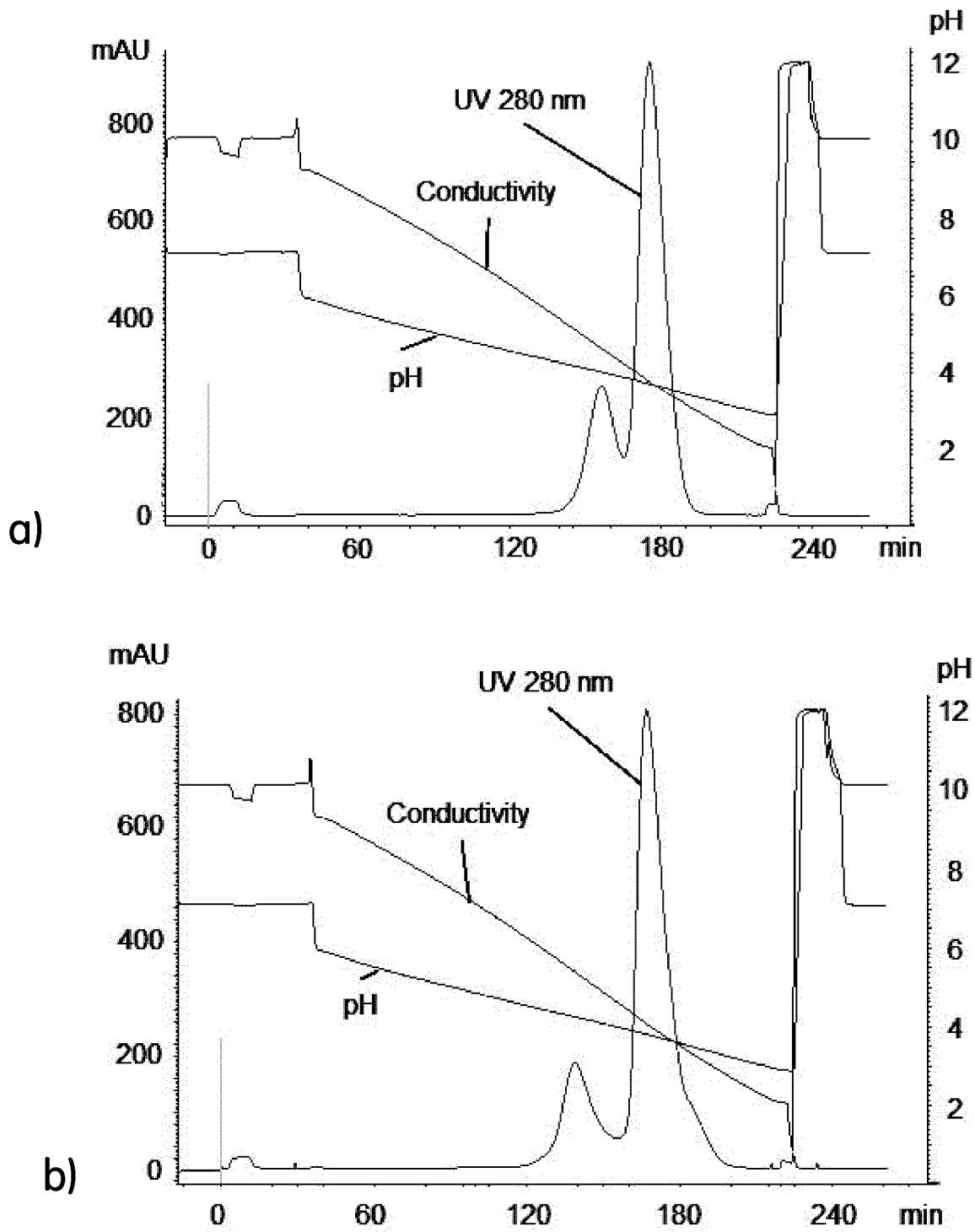


Fig. 7.

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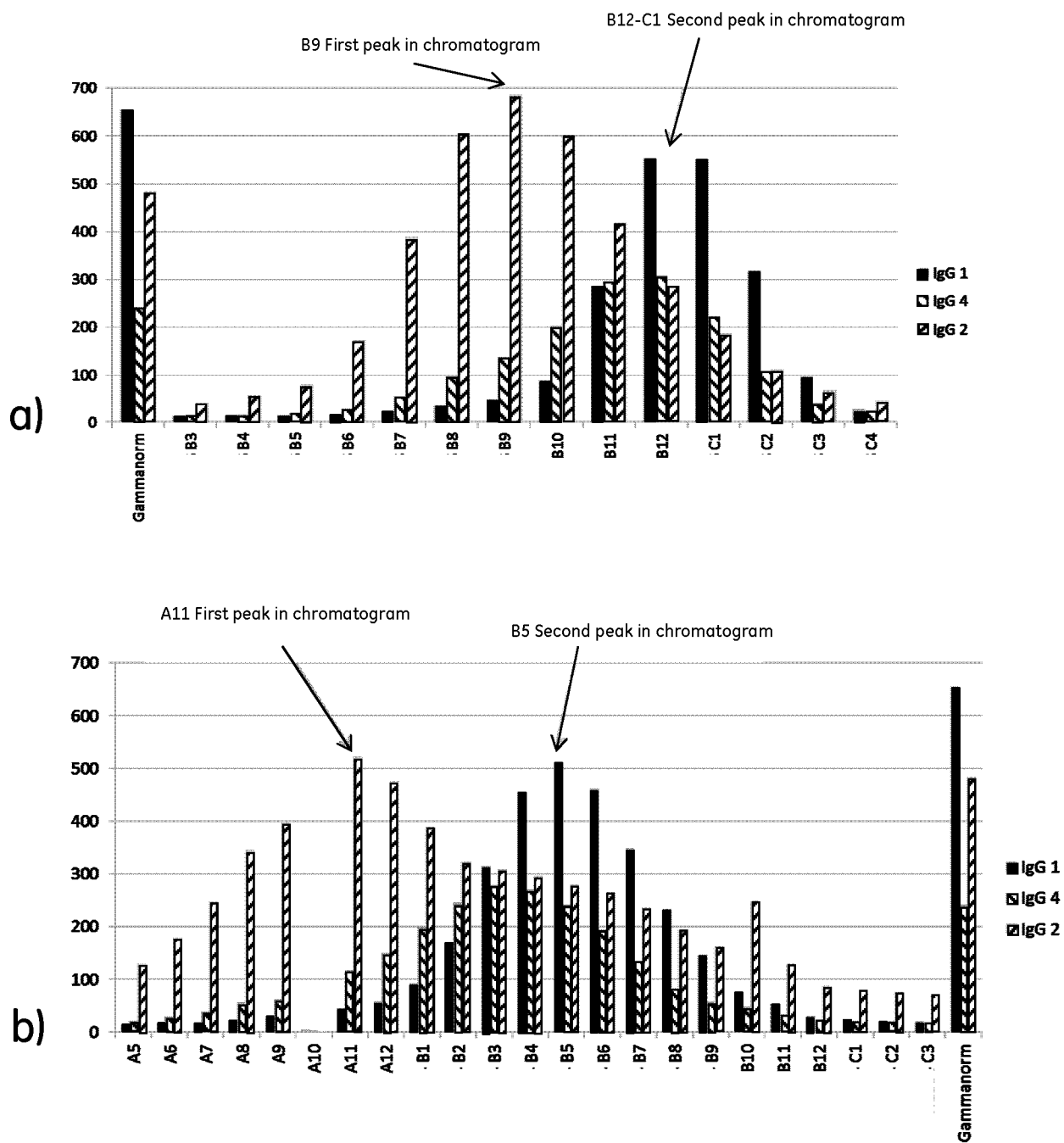


Fig. 8.

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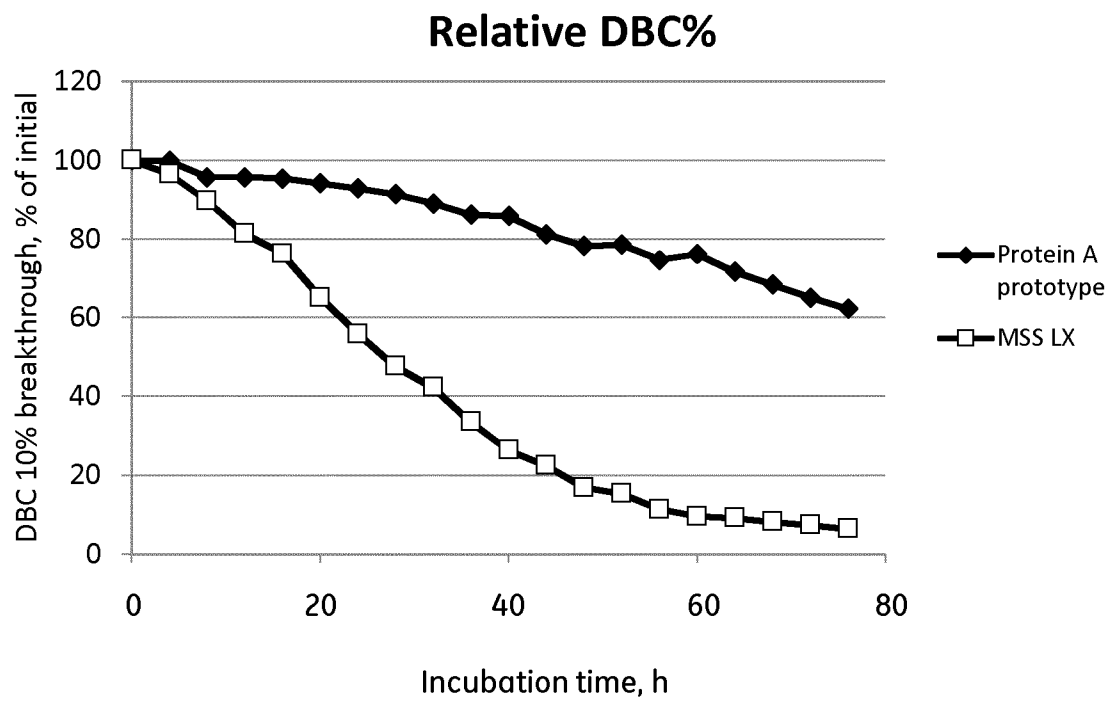


Fig. 9.

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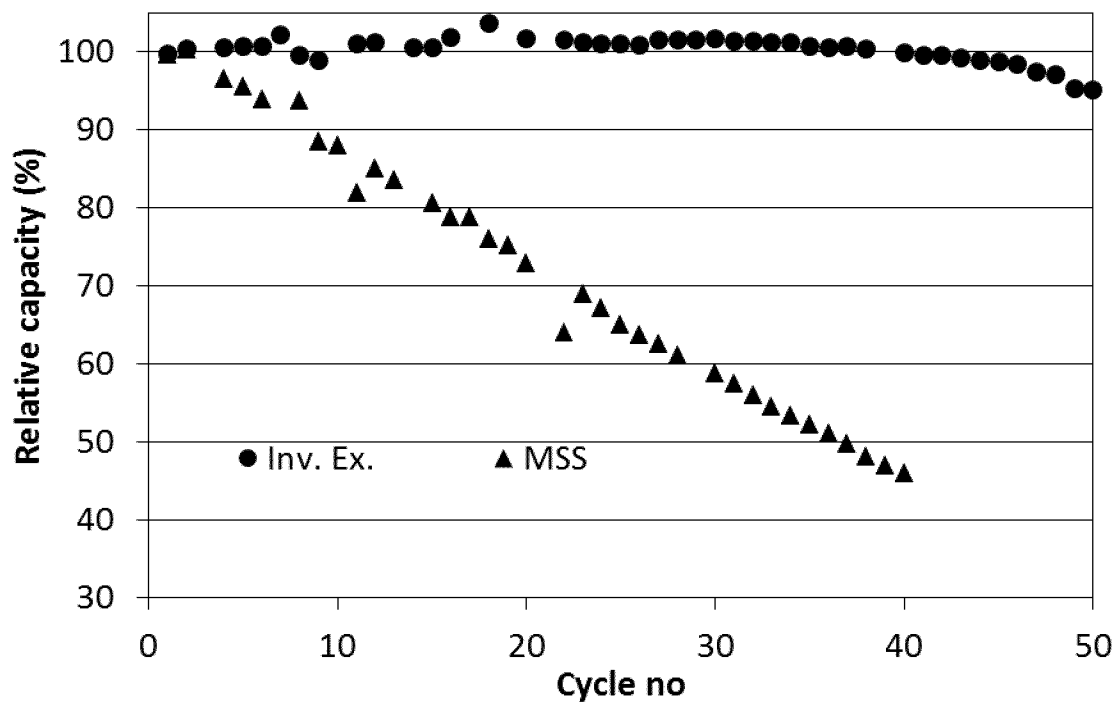


Fig. 10.

11/13

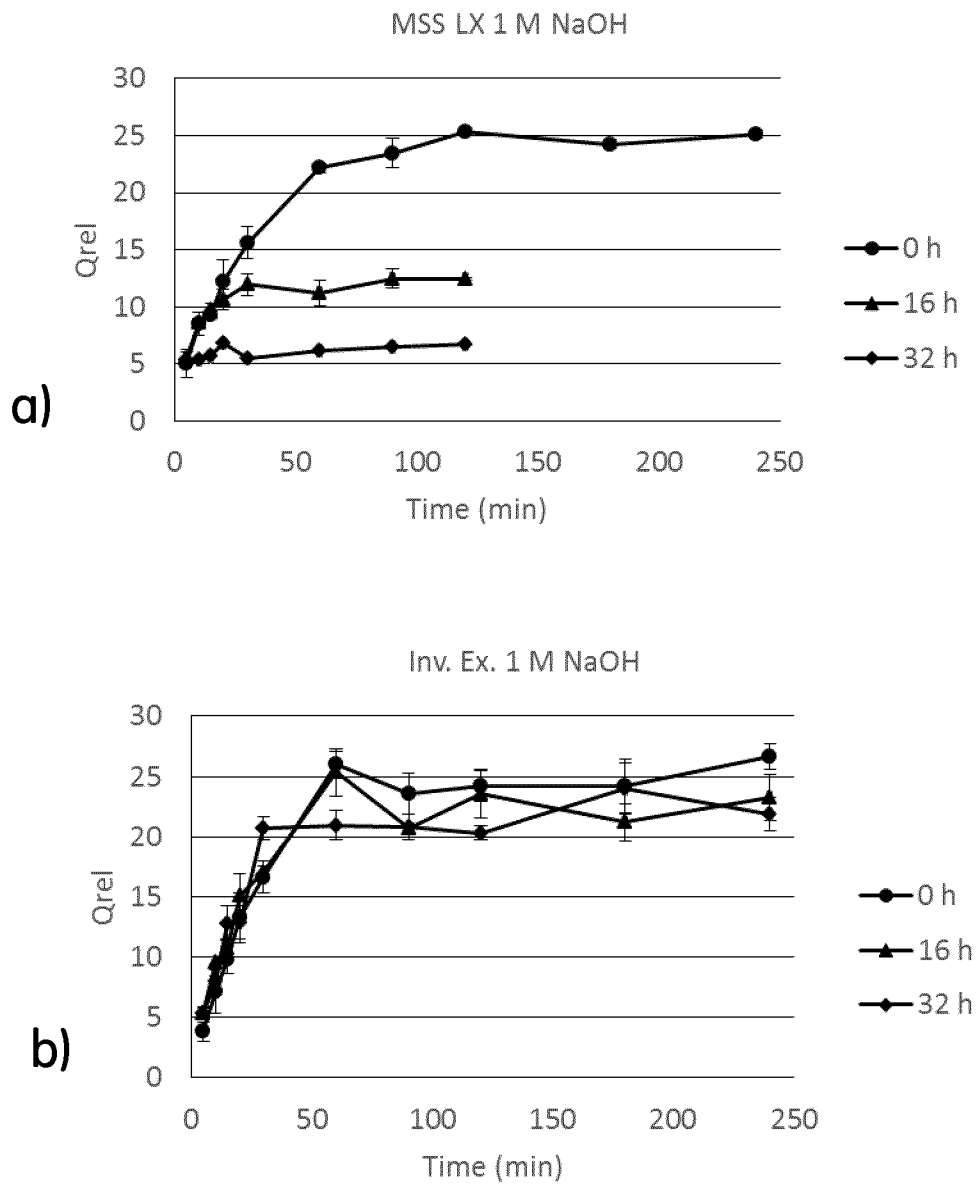


Fig. 11.

12/13

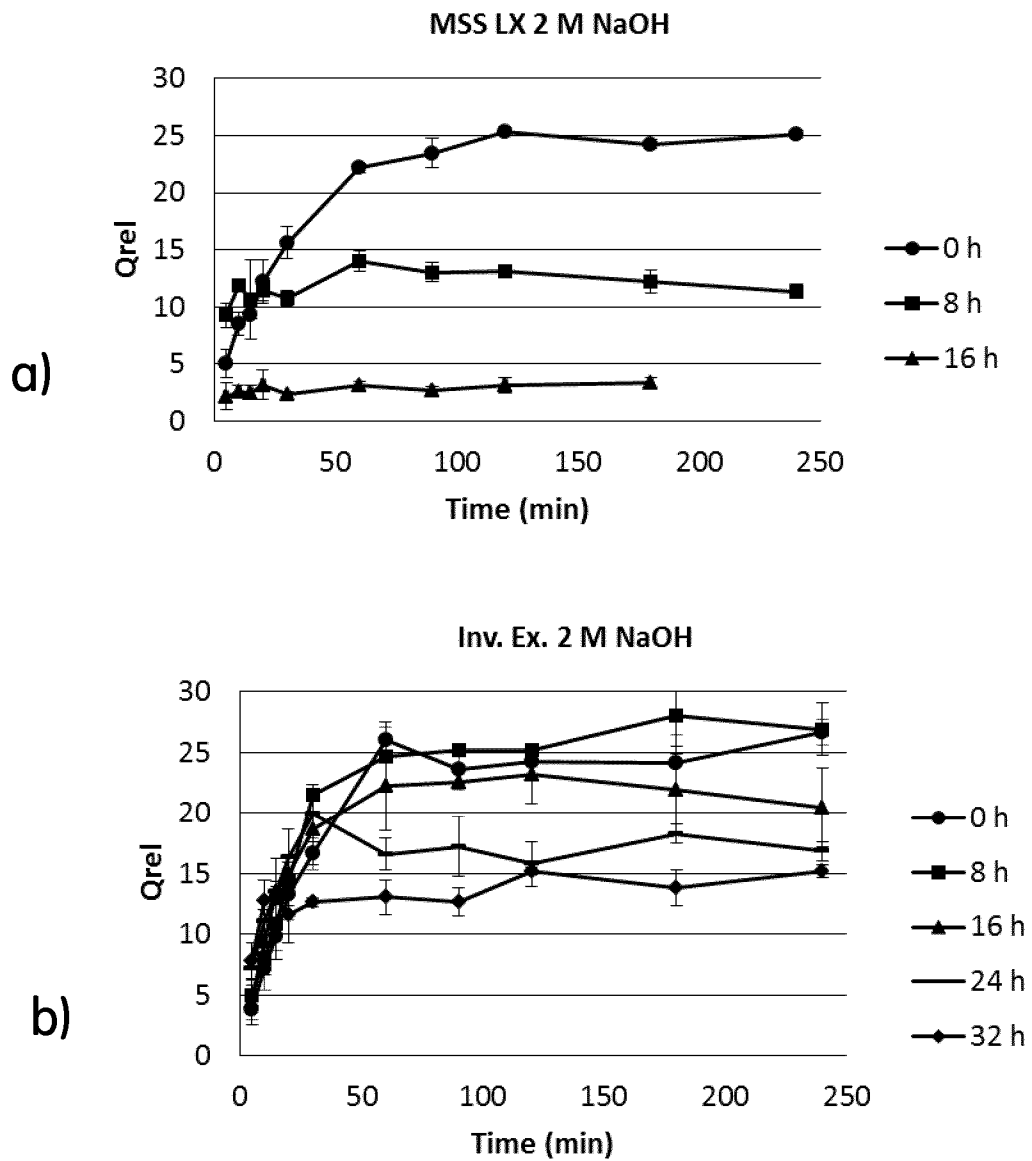


Fig. 12.

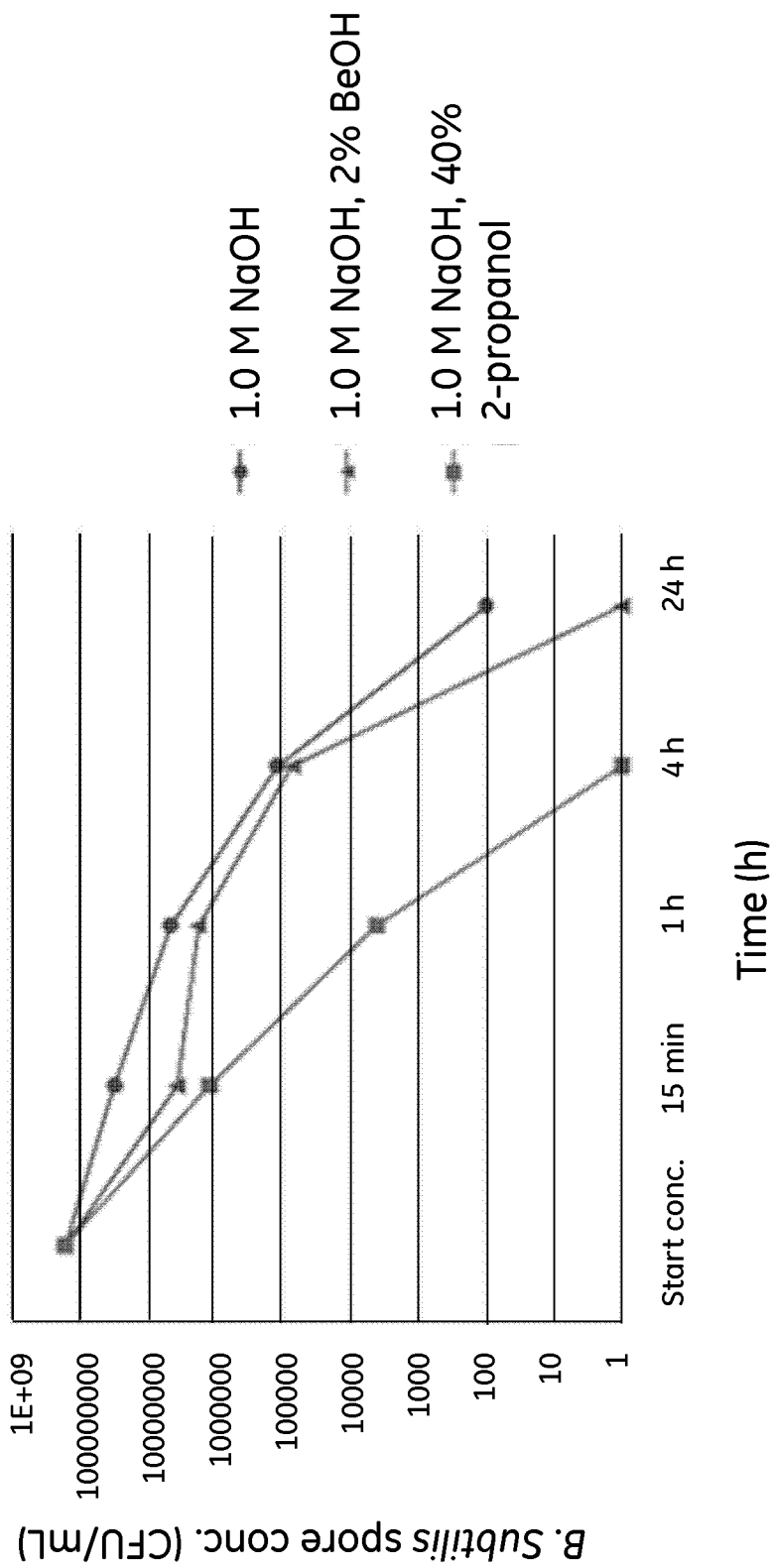


Fig. 13.

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/EP2017/061159

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

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:
- a. ☒ forming part of the international application as filed:
- ☒ in the form of an Annex C/ST.25 text file.
- ☐ on paper or in the form of an image file.
- b. ☐ furnished together with the international application under PCT Rule 13~~ter~~.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.
- c. ☐ furnished subsequent to the international filing date for the purposes of international search only:
- ☐ in the form of an Annex C/ST.25 text file (Rule 13~~ter~~.1(a)).
- ☐ on paper or in the form of an image file (Rule 13~~ter~~.1(b) and Administrative Instructions, Section 713).
2. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.
3. Additional comments:

## INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2017/061159

## A. CLASSIFICATION OF SUBJECT MATTER

INV. C07K14/31

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

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, BIOSIS, EMBASE, WPI Data

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages                                                                                                                                                                                                                                                                                                                                                                                                   | Relevant to claim No. |
|-----------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------|
| X         | <p>WO 2015/005859 A1 (GE HEALTHCARE BIO SCIENCES AB [SE])<br/> 15 January 2015 (2015-01-15)<br/> abstract<br/> page 3<br/> page 5, lines 6-7<br/> page 5, line 32 - page 6, line 2<br/> page 7, line 29 - page 9, line 18<br/> page 10, line 26 - page 11, line 10<br/> page 12, line 32<br/> page 13, lines 20, 21<br/> page 13, line 28 - page 14, line 2<br/> page 14, line 19 - line 20<br/> examples 1-2<br/> claims 31-33</p> <p style="text-align: center;">-----<br/>-/-</p> | 1-19                  |



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

"&amp;" document member of the same patent family

Date of the actual completion of the international search

20 July 2017

Date of mailing of the international search report

01/08/2017

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## INTERNATIONAL SEARCH REPORT

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PCT/EP2017/061159

| C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT |                                                                                                                                                                                                                                                                                                                                     |                       |
|------------------------------------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------|
| Category*                                            | Citation of document, with indication, where appropriate, of the relevant passages                                                                                                                                                                                                                                                  | Relevant to claim No. |
| X                                                    | WO 2012/083425 A1 (UNIV WESTERN ONTARIO<br>[CA]; LI SHUN-CHENG [CA]; LI XING [CA];<br>VOSS COUR) 28 June 2012 (2012-06-28)<br>abstract<br>paragraphs [0001], [0006], [0007],<br>[0011], [0030], [0034] - [0036],<br>[0043], [0047] - [0049], [0055]<br>examples 1, 2<br>-----                                                       | 1-19                  |
| X,P                                                  | WO 2016/079033 A1 (GE HEALTHCARE<br>BIOPROCESS R&D AB [SE])<br>26 May 2016 (2016-05-26)<br>abstract<br>sentences 5-8<br>page 3, line 26 - page 4, line 11<br>page 6, line 8 - page 15, line 16<br>page 16, lines 23, 28<br>page 18, line 10 - line 13<br>page 19, lines 6, 7<br>page 20, line 17 - line 20<br>examples 1-5<br>----- | 1-19                  |

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/EP2017/061159

| Patent document<br>cited in search report | Publication<br>date | Patent family<br>member(s) | Publication<br>date |
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