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(54) Title: ALBUMIN VARIANTS

(57) Abstract: The present invention relates to variants of a parent albumin having altered plasma half-life compared with the parent albumin. The present invention also relates to fusion polypeptides and conjugates comprising said variant albumin.



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ALBUMIN VARIANTS

Reference to a Sequence Listing

This application contains a Sequence Listing in computer readable form, which is
5 incorporated herein by reference.

Background of the Invention

Field of the Invention

The present invention relates to variants of albumin or fragments thereof or fusion
10 polypeptides comprising variant albumin or fragments thereof having a change in half-life compared to the albumin, fragment thereof or fusion polypeptide comprising albumin or a fragment thereof.

Description of the Related Art

Albumin is a protein naturally found in the blood plasma of mammals where it is the most
15 abundant protein. It has important roles in maintaining the desired osmotic pressure of the blood and also in transport of various substances in the blood stream.

Albumins have been characterized from many species including human, pig, mouse, rat, rabbit and goat and they share a high degree of sequence and structural homology.

Albumin binds *in vivo* to its receptor, the neonatal Fc receptor (FcRn) "Brambell" and this
20 interaction is known to be important for the plasma half-life of albumin. FcRn is a membrane bound protein, expressed in many cell and tissue types. FcRn has been found to salvage albumin from intracellular degradation (Roopenian D. C. and Akilesh, S. (2007), *Nat. Rev. Immunol* **7**, 715-725.). FcRn is a bifunctional molecule that contributes to maintaining a high level of IgGs and albumin in serum in mammals such as human beings.

25 Whilst the FcRn-immunoglobulin (IgG) interaction has been characterized in the prior art, the FcRn-albumin interaction is less well characterized. The major FcRn binding site is localized within DIII (381-585). Andersen *et al* (2010). *Clinical Biochemistry* **43**, 367-372. Data indicates that IgG and albumin bind non-cooperatively to distinct sites on FcRn (Andersen *et al.* (2006), *Eur. J. Immunol* **36**, 3044-3051; Chaudhury *et al.* (2006), *Biochemistry* **45**, 4983-4990.).

30 It is known that mouse FcRn binds IgG from mice and humans whereas human FcRn appears to be more discriminating (Ober *et al.* (2001) *Int. Immunol* **13**, 1551-1559). Andersen *et al.* (2010). *Journal of Biological Chemistry* 285(7):4826-36, describes the affinity of human and mouse FcRn for each mouse and human albumin (all possible combinations). No binding of albumin from either species was observed at physiological pH to either receptor. At acidic pH, a 100-fold

difference in binding affinity was observed. In all cases, binding of albumin and IgG from either species to both receptors were additive.

Human serum albumin (HSA) has been well characterized as a polypeptide of 585 amino acids, the sequence of which can be found in Peters, T., Jr. (1996) *All about Albumin: Biochemistry, Genetics and Medical, Applications pp10*, Academic Press, Inc., Orlando (ISBN 0-12-552110-3). It has a characteristic binding to its receptor FcRn, where it binds at pH 6.0 but not at pH 7.4.

The plasma half-life of HSA has been found to be approximately 19 days. A natural variant having lower plasma half-life has been identified (Peach, R. J. and Brennan, S. O., (1991) *Biochim Biophys Acta*. **1097**:49-54) having the substitution D494N. This substitution generated an N-glycosylation site in this variant, which is not present in the wild-type albumin. It is not known whether the glycosylation or the amino acid change is responsible for the change in plasma half-life.

Albumin has a long plasma half-life and because of this property it has been suggested for use in drug delivery. Albumin has been conjugated to pharmaceutically beneficial compounds (WO 2000/69902A), and it was found that the conjugate - maintained the long plasma half-life of albumin. The resulting plasma half-life of the conjugate was generally considerably longer than the plasma half-life of the beneficial therapeutic compound alone.

Further, albumin has been fused to therapeutically beneficial peptides (WO 2001/79271 A and WO 2003/59934 A) with the typical result that the fusion has the activity of the therapeutically beneficial peptide and a considerably longer plasma half-life than the plasma half-life of the therapeutically beneficial peptides alone.

Otagiri *et al* (2009), *Biol. Pharm. Bull.* **32**(4), 527-534, discloses that 77 albumin variant are known, of these 25 are found in domain III. A natural variant lacking the last 175 amino acids at the carboxy termini has been shown to have reduced half-life (Andersen *et al* (2010), *Clinical Biochemistry* **43**, 367-372). Iwao *et al.* (2007) studied the half-life of naturally occurring human albumin variants using a mouse model, and found that K541E and K560E had reduced half-life, E501K and E570K had increased half-life and K573E had almost no effect on half-life (Iwao, *et. al.* (2007) *B.B.A. Proteins and Proteomics* **1774**, 1582-1590).

Galliano *et al* (1993) *Biochim. Biophys. Acta* 1225, 27-32 discloses a natural variant E505K. Minchiotti *et al.* (1990) discloses a natural variant K536E. Minchiotti *et al* (1987) *Biochim. Biophys. Acta* 916, 411-418 discloses a natural variant K574N. Takahashi *et al* (1987) *Proc. Natl. Acad. Sci. USA* 84, 4413-4417, discloses a natural variant D550G. Carlson *et al* (1992). *Proc. Nat. Acad. Sci. USA* 89, 8225- 8229, discloses a natural variant D550A.

Albumin has the ability to bind a number of ligands and these become associated (associates) with albumin. This property has been utilized to extend the plasma half-life of drugs

having the ability to noncovalently bind to albumin. This can also be achieved by binding a pharmaceutical beneficial compound, which has little or no albumin binding properties, to a moiety having albumin binding properties. See review article and reference therein, Kratz (2008). *Journal of Controlled Release* **132**, 171-183.

Albumin is used in preparations of pharmaceutically beneficial compounds, in which such a preparation maybe for example, but not limited to, a nano particle or micro particle of albumin. In these examples the delivery of a pharmaceutically beneficial compound or mixture of compounds may benefit from alteration in the albumins affinity to its receptor where the beneficial compound has been shown to associate with albumin for the means of delivery.

It is not clear what determines the plasma half-life of the formed associates (for example but not limited to Levemir®, Kurtzhals P *et al.* *Biochem. J.* 1995; **312**:725-731) conjugates or fusion polypeptides but it appears to be a result of the combination of the albumin and the selected pharmaceutically beneficial compound/polypeptide. It would be desirable to be able to control the plasma half-life of given albumin conjugates, associates or albumin fusion polypeptides so that a longer or shorter plasma half-life can be achieved than given by the components of the association, conjugation or fusion, in order to be able to design a particular drug according to the particulars of the indication intended to be treated.

Albumin is known to accumulate and be catabolised in tumours, it has also been shown to accumulate in inflamed joints of rheumatoid arthritis sufferers. See review article and reference therein, Kratz (2008). *Journal of Controlled Release* **132**, 171-183. It is envisaged that HSA variants with increased affinity for FcRn would be advantageous for the delivery of pharmaceutically beneficial compounds.

It may even be desirable to have variants of albumin that have little or no binding to FcRn in order to provide shorter half-lives or controlled serum pharmacokinetics as described by Kenanova *et al* (2009) *J. Nucl. Med.*; **50** (Supplement 2):1582).

Summary of the Invention

The present invention provides variants of a parent albumin with improved properties compared to its parent. In particular the invention provides variants of a parent albumin having altered plasma half-life compare to its parent.

The present invention relates to isolated variants of albumin or fragments thereof, or fusion polypeptides comprising variant albumin or fragments thereof, of a parent albumin, comprising an alteration at one or more (several) positions corresponding to positions 417, 440, 464, 490, 492, 493, 494, 495, 496, 499, 500, 501, 503, 504, 505, 506, 510, 535, 536, 537, 538, 540, 541, 542, 550, 573, 574, 575, 577, 578, 579, 580, 581, 582 and 584 of the mature polypeptide of SEQ ID

NO: 2, wherein the variant is not the variant consisting of SEQ ID NO: 2 with the substitution D494N, E501K, K541E, D550G,A, K573E or K574N.

The alteration at one or more position may independently be selected among substitutions, insertions and deletions, where substitution are preferred.

5 The present invention also relates to isolated polynucleotides encoding the variants; nucleic acid constructs, vectors, and host cells comprising the polynucleotides; and methods of producing the variants.

The present invention also relates to conjugates or associates comprising the variant albumin or fragment thereof according to the invention and a beneficial therapeutic moiety or to a
10 fusion polypeptide comprising a variant albumin or fragment thereof of the invention and a fusion partner polypeptide.

The invention further relates to compositions comprising the variant albumin, fragment thereof, fusion polypeptide comprising variant albumin or fragment thereof or conjugates comprising the variant albumin or fragment thereof, according to the invention or associates
15 comprising the variant albumin or fragment thereof, according to the invention. The compositions are preferably pharmaceutical compositions.

The invention further relates to a pharmaceutical composition comprising a variant albumin, fragment thereof, fusion polypeptide comprising variant albumin or fragment thereof or conjugates comprising the variant albumin or fragment thereof, or associates comprising the variant albumin or
20 fragment thereof, wherein said variant albumin, fragment thereof, fusion polypeptide comprising variant albumin or fragment thereof or conjugates comprising the variant albumin or fragment or associates of variant albumin or fragment thereof has altered plasma half-life compared to the corresponding plasma half-life of the HSA or fragment thereof, fusion polypeptide comprising HSA or fragment thereof or conjugates or associates of HSA or, fragment thereof, comprising HSA or
25 fragment thereof.

Brief Description of the Figures

Figure 1 shows a restriction map of the expression plasmid pDB4082.

Figure 2 shows a restriction map of the expression plasmid pDB2305

30 **Figure 3** shows a restriction map of the expression plasmid pDB4005

Figure 4 shows SPR sensorgrams 10 μ M albumin injected over shFcRn HSA (JTA) = fatty acid free HSA obtained from Sigma-Aldrich (A3782), HSA (Novozymes) = Commercial Recombinant human serum albumin (RECOMBUMIN).

Figure 5 shows ELISA binding of shFcRn-GST to human serum albumin (HSA) variants
35 (100-0.045 μ g/ml). Binding of WT, D494N, D494Q and D494A pH 6.0 and pH 7.4. Binding of WT,

D494N, D494N/T496A and T496A at pH 6.0 and pH 7.4. Binding of WT, E495Q and E495A at pH 6.0 and pH 7.4.

Figure 6 shows representative sensorgrams of binding of 0.2 μ M of HSA variants to immobilized shFcRn (\sim 4600 RU). WT, D494N, D494Q, D494A, D494N/T496A and T496A.

Figure 7 shows representative sensorgrams of binding of 1 μ M of HSA variants to immobilized shFcRn (\sim 1400 RU). WT, D494N, D494Q, D494A, D494N/T496A and T496A.

Figure 8 shows relative binding of the HSA variants compared to WT based on two independent SPR experiments as shown (A) Figure 6 and (B) Figure 7.

Figure 9 shows ELISA: (A) binding of shFcRn to albumins from human, donkey, bovine, sheep, goat and rabbit at pH 6.0. (B) binding of shFcRn to albumin from guinea pig, hamster, rat and chicken at pH 6.0. (C) binding of shFcRn to albumin from human, donkey, bovine, sheep, goat and rabbit at pH 7.4. (D) binding of shFcRn to albumin from guinea pig, hamster, rat and chicken at pH 7.4. (E) relative binding of the different albumins. Relative binding of human albumin to shFcRn is defined as 1.0. The ELISA values represent the mean of duplicates.

Figure 10 shows SPR: Binding of shFcRn-GST to albumin from several species at pH 6.0 and pH 7.4. Representative sensorgrams showing binding of 5.0 μ M of albumin from different species; (A) human, (B) donkey, (C) bovine, (D) goat, (E) sheep, (F) rabbit, (G) dog, (H) guinea pig, (I) hamster, (J) rat, (K) mouse and (L) chicken. The albumin variants were injected over immobilized GST-tagged shFcRn (\sim 2100 RU). Injections were performed at 25°C at a rate of 40 μ l/min.

Figure 11 shows SPR sensorgrams of selected HSA mutants compared with wild-type HSA. 20 μ M of (A) WT and P499A (B) WT and K500A, (C) WT and K536A, (D) WT and P537A and (E) WT and K538A and (F) WT and K537A were injected over immobilized shFcRn at pH 6.0 (\sim 1500 RU)

Figure 12 shows SPR sensorgrams of HSA mutants compared with WT HSA. 10 μ M of (A) WT and K573A (B) WT and K573C, (C) WT and K573F, (D) WT and K573G and (E) WT and K573L and (F) WT and K573M, (G) WT and K573Q, (H) WT and K573R and (I) WT and K573T and (J) WT and K573V injected over immobilized shFcRn at pH 5.5 and pH7.4. Injections were performed at 25°C at a flow rate of 80 μ l/min.

Figure 13 shows SPR sensorgrams of HSA mutants compared with wild-type HSA. 10 μ M of (A) WT and K573D (B) WT and K573E, (C) WT and K573H, (D) WT and K573I and (E) WT and K573N and (F) WT and K573P, (G) WT and K573S, (H) WT and K573* and (I) WT and K573W and (J) WT and K573Y injected over immobilized shFcRn at pH 5.5 and pH7.4. Injections were performed at 25°C at a flow rate of 80 μ l/min.

Figure 14 shows SPR sensorgrams of HSA mutants compared with wild-type HSA. 20 μ M of (A) WT and E492G+K538H+K541N+E542D (B) WT and E492T+N503K+K541A, (C) WT and E492P+N503K+K541G+E542P, (D) WT and E492H+E501P+N503H+E505D+T506S+T540S+K541E and (E) WT and A490D+E492T+V493L+E501P+N503D+A504E+5 +E505K+T506F+K541D and (F) WT and E492G+V493P+K538H+K541N+E542D injected over immobilized shFcRn at pH 6.0. Injections were performed at 25°C at a flow rate of 80 μ l/min.

Figure 15 shows SPR sensorgrams of HSA mutants compared with wild-type HSA. Twenty μ M of (A) WT, (B) H440Q, (C) H464Q and (D) H535Q injected over immobilized shFcRn at pH 6.0. Injections were performed at 25°C at a flow rate of 80 μ l/min.

Figure 16 shows SPR sensorgrams of HSA mutant K500E compared with wild-type HSA. Ten μ M of HSA mutant K500E injected over immobilized shFcRn at pH 5.75. Injections were performed at 25°C at a flow rate of 30 μ l/min.

Figure 17 shows a restriction map of the expression plasmid pDB3017

Figure 18 shows a restriction map of the expression plasmid pDB3021

Figure 19 shows a restriction map of the expression plasmid pDB3056

Figure 20 shows a restriction map of the expression plasmid pDB3165

Figure 21 shows a restriction map of the expression plasmid pDB4172

Figure 22 shows a restriction map of the expression plasmid pDB4267

Figure 23 shows a restriction map of the expression plasmid pDB4285

Figure 24 shows a GP-HPLC chromatogram of WT HSA and mutant K573P HRP conjugates for shFcRn analysis. Injections of 25 μ L were made onto a TSK G3000SWXL column (Tosoh Bioscience) as described in materials and methods.

Figure 25 shows SDS PAGE separation followed by both visual (A) and ultraviolet (B) detection of the Fluorescein conjugated albumin. HSA::F5M (Lane 1), K573P::F5M (Lane 2) and rHA standard (Lane 3).

Figure 26 shows shFcRn binding properties of HSA variants. 10 μ M of WT rHA and E492T(A), WT rHA and D494N/E495Q/T496A(B), WT rHA and N503D(C), WT rHA and N503K(D), WT rHA and E492T/N503D(E), WT rHA and E495Q/T496A(F), WT rHA and K538H(G), WT rHA and E492D(H) injected over immobilised shFcRn at pH5.5

Figure 27 shows shFcRn binding properties of HSA variants. 10 μ M of WT rHA and K541A(I) and WT rHA and K541N(J) were injected over immobilised shFcRn at pH5.5.

Figure 28 shows competitive binding of K573A and K573P measured by injecting shFcRn (100 nM) alone or pre-incubated with different amounts of HSA K573A and K573P over immobilized HSA (~2500 RU) at pH6.0

Figure 29 shows competitive binding of HSA-FLAG variants measured by injecting shFcRn (100 nM) alone or together with different amounts of HSA-FLAG variants over immobilized HSA (~2500 RU) at pH6.0.

Figure 30 shows competitive binding of HSA-IL1Ra variants measured by injecting shFcRn (100 nM) alone or together with different amounts of HSA-IL1Ra variants over immobilized HSA (~2500 RU) at pH6.0

Figure 31 shows competitive binding of scFv-fused HSA variants measured by injecting shFcRn (100 nM) alone or together with different amounts of (A) scFv-HSA-FLAG variants or (B) HSA-scFv-FLAG variants over immobilized HSA (~2500 RU) at pH6.0.

Figure 32 shows binding of HSA, single, double and triple mutant variants to shFcRn. Samples of 10 µM of each HSA variant were injected over immobilized shFcRn at pH 5.5 or pH 7.4.

Detailed Description of the Invention

The present invention relates to isolated variants of albumin or fragments thereof, or fusion polypeptides comprising variant albumin or fragments thereof, of a parent albumin, comprising an alteration at one or more (several) positions corresponding to positions 417, 440, 464, 490, 492, 493, 494, 495, 496, 499, 500, 501, 503, 504, 505, 506, 510, 535, 536, 537, 538, 540, 541, 542, 550, 573, 574, 575, 577, 578, 579, 580, 581, 582 and 584 of the mature polypeptide of SEQ ID NO: 2, wherein the variant is not the variant consisting of SEQ ID NO: 2 with the substitution D494N, E501K, K541E, D550G,A, K573E or K574N..

The alteration at one or more position may independently be selected among substitutions, insertions and deletions, where substitution are preferred.

Definitions

Variant: The term “variant” means a polypeptide derived from a parent albumin by one or more alteration(s), *i.e.*, a substitution, insertion, and/or deletion, at one or more (several) positions. A substitution means a replacement of an amino acid occupying a position with a different amino acid; a deletion means removal of an amino acid occupying a position; and an insertion means adding 1 or more, preferably 1-3 amino acids immediately adjacent to an amino acid occupying a position.

Mutant: The term “mutant” means a polynucleotide encoding a variant.

Wild-Type Albumin: The term “wild-type” (WT) albumin means albumin having the same amino acid sequence as naturally found in an animal or in a human being.

Parent or Parent albumin The term “parent” or “parent albumin” means an albumin to which an alteration is made by the hand of man to produce the albumin variants of the present

invention. The parent may be a naturally occurring (wild-type) polypeptide or an allele thereof, or even a variant thereof.

FcRn and shFcRn: The term “FcRn” means the human neonatal Fc receptor (FcRn). shFcRn is a soluble recombinant form of FcRn.

5 **smFcRn:** The term “smFcRn” is a soluble recombinant form of the mouse neonatal Fc Receptor.

Isolated variant: The term “isolated variant” means a variant that is modified by the hand of man and separated completely or partially from at least one component with which it naturally occurs. In one aspect, the variant is at least 1% pure, e.g., at least 5% pure, at least 10% pure, at
10 least 20% pure, at least 40% pure, at least 60% pure, at least 80% pure, and at least 90% pure, as determined by SDS-PAGE or GP-HPLC.

Substantially pure variant: The term “substantially pure variant” means a preparation that contains at most 10%, at most 8%, at most 6%, at most 5%, at most 4%, at most 3%, at most 2%, at most 1%, and at most 0.5% by weight of other polypeptide material with which it is natively or
15 recombinantly associated. Preferably, the variant is at least 92% pure, e.g., at least 94% pure, at least 95% pure, at least 96% pure, at least 97% pure, at least 98% pure, at least 99%, at least 99.5% pure, and 100% pure by weight of the total polypeptide material present in the preparation. The variants of the present invention are preferably in a substantially pure form. This can be accomplished, for example, by preparing the variant by well known recombinant methods and by
20 purification methods.

Mature polypeptide: The term “mature polypeptide” means a polypeptide in its final form following translation and any post-translational modifications, such as N-terminal processing, C-terminal truncation, glycosylation, phosphorylation, etc. In one aspect, the mature polypeptide is amino acids 1 to 585 of SEQ ID NO: 2, with the inclusion of any post-translational modifications.

25 **Mature polypeptide coding sequence:** The term “mature polypeptide coding sequence” means a polynucleotide that encodes a mature albumin polypeptide. In one aspect, the mature polypeptide coding sequence is nucleotides 1 to 1758 of SEQ ID NO: 1.

Sequence Identity: The relatedness between two amino acid sequences or between two nucleotide sequences is described by the parameter “sequence identity”.

30 For purposes of the present invention, the degree of sequence identity between two amino acid sequences is determined using the Needleman-Wunsch algorithm (Needleman and Wunsch, 1970, *J. Mol. Biol.* 48: 443-453) as implemented in the Needle program of the EMBOSS package (EMBOSS: The European Molecular Biology Open Software Suite, Rice *et al.*, 2000, *Trends Genet.* 16: 276-277), preferably version 3.0.0 or later. The optional parameters used are gap open penalty
35 of 10, gap extension penalty of 0.5, and the EBLOSUM62 (EMBOSS version of BLOSUM62)

substitution matrix. The output of Needle labelled "longest identity" (obtained using the –nobrief option) is used as the percent identity and is calculated as follows:

$$(\text{Identical Residues} \times 100) / (\text{Length of Alignment} - \text{Total Number of Gaps in Alignment})$$

For purposes of the present invention, the degree of sequence identity between two deoxyribonucleotide sequences is determined using the Needleman-Wunsch algorithm (Needleman and Wunsch, 1970, *supra*) as implemented in the Needle program of the EMBOSS package (EMBOSS: The European Molecular Biology Open Software Suite, Rice *et al.*, 2000, *supra*), preferably version 3.0.0 or later. The optional parameters used are gap open penalty of 10, gap extension penalty of 0.5, and the EDNAFULL (EMBOSS version of NCBI NUC4.4) substitution matrix. The output of Needle labeled "longest identity" (obtained using the –nobrief option) is used as the percent identity and is calculated as follows:

$$(\text{Identical Deoxyribonucleotides} \times 100) / (\text{Length of Alignment} - \text{Total Number of Gaps in Alignment})$$

Fragment: The term "fragment" means a polypeptide having one or more (several) amino acids deleted from the amino and/or carboxyl terminus of an albumin and/or an internal region of albumin that has retained the ability to bind to FcRn. Fragments may consist of one uninterrupted sequence derived from HSA or it may comprise two or more sequences derived from HSA. The fragments according to the invention have a size of more than approximately 20 amino acid residues, preferably more than 30 amino acid residues, more preferred more than 40 amino acid residues, more preferred more than 50 amino acid residues, more preferred more than 75 amino acid residues, more preferred more than 100 amino acid residues, more preferred more than 200 amino acid residues, more preferred more than 300 amino acid residues, even more preferred more than 400 amino acid residues and most preferred more than 500 amino acid residues.

Allelic variant: The term "allelic variant" means any of two or more alternative forms of a gene occupying the same chromosomal locus. Allelic variation arises naturally through mutation, and may result in polymorphism within populations. Gene mutations can be silent (no change in the encoded polypeptide) or may encode polypeptides having altered amino acid sequences. An allelic variant of a polypeptide is a polypeptide encoded by an allelic variant of a gene.

Coding sequence: The term "coding sequence" means a polynucleotide, which directly specifies the amino acid sequence of its translated polypeptide product. The boundaries of the coding sequence are generally determined by an open reading frame, which usually begins with the ATG start codon or alternative start codons such as GTG and TTG and ends with a stop codon such as TAA, TAG, and TGA. The coding sequence may be a DNA, cDNA, synthetic, or recombinant polynucleotide.

cDNA: The term "cDNA" means a DNA molecule that can be prepared by reverse transcription from a mature, spliced, mRNA molecule obtained from a eukaryotic cell. cDNA lacks

intron sequences that may be present in the corresponding genomic DNA. The initial, primary RNA transcript is a precursor to mRNA that is processed through a series of steps, including splicing, before appearing as mature spliced mRNA.

Nucleic acid construct: The term "nucleic acid construct" means a nucleic acid molecule, either single- or double-stranded, which is isolated from a naturally occurring gene or is modified to contain segments of nucleic acids in a manner that would not otherwise exist in nature or which is synthetic. The term nucleic acid construct is synonymous with the term "expression cassette" when the nucleic acid construct contains the control sequences required for expression of a coding sequence of the present invention.

Control sequences: The term "control sequences" means all components necessary for the expression of a polynucleotide encoding a variant of the present invention. Each control sequence may be native or foreign to the polynucleotide encoding the variant or native or foreign to each other. Such control sequences include, but are not limited to, a leader, polyadenylation sequence, propeptide sequence, promoter, signal peptide sequence, and transcription terminator. At a minimum, the control sequences include a promoter, and transcriptional and translational stop signals. The control sequences may be provided with linkers for the purpose of introducing specific restriction sites facilitating ligation of the control sequences within the coding region of the polynucleotide encoding a variant.

Operably linked: The term "operably linked" means a configuration in which a control sequence is placed at an appropriate position relative to the coding sequence of a polynucleotide such that the control sequence directs the expression of the coding sequence.

Expression: The term "expression" includes any step involved in the production of the variant including, but not limited to, transcription, post-transcriptional modification, translation, post-translational modification, and secretion.

Expression vector: The term "expression vector" means a linear or circular DNA molecule that comprises a polynucleotide encoding a variant and is operably linked to additional nucleotides that provide for its expression.

Host cell: The term "host cell" means any cell type that is susceptible to transformation, transfection, transduction, and the like with a nucleic acid construct or expression vector comprising a polynucleotide of the present invention. The term "host cell" encompasses any progeny of a parent cell that is not identical to the parent cell due to mutations that occur during replication.

Plasma half-life: Plasma half-life is ideally determined *in vivo* in suitable individuals. However, since it is time consuming and expensive and there inevitable are ethical concerns connected with doing experiments in animals or man it is desirable to use an *in vitro* assay for determining whether plasma half-life is extended or reduced. It is known that the binding of albumin

to its receptor FcRn is important for plasma half-life and the correlation between receptor binding and plasma half-life is that a higher affinity of albumin to its receptor leads to longer plasma half-life. Thus for the present invention a higher affinity of albumin to FcRn is considered indicative of an increased plasma half-life and a lower affinity of albumin to its receptor is considered indicative of a reduced plasma half-life.

In this application and claims the binding of albumin to its receptor FcRn is described using the term affinity and the expressions "stronger" or "weaker". Thus, it should be understood that a molecule having a higher affinity to FcRn than HSA is considered to bind stronger to FcRn than HSA and a molecule having a lower affinity to FcRn than HSA is considered to bind weaker to FcRn than HSA.

The terms "longer plasma half-life" or "shorter plasma half-life" and similar expressions are understood to be in relationship to the corresponding parent albumin molecule. Thus, a longer plasma half-life with respect to a variant albumin of the invention means that the variant has longer plasma half-life than the corresponding albumin having the same sequences except for the alteration(s) in positions corresponding to 417, 440, 464, 490, 492, 493, 494, 495, 496, 499, 500, 501, 503, 504, 505, 506, 510, 535, 536, 537, 538, 540, 541, 542, 550, 573, 574, 575, 577, 578, 579, 580, 581, 582 and 584 in SEQ ID NO: 2.

Conventions for Designation of Variants

For purposes of the present invention, the mature polypeptide disclosed in SEQ ID NO: 2 is used to determine the corresponding amino acid residue in another albumin. The amino acid sequence of another albumin is aligned with the mature polypeptide disclosed in SEQ ID NO: 2, and based on the alignment, the amino acid position number corresponding to any amino acid residue in the mature polypeptide disclosed in SEQ ID NO: 2 is determined using the Needleman-Wunsch algorithm (Needleman and Wunsch, 1970, *J. Mol. Biol.* **48**: 443-453) as implemented in the Needle program of the EMBOSS package (EMBOSS: The European Molecular Biology Open Software Suite, Rice *et al.*, 2000, *Trends Genet.* **16**: 276-277), preferably version 3.0.0 or later.

Identification of the corresponding amino acid residue in another albumin can be confirmed by an alignment of multiple polypeptide sequences using "ClustalW" (Larkin *et al.*, 2007, *Bioinformatics* **23**: 2947-2948).

When the other polypeptide (or protein) has diverged from the mature polypeptide of SEQ ID NO: 2 such that traditional sequence-based comparison fails to detect their relationship (Lindahl and Elofsson, 2000, *J. Mol. Biol.* **295**: 613-615), other pairwise sequence comparison algorithms can be used. Greater sensitivity in sequence-based searching can be attained using search programs that utilize probabilistic representations of polypeptide families (profiles) to search

databases. For example, the PSI-BLAST program generates profiles through an iterative database search process and is capable of detecting remote homologs (Atschul *et al.*, 1997, *Nucleic Acids Res.* **25**: 3389-3402). Even greater sensitivity can be achieved if the family or superfamily for the polypeptide has one or more representatives in the protein structure databases. Programs such as

5 GenTHREADER (Jones, 1999, *J. Mol. Biol.* **287**: 797-815; McGuffin and Jones, 2003, *Bioinformatics* **19**: 874-881) utilize information from a variety of sources (PSI-BLAST, secondary structure prediction, structural alignment profiles, and solvation potentials) as inputs to a neural network that predicts the structural fold for a query sequence. Similarly, the method of Gough *et al.*, 2000, *J. Mol. Biol.* **313**: 903-919, can be used to align a sequence of unknown structure within the

10 superfamily models present in the SCOP database. These alignments can in turn be used to generate homology models for the polypeptide, and such models can be assessed for accuracy using a variety of tools developed for that purpose.

For proteins of known structure, several tools and resources are available for retrieving and generating structural alignments. For example the SCOP superfamilies of proteins have been

15 structurally aligned, and those alignments are accessible and downloadable. Two or more protein structures can be aligned using a variety of algorithms such as the distance alignment matrix (Holm and Sander, 1998, *Proteins* **33**: 88-96) or combinatorial extension (Shindyalov and Bourne, 1998, *Protein Engineering* **11**: 739-747), and implementations of these algorithms can additionally be utilized to query structure databases with a structure of interest in order to discover possible

20 structural homologs (e.g., Holm and Park, 2000, *Bioinformatics* **16**: 566-567).

In describing the albumin variants of the present invention, the nomenclature described below is adapted for ease of reference. The accepted IUPAC single letter or three letter amino acid abbreviation is employed.

Substitutions. For an amino acid substitution, the following nomenclature is used: Original

25 amino acid, position, substituted amino acid. Accordingly, for example the substitution of threonine with alanine at position 226 is designated as "Thr226Ala" or "T226A". Multiple mutations are separated by addition marks ("+"), e.g., "Gly205Arg + Ser411Phe" or "G205R + S411F", representing substitutions at positions 205 and 411 of glycine (G) with arginine (R) and serine (S) with phenylalanine (F), respectively. The Figures also use ("/"), e.g., "E492T/N503D" this should be

30 viewed as interchangeable with ("+").

Deletions. For an amino acid deletion, the following nomenclature is used: Original amino acid, position*. Accordingly, the deletion of glycine at position 195 is designated as "Gly195*" or "G195*". Multiple deletions are separated by addition marks ("+"), e.g., "Gly195* + Ser411*" or "G195* + S411*".

Insertions. For an amino acid insertion, the following nomenclature is used: Original amino acid, position, original amino acid, inserted amino acid. Accordingly the insertion of lysine after glycine at position 195 is designated “Gly195GlyLys” or “G195GK”. An insertion of multiple amino acids is designated [Original amino acid, position, original amino acid, inserted amino acid #1, inserted amino acid #2; etc.]. For example, the insertion of lysine and alanine after glycine at position 195 is indicated as “Gly195GlyLysAla” or “G195GKA”.

In such cases the inserted amino acid residue(s) are numbered by the addition of lower case letters to the position number of the amino acid residue preceding the inserted amino acid residue(s). In the above example, the sequence would thus be:

<u>Parent:</u>	<u>Variant:</u>
195	195 195a 195b
G	G - K - A

Multiple alterations. Variants comprising multiple alterations are separated by addition marks (“+”), e.g., “Arg170Tyr+Gly195Glu” or “R170Y+G195E” representing a substitution of tyrosine and glutamic acid for arginine and glycine at positions 170 and 195, respectively.

Different substitutions. Where different substitutions can be introduced at a position, the different substitutions are separated by a comma, e.g., “Arg170Tyr,Glu” represents a substitution of arginine with tyrosine or glutamic acid at position 170. Thus, “Tyr167Gly,Ala + Arg170Gly,Ala” designates the following variants:

“Tyr167Gly+Arg170Gly”, “Tyr167Gly+Arg170Ala”, “Tyr167Ala+Arg170Gly”, and “Tyr167Ala+Arg170Ala”.

Parent albumin

Albumins are proteins and constitute the most abundant protein in plasma in mammals and albumins from a long number of mammals have been characterized by biochemical methods and/or by sequence information. Several albumins, e.g., human serum albumin (HSA), have also been characterized crystallographically and the structure determined.

HSA is a preferred albumin according to the invention and is a protein consisting of 585 amino acid residues and has a molecular weight of 67 kDa. In its natural form it is not glycosylated. The amino acid sequence of HSA is shown in SEQ ID NO: 2. The skilled person will appreciate that natural alleles may exist having essentially the same properties as HSA but having one or more amino acid changes compared to SEQ ID NO: 2, and the inventors also contemplate the use of such natural alleles as parent albumin according to the invention.

Albumins have generally a long plasma half-life of approximately 20 days or longer, e.g., HSA has a plasma half-life of 19 days. It is known that the long plasma half-life of HSA is mediated via interaction with its receptor FcRn, however, an understanding or knowledge of the exact mechanism behind the long half-life of HSA is not essential for the present invention.

According to the invention the term "albumin" means a protein having the same, or very similar three dimensional structure as HSA and having a long plasma half-life. As examples of albumin proteins according to the invention can be mentioned human serum albumin, primate serum albumin, (such as chimpanzee serum albumin, gorilla serum albumin), rodent serum albumin (such as hamster serum albumin, guinea pig serum albumin, mouse albumin and rat serum albumin), bovine serum albumin, equine serum albumin, donkey serum albumin, rabbit serum albumin, goat serum albumin, sheep serum albumin, dog serum albumin, chicken serum albumin and pig serum albumin. HSA as disclosed in SEQ ID NO: 2 or any naturally occurring allele thereof, is the preferred albumin according to the invention.

The parent albumin, a fragment thereof, or albumin part of a fusion polypeptide comprising albumin or a fragment thereof according to the invention has generally a sequence identity to the sequence of HSA shown in SEQ ID NO: 2 of at least 60%, preferably at least 70%, preferably at least 80%, preferably at least 85%, preferably at least 86%, preferably at least 87%, preferably at least 88%, preferably at least 89%, preferably at least 90%, preferably at least 91%, preferably at least 92%, preferably at least 93%, preferably at least 94%, preferably at least 95%, more preferred at least 96%, more preferred at least 97%, more preferred at least 98% and most preferred at least 99%.

The parent preferably comprises or consists of the amino acid sequence of SEQ ID NO: 2. In another aspect, the parent comprises or consists of the mature polypeptide of SEQ ID NO: 2.

In another embodiment, the parent is an allelic variant of the mature polypeptide of SEQ ID NO: 2.

In a second aspect, the parent is encoded by a polynucleotide that hybridizes under very low stringency conditions, low stringency conditions, medium stringency conditions, medium-high stringency conditions, high stringency conditions, or very high stringency conditions with (i) the mature polypeptide coding sequence of SEQ ID NO: 1, (ii) the mature polypeptide coding sequence of SEQ ID NO: 1, or (iii) the full-length complementary strand of (i) or (ii) (J. Sambrook, E.F. Fritsch, and T. Maniatis, 1989, *Molecular Cloning, A Laboratory Manual*, 2d edition, Cold Spring Harbor, New York).

The polynucleotide of SEQ ID NO: 1 or a subsequence thereof, as well as the amino acid sequence of SEQ ID NO: 2 or a fragment thereof, may be used to design nucleic acid probes to identify and clone DNA encoding a parent from strains of different genera or species according to

methods well known in the art. In particular, such probes can be used for hybridization with the genomic or cDNA of the genus or species of interest, following standard Southern blotting procedures, in order to identify and isolate the corresponding gene therein. Such probes can be considerably shorter than the entire sequence, but should be at least 14, e.g., at least 25, at least 35, or at least 70 nucleotides in length. Preferably, the nucleic acid probe is at least 100 nucleotides in length, e.g., at least 200 nucleotides, at least 300 nucleotides, at least 400 nucleotides, at least 500 nucleotides, at least 600 nucleotides, at least 700 nucleotides, at least 800 nucleotides, or at least 900 nucleotides in length. Both DNA and RNA probes can be used. The probes are typically labelled for detecting the corresponding gene (for example, with ^{32}P , ^3H , ^{35}S , biotin, or avidin). Such probes are encompassed by the present invention.

A genomic DNA or cDNA library prepared from such other organisms may be screened for DNA that hybridizes with the probes described above and encodes a parent. Genomic or other DNA from such other organisms may be separated by agarose or polyacrylamide gel electrophoresis, or other separation techniques. DNA from the libraries or the separated DNA may be transferred to and immobilized on nitrocellulose or other suitable carrier material. In order to identify a clone or DNA that is homologous with SEQ ID NO: 1 or a subsequence thereof, the carrier material is used in a Southern blot.

For purposes of the present invention, hybridization indicates that the polynucleotide hybridizes to a labelled nucleotide probe corresponding to the polynucleotide shown in SEQ ID NO: 1, its complementary strand, or a subsequence thereof, under low to very high stringency conditions. Molecules to which the probe hybridizes can be detected using, for example, X-ray film or any other detection means known in the art.

In one aspect, the nucleic acid probe is the mature polypeptide coding sequence of SEQ ID NO: 1. In another aspect, the nucleic acid probe is nucleotides 1 to 1785 of SEQ ID NO: 1. In another aspect, the nucleic acid probe is a polynucleotide that encodes the polypeptide of SEQ ID NO: 2 or a fragment thereof. In another aspect, the nucleic acid probe is SEQ ID NO: 1.

For long probes of at least 100 nucleotides in length, very low to very high stringency conditions are defined as prehybridization and hybridization at 42°C in 5X SSPE, 0.3% SDS, 200 micrograms/ml sheared and denatured salmon sperm DNA, and either 25% formamide for very low and low stringencies, 35% formamide for medium and medium-high stringencies, or 50% formamide for high and very high stringencies, following standard Southern blotting procedures for 12 to 24 hours optimally. The carrier material is finally washed three times each for 15 minutes using 2X SSC, 0.2% SDS at 45°C (very low stringency), 50°C (low stringency), 55°C (medium stringency), 60°C (medium-high stringency), 65°C (high stringency), or 70°C (very high stringency).

For short probes that are about 15 nucleotides to about 70 nucleotides in length, stringency conditions are defined as prehybridization and hybridization at about 5°C to about 10°C below the calculated T_m using the calculation according to Bolton and McCarthy (1962, *Proc. Natl. Acad. Sci. USA* 48: 1390) in 0.9 M NaCl, 0.09 M Tris-HCl pH 7.6, 6 mM EDTA, 0.5% NP-40, 1X Denhardt's solution, 1 mM sodium pyrophosphate, 1 mM sodium monobasic phosphate, 0.1 mM ATP, and 0.2 mg of yeast RNA per ml following standard Southern blotting procedures for 12 to 24 hours optimally. The carrier material is finally washed once in 6X SSC plus 0.1% SDS for 15 minutes and twice each for 15 minutes using 6X SSC at 5°C to 10°C below the calculated T_m .

In a third aspect, the parent is encoded by a polynucleotide with a sequence identity to the mature polypeptide coding sequence of SEQ ID NO: 1 of at least 60%, e.g., at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100%, which encodes a polypeptide which is able to function as an albumin. In an embodiment, the parent is encoded by a polynucleotide comprising or consisting of SEQ ID NO: 1.

Preparation of Variants

In a further aspect the invention relates to a method for preparing a variant albumin, fragment thereof, or fusion polypeptide comprising variant albumin or a fragment thereof comprising the steps of:

- a. Identifying one or more amino acid residue positions being important for the binding of albumin to FcRn, in an albumin or a fragment thereof or the albumin part of a fusion polypeptide comprising albumin or a fragment thereof;
- b. Providing a nucleic acid encoding said albumin, the fragment thereof or the albumin part of a fusion polypeptide comprising albumin or the fragment thereof;
- c. Modifying the nucleic acid provided in b., so that the one or more (several) amino acid residue located at the positions identified in a., are deleted or substituted or inserted with a different amino acid;
- d. Expressing the modified nucleic acid in a suitable host cell; and
- e. Recovering the variant albumin, the fragment thereof or the fusion polypeptide comprising variant albumin or the fragment thereof.

The identification of one or more amino acid residue positions being important for the binding of albumin to FcRn, in albumin, fragment thereof or the albumin part of a fusion polypeptide can be done in several ways including, but not limited to, random mutagenesis followed by analysis of the generated mutants and comparison with the non-mutated parent molecule, and identification

based on structural considerations optionally followed by generation of variants having the identified alterations and comparison with the non-mutated patent molecule.

A preferred method for identification of one or more amino acid residue positions to be changed to in order to prepare a variant HSA having an altered binding to FcRn compared with natural HSA, comprises the following steps:

- i) Identifying a non-human albumin having a different binding property to FcRn;
- ii) Identifying the amino acid residues of the human serum albumin interacting with FcRn;
- iii) Comparing the primary and/or the tertiary structure of the identified non-human albumin and human serum albumin with respect to the amino acid residues identified in step ii) and identifying the amino acid residues that differ between said non-human albumin and human serum albumin as being responsible for the observed binding difference; and
- iv) Optionally preparing variants of HSA at the positions identified in step iii) and confirming that the prepared variants have altered binding to FcRn compared with HSA.

Step i) above may be done using the SPR assay described below. However, the skilled person will appreciate that other methods may be used to identify non-human albumins having different binding properties to FcRn than HSA, and that the method is not dependent on how the non-human albumin, having different binding properties to FcRn, has been identified.

In one preferred embodiment the identified non-human albumin has a stronger binding to FcRn than HSA. Examples of non-human albumins having stronger binding to FcRn than HSA include donkey serum albumin, rabbit serum albumin, dog serum albumin, hamster serum albumin, guinea pig serum albumin, mouse serum albumin and rat serum albumin. Step ii) may be accomplished by considering the structure of FcRn, HSA and the binding complex of these two. In the absence of an available structure of the binding complex it is possible to use a model where the HSA structure is docked into the structure of the FcRn structure and thereby identify amino acid residues of HSA interacting with FcRn.

In another preferred embodiment the identified non-human albumin has a weaker binding to FcRn than HSA. Examples of non-human albumins having weaker binding to FcRn than HSA include bovine serum albumin, goat serum albumin, sheep serum albumin and chicken serum albumin. Step ii) may be accomplished by considering the structure of FcRn, HSA and the binding complex of these two. In absence of an available structure of the binding complex it is possible to use a model where the HSA structure is docked into the structure of the FcRn structure and thereby identify residues of HSA interacting with FcRn.

In this invention and claims, an amino acid residues of HSA interacting with FcRn is considered any amino acid residues of HSA being located less than 10Å from an amino acid in the FcRn or any amino acid residue that is involved in a hydrogen bond, a salt bridge or a polar or nonpolar interaction with an amino acid residue that is located less than 10Å from an amino acid in the FcRn. Preferably the amino acid in HSA residues are located less than 10Å from amino acids in the FcRn, more preferred less than 6Å from amino acids in the FcRn and most preferred less than 3Å from amino acids in the FcRn.

Step iii) and iv) can be done using techniques well known to the skilled person.

The present invention also relates to methods for obtaining a variant albumin or fragments thereof, or fusion polypeptides comprising the variant albumin or fragments thereof, or associates of variant albumin or fragment thereof comprising: (a) introducing into a parent albumin or fragments thereof, or fusion polypeptides comprising the parent albumin or fragments thereof an alteration at one or more (several) positions corresponding to positions 417, 440, 464, 490, 492, 493, 494, 495, 496, 499, 500, 501, 503, 504, 505, 506, 510, 535, 536, 537, 538, 540, 541, 542, 550, 573, 574, 575, 577, 578, 579, 580, 581, 582 and 584 of the mature polypeptide of SEQ ID NO: 2; and (b) recovering the variant albumin or fragments thereof, or fusion polypeptides comprising the variant albumin or fragments thereof.

The variants can be prepared by those skilled persons using any mutagenesis procedure known in the art, such as site-directed mutagenesis, synthetic gene construction, semi-synthetic gene construction, random mutagenesis, shuffling, etc.

Site-directed mutagenesis is a technique in which one or more (several) mutations are created at one or more defined sites in a polynucleotide encoding the parent.

Site-directed mutagenesis can be accomplished *in vitro* by PCR involving the use of oligonucleotide primers containing the desired mutation. Site-directed mutagenesis can also be performed *in vitro* by cassette mutagenesis involving the cleavage by a restriction enzyme at a site in the plasmid comprising a polynucleotide encoding the parent and subsequent ligation of an oligonucleotide containing the mutation in the polynucleotide. Usually the restriction enzyme that digests at the plasmid and the oligonucleotide is the same, permitting ligation of the plasmid and insert to one another. See, e.g., Scherer and Davis, 1979, *Proc. Natl. Acad. Sci. USA* **76**: 4949-4955; and Barton *et al.*, 1990, *Nucleic Acids Res.* **18**: 7349-4966.

Site-directed mutagenesis can also be accomplished *in vivo* by methods known in the art. See, e.g., U.S. Patent Application Publication No. 2004/0171154; Storici *et al.*, 2001, *Nature Biotechnol.* **19**: 773-776; Kren *et al.*, 1998, *Nat. Med.* **4**: 285-290; and Calissano and Macino, 1996, *Fungal Genet. Newslett.* **43**: 15-16.

Any site-directed mutagenesis procedure can be used in the present invention. There are many commercial kits available that can be used to prepare variants.

Synthetic gene construction entails *in vitro* synthesis of a designed polynucleotide molecule to encode a polypeptide of interest. Gene synthesis can be performed utilizing a number of techniques, such as the multiplex microchip-based technology described by Tian *et al.* (2004, *Nature* **432**: 1050-1054) and similar technologies wherein oligonucleotides are synthesized and assembled upon photo-programable microfluidic chips.

Single or multiple amino acid substitutions, deletions, and/or insertions can be made and tested using known methods of mutagenesis, recombination, and/or shuffling, followed by a relevant screening procedure, such as those disclosed by Reidhaar-Olson and Sauer, 1988, *Science* **241**: 53-57; Bowie and Sauer, 1989, *Proc. Natl. Acad. Sci. USA* **86**: 2152-2156; WO 95/17413; or WO 95/22625. Other methods that can be used include error-prone PCR, phage display (e.g., Lowman *et al.*, 1991, *Biochemistry* **30**: 10832-10837; U.S. Patent No. 5,223,409; WO 92/06204) and region-directed mutagenesis (Derbyshire *et al.*, 1986, *Gene* **46**: 145; Ner *et al.*, 1988, *DNA* **7**: 127).

Mutagenesis/shuffling methods can be combined with high-throughput, automated screening methods to detect activity of cloned, mutagenized polypeptides expressed by host cells (Ness *et al.*, 1999, *Nature Biotechnology* **17**: 893-896). Mutagenized DNA molecules that encode active polypeptides can be recovered from the host cells and rapidly sequenced using standard methods in the art. These methods allow the rapid determination of the importance of individual amino acid residues in a polypeptide.

Semi-synthetic gene construction is accomplished by combining aspects of synthetic gene construction, and/or site-directed mutagenesis, and/or random mutagenesis, and/or shuffling. Semi-synthetic construction is typified by a process utilizing polynucleotide fragments that are synthesized, in combination with PCR techniques. Defined regions of genes may thus be synthesized *de novo*, while other regions may be amplified using site-specific mutagenic primers, while yet other regions may be subjected to error-prone PCR or non-error prone PCR amplification. Polynucleotide sub sequences may then be shuffled.

30 Variants

The present invention also provides variant albumins or fragments thereof, or fusion polypeptides comprising the variant albumin or fragments thereof, of a parent albumin, comprising an alteration at one or more (several) positions corresponding to positions 417, 440, 464, 490, 492, 493, 494, 495, 496, 499, 500, 501, 503, 504, 505, 506, 510, 535, 536, 537, 538, 540, 541, 542, 550, 573, 574, 575, 577, 578, 579, 580, 581, 582 and 584 in SEQ ID NO: 2, wherein each

alteration is independently a substitution, insertion or deletion with the provision that the and the variant is not SEQ ID NO: 2 having the substitution D494N, E501K, K541E, D550G,A, K573E or K574N..

The variant albumin, a fragment thereof, or albumin part of a fusion polypeptide comprising
5 variant albumin or a fragment thereof according to the invention has generally a sequence identity the sequence of HSA shown in SEQ ID NO: 2 of at least 60%, preferably at least 70%, preferably at least 80%, preferably at least 85%, preferably at least 90 %, more preferred at least 95%, more preferred at least 96%, more preferred at least 97%, more preferred at least 98% and most preferred at least 99%.

10 In one aspect, the number of alterations in the variants of the present invention is 1-20, e.g., 1-10 and 1-5, such as 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10 alterations.

The variant albumin, a fragment thereof or fusion polypeptide comprising the variant albumin or fragment thereof has altered plasma half-life compared with the corresponding parent albumin, fragment thereof, or fusion polypeptide comprising the variant albumin or fragment
15 thereof.

In a particular preferred embodiment the parent albumin is HSA and the variant albumin, a fragment thereof or fusion polypeptide comprising the variant albumin or fragment thereof has altered plasma half-life compared with the HSA, the corresponding fragment or fusion polypeptide comprising HSA or fragment thereof.

20 The correlation between binding of albumin to its receptor and plasma half-life has been realized by the present inventors based on the natural occurring allele of HSA D494N. The inventors have analyzed this allele and found that it has a lower affinity to its receptor FcRn.

Further, it has been disclosed that a transgenic mouse having the natural mouse FcRn replaced with human FcRn has a higher serum albumin level than normal mouse; see (J Exp Med.
25 (2003) **197**(3):315-22). The inventors have discovered that human FcRn has a higher affinity to mouse serum albumin than mouse FcRn has to mouse serum albumin and, therefore, the observed increase in serum albumin in the transgenic mice corresponds with a higher affinity between serum albumin and its receptor, confirming the correlation between albumin binding to FcRn and plasma half-life. In addition, variants of albumin that have little or no binding to FcRn have been shown to
30 have reduced half-life in a mouse model, Kenanova *et al* (2009) *J. Nucl. Med.*; **50** (Supplement 2):1582).

One way to determine whether the affinity of a variant albumin to FcRn is higher or lower than the parent albumin is to use the Surface Plasmon Resonance assay (SPR) as described below. The skilled person will understand that other methods might be useful to determine whether
35 the affinity of a variant albumin to FcRn is higher or lower than the affinity of the parent albumin to

FcRn, e.g., determination and comparison of the binding constants K_D . Thus, according to the invention variant albumins having a K_D that is lower than the K_D for natural HSA is considered to have a higher plasma half-life than HSA and variant albumins having a K_D that is higher than the K_D for natural HSA is considered to have a lower plasma half-life than HSA.

5 The variants of albumin or fragments thereof or fusion polypeptides comprising albumin or fragments thereof comprise one or more alterations, such as substitutions, deletions or insertions at one or more (several) positions corresponding to the positions in HSA selected from the group consisting of 417, 440, 464, 490, 492, 493, 494, 495, 496, 499, 500, 501, 503, 504, 505, 506, 510, 535, 536, 537, 538, 540, 541, 542, 550, 573, 574, 575, 577, 578, 579, 580, 581, 582 and 584. The
10 substitution may be any substitution where the amino acid in the natural albumin sequence is substituted with a different amino acid selected among the remaining 19 natural occurring amino acids.

In one aspect, a variant comprises an alteration at one or more (several) positions corresponding to positions 417, 440, 464, 490, 492, 493, 494, 495, 496, 499, 500, 501, 503, 504,
15 505, 506, 510, 535, 536, 537, 538, 540, 541, 542, 550, 573, 574, 575, 577, 578, 579, 580, 581, 582 and 584 in SEQ ID NO: 2. In another aspect, a variant comprises an alteration at two positions corresponding to any of 417, 440, 464, 490, 492, 493, 494, 495, 496, 499, 500, 501, 503, 504, 505, 506, 510, 535, 536, 537, 538, 540, 541, 542, 550, 573, 574, 575, 577, 578, 579, 580, 581, 582 and 584 in SEQ ID NO: 2. In another aspect, a variant comprises an alteration at three positions
20 corresponding to any of positions 417, 440, 464, 490, 492, 493, 494, 495, 496, 499, 500, 501, 503, 504, 505, 506, 510, 535, 536, 537, 538, 540, 541, 542, 550, 573, 574, 575, 577, 578, 579, 580, 581, 582 and 584 in SEQ ID NO: 2. In another aspect, a variant comprises an alteration at each position corresponding to positions 417, 440, 464, 490, 492, 493, 494, 495, 496, 499, 500, 501, 503, 504, 505, 506, 510, 535, 536, 537, 538, 540, 541, 542, 550, 573, 574, 575, 577, 578, 579,
25 580, 581, 582 and 584 in SEQ ID NO: 2.

In another aspect, the variant comprises the substitution Q417A,H of the mature polypeptide of SEQ ID NO: 2. In another aspect, the variant comprises the substitution H440Q of the mature polypeptide of SEQ ID NO: 2. In another aspect, the variant comprises the substitution H464Q of the mature polypeptide of SEQ ID NO: 2. In another aspect, the variant comprises the substitution
30 A490D of the mature polypeptide of SEQ ID NO: 2. In another aspect, the variant comprises the substitution E492G, T,P,H of the mature polypeptide of SEQ ID NO: 2. In another aspect, the variant comprises the substitution V493P,L of the mature polypeptide of SEQ ID NO: 2. In another aspect, the variant comprises the substitution D494N,Q,A,E,P of the mature polypeptide of SEQ ID NO: 2. In another aspect, the variant comprises the substitution E495Q,A of the mature polypeptide
35 of SEQ ID NO: 2. In another aspect, the variant comprises the substitution T496A of the mature

polypeptide of SEQ ID NO: 2. In another aspect, the variant comprises the substitution P499A of the mature polypeptide of SEQ ID NO: 2. In another aspect, the variant comprises the substitution K500E,G,D,A,S,C,P,H,F,N,W,T,M,Y,V,Q,L,I,R of the mature polypeptide of SEQ ID NO: 2. In another aspect, the variant comprises the substitution E501A,P,Q of the mature polypeptide of SEQ ID NO: 2. In another aspect, the variant comprises the substitution N503K,D,H of the mature polypeptide of SEQ ID NO: 2. In another aspect, the variant comprises the substitution A504E of the mature polypeptide of SEQ ID NO: 2. In another aspect, the variant comprises the substitution E505K, D of the mature polypeptide of SEQ ID NO: 2. In another aspect, the variant comprises the substitution T506F, S of the mature polypeptide of SEQ ID NO: 2. In another aspect, the variant comprises the substitution H510Q of the mature polypeptide of SEQ ID NO: 2. In another aspect, the variant comprises the substitution H535Q of the mature polypeptide of SEQ ID NO: 2. In another aspect, the variant comprises the substitution K536A of the mature polypeptide of SEQ ID NO: 2. In another aspect, the variant comprises the substitution P537A of the mature polypeptide of SEQ ID NO: 2. In another aspect, the variant comprises the substitution K538A,H of the mature polypeptide of SEQ ID NO: 2. In another aspect, the variant comprises the substitution T540S of the mature polypeptide of SEQ ID NO: 2. In another aspect, the variant comprises the substitution K541A,D,G,N,E of the mature polypeptide of SEQ ID NO: 2. In another aspect, the variant comprises the substitution E542P,D of the mature polypeptide of SEQ ID NO: 2. In another aspect, the variant comprises the substitution D550N of the mature polypeptide of SEQ ID NO: 2. In another aspect, the variant comprises the substitution K573Y,W,P,H,F,V,I,T,N,S,G,M,C,A,E,Q,R,L,D of the mature polypeptide of SEQ ID NO: 2. In another aspect, the variant comprises the substitution K574N of the mature polypeptide of SEQ ID NO: 2. In another aspect, the variant comprises the substitution Q580K of the mature polypeptide of SEQ ID NO: 2. In another aspect, the variant comprises the substitution L575F of the mature polypeptide of SEQ ID NO: 2. In another aspect, the variant comprises the substitution A577T,E of the mature polypeptide of SEQ ID NO: 2. In another aspect, the variant comprises the substitution A578R,S of the mature polypeptide of SEQ ID NO: 2. In another aspect, the variant comprises the substitution S579C,T of the mature polypeptide of SEQ ID NO: 2. In another aspect, the variant comprises the substitution Q580K of the mature polypeptide of SEQ ID NO: 2. In another aspect, the variant comprises the substitution A581D of the mature polypeptide of SEQ ID NO: 2. In another aspect, the variant comprises the substitution A582T of the mature polypeptide of SEQ ID NO: 2. In another aspect, the variant comprises the substitution G584A of the mature polypeptide of SEQ ID NO: 2.

In one aspect, the variant comprises an alteration at a position corresponding to position 417. In another aspect, the amino acid at a position corresponding to position 417 is substituted

with Ala, Arg, Asn, Asp, Cys, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, or Val, preferably with Ala or His. In another aspect, the variant comprises the substitution Q417A, H of the mature polypeptide of SEQ ID NO: 2.

In another aspect, the variant comprises an alteration at a position corresponding to position 440. In another aspect, the amino acid at a position corresponding to position 440 is substituted with Ala, Arg, Asn, Asp, Cys, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, or Val, preferably with Ala. In another aspect, the variant comprises the substitution H440Q of the mature polypeptide of SEQ ID NO: 2.

In another aspect, the variant comprises an alteration at a position corresponding to position 464. In another aspect, the amino acid at a position corresponding to position 464 is substituted with Ala, Arg, Asn, Asp, Cys, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, or Val, preferably with Ala. In another aspect, the variant comprises the substitution H464Q of the mature polypeptide of SEQ ID NO: 2.

In another aspect, the variant comprises an alteration at a position corresponding to position 490. In another aspect, the amino acid at a position corresponding to position 490 is substituted with Ala, Arg, Asn, Asp, Cys, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, or Val. In another aspect, the variant comprises the substitution A490G of the mature polypeptide of SEQ ID NO: 2.

In another aspect, the variant comprises an alteration at a position corresponding to position 492. In another aspect, the amino acid at a position corresponding to position 492 is substituted with Ala, Arg, Asn, Asp, Cys, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, or Val, preferably with Gly. In another aspect, the variant comprises the substitution E492G of the mature polypeptide of SEQ ID NO: 2.

In another aspect, the variant comprises an alteration at a position corresponding to position 493. In another aspect, the amino acid at a position corresponding to position 493 is substituted with Ala, Arg, Asn, Asp, Cys, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, or Val, preferably with Pro. In another aspect, the variant comprises the substitution V493P of the mature polypeptide of SEQ ID NO: 2.

In another aspect, the variant comprises an alteration at a position corresponding to position 494. In another aspect, the amino acid at a position corresponding to position 494 is substituted with Ala, Arg, Asn, Asp, Cys, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, or Val, preferably with Asn, Gln or Ala. In another aspect, the variant comprises the substitution D494N,Q, A of the mature polypeptide of SEQ ID NO: 2.

In another aspect, the variant comprises an alteration at a position corresponding to position 495. In another aspect, the amino acid at a position corresponding to position 495 is substituted

with Ala, Arg, Asn, Asp, Cys, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, or Val, preferably with Gln or Ala. In another aspect, the variant comprises the substitution E495Q or A of the mature polypeptide of SEQ ID NO: 2.

In another aspect, the variant comprises an alteration at a position corresponding to position 496. In another aspect, the amino acid at a position corresponding to position 496 is substituted with Ala, Arg, Asn, Asp, Cys, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, or Val, preferably with Ala. In another aspect, the variant comprises the substitution T496A of the mature polypeptide of SEQ ID NO: 2.

In another aspect, the variant comprises an alteration at a position corresponding to position 499. In another aspect, the amino acid at a position corresponding to position 499 is substituted with Ala, Arg, Asn, Asp, Cys, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, or Val, preferably with Ala. In another aspect, the variant comprises the substitution P499A of the mature polypeptide of SEQ ID NO: 2.

In another aspect, the variant comprises an alteration at a position corresponding to position 500. In another aspect, the amino acid at a position corresponding to position 500 is substituted with Ala, Arg, Asn, Asp, Cys, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, or Val, preferably with Ala. In another aspect, the variant comprises the substitution K500E,G,D,A,S,C,P,H,F,N,W,T,M,Y,V,Q,L,I,R of the mature polypeptide of SEQ ID NO: 2.

In another aspect, the variant comprises an alteration at a position corresponding to position 501. In another aspect, the amino acid at a position corresponding to position 501 is substituted with Ala, Arg, Asn, Asp, Cys, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, or Val, preferably with Ala or Gln to reduce affinity and Pro to increase affinity. In another aspect, the variant comprises the substitution E501A, Q, P of the mature polypeptide of SEQ ID NO: 2.

In another aspect, the variant comprises an alteration at a position corresponding to position 503. In another aspect, the amino acid at a position corresponding to position 503 is substituted with Ala, Arg, Asn, Asp, Cys, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, or Val, preferably with Asp or Lys or His. In another aspect, the variant comprises the substitution N503D, K, H of the mature polypeptide of SEQ ID NO: 2.

In another aspect, the variant comprises an alteration at a position corresponding to position 504. In another aspect, the amino acid at a position corresponding to position 504 is substituted with Ala, Arg, Asn, Asp, Cys, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, or Val. In another aspect, the variant comprises the substitution A504 of the mature polypeptide of SEQ ID NO: 2.

In another aspect, the variant comprises an alteration at a position corresponding to position 505. In another aspect, the amino acid at a position corresponding to position 505 is substituted

with Ala, Arg, Asn, Asp, Cys, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, or Val. In another aspect, the variant comprises the substitution E505D of the mature polypeptide of SEQ ID NO: 2.

In another aspect, the variant comprises an alteration at a position corresponding to position 506. In another aspect, the amino acid at a position corresponding to position 506 is substituted with Ala, Arg, Asn, Asp, Cys, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, or Val. In another aspect, the variant comprises the substitution T506S,F of the mature polypeptide of SEQ ID NO: 2.

In another aspect, the variant comprises an alteration at a position corresponding to position 510. In another aspect, the amino acid at a position corresponding to position 510 is substituted with Ala, Arg, Asn, Asp, Cys, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, or Val, preferably with Gln. In another aspect, the variant comprises the substitution H510Q of the mature polypeptide of SEQ ID NO: 2.

In another aspect, the variant comprises an alteration at a position corresponding to position 535. In another aspect, the amino acid at a position corresponding to position 535 is substituted with Ala, Arg, Asn, Asp, Cys, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, or Val, preferably with Gln. In another aspect, the variant comprises the substitution H535Q of the mature polypeptide of SEQ ID NO: 2.

In another aspect, the variant comprises an alteration at a position corresponding to position 536. In another aspect, the amino acid at a position corresponding to position 536 is substituted with Ala, Arg, Asn, Asp, Cys, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, or Val, preferably with Ala. In another aspect, the variant comprises the substitution K536A of the mature polypeptide of SEQ ID NO: 2.

In another aspect, the variant comprises an alteration at a position corresponding to position 537. In another aspect, the amino acid at a position corresponding to position 537 is substituted with Ala, Arg, Asn, Asp, Cys, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, or Val, preferably with Ala. In another aspect, the variant comprises the substitution P537A of the mature polypeptide of SEQ ID NO: 2.

In another aspect, the variant comprises an alteration at a position corresponding to position 538. In another aspect, the amino acid at a position corresponding to position 538 is substituted with Ala, Arg, Asn, Asp, Cys, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, or Val, preferably with Ala. In another aspect, the variant comprises the substitution K538H, A of the mature polypeptide of SEQ ID NO: 2.

In another aspect, the variant comprises an alteration at a position corresponding to position 540. In another aspect, the amino acid at a position corresponding to position 540 is substituted

with Ala, Arg, Asn, Asp, Cys, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, or Val. In another aspect, the variant comprises the substitution T540S of the mature polypeptide of SEQ ID NO: 2.

In another aspect, the variant comprises an alteration at a position corresponding to position 541. In another aspect, the amino acid at a position corresponding to position 541 is substituted with Ala, Arg, Asn, Asp, Cys, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, or Val, preferably with Gly, Asp or Ala. In another aspect, the variant comprises the substitution K541G, D A, N of the mature polypeptide of SEQ ID NO: 2.

In another aspect, the variant comprises an alteration at a position corresponding to position 542. In another aspect, the amino acid at a position corresponding to position 542 is substituted with Ala, Arg, Asn, Asp, Cys, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, or Val, preferably with Asp or Pro. In another aspect, the variant comprises the substitution E542D, P of the mature polypeptide of SEQ ID NO: 2.

In another aspect, the variant comprises an alteration at a position corresponding to position 550. In another aspect, the amino acid at a position corresponding to position 550 is substituted with Ala, Arg, Asn, Asp, Cys, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, or Val, preferably with Asn to reduce affinity, preferably with Glu to increase affinity.

In another aspect, the variant comprises an alteration at a position corresponding to position 573. In another aspect, the amino acid at a position corresponding to position 573 is substituted with Ala, Arg, Asn, Asp, Cys, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, or Val, preferably with Tyr, Trp, Pro, His, Phe, Val, Ile, Thr, Asn, Ser, Gly, Met, Cys, Ala, Glu, Gln, Arg, Leu, Asp. In another aspect, the variant comprises the substitution K573Y,W,P,H,F,V,I,T,N,S,G,M,C,A,E,Q,R,L,D of the mature polypeptide of SEQ ID NO: 2.

In another aspect, the variant comprises an alteration at a position corresponding to position 574. In another aspect, the amino acid at a position corresponding to position 574 is substituted with Ala, Arg, Asn, Asp, Cys, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, or Val, preferably with Asn. In another aspect, the variant comprises the substitution K574N of the mature polypeptide of SEQ ID NO: 2.

In another aspect, the variant comprises an alteration at a position corresponding to position 575. In another aspect, the amino acid at a position corresponding to position 575 is substituted with Ala, Arg, Asn, Asp, Cys, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, or Val, preferably with Phe. In another aspect, the variant comprises the substitution L575F of the mature polypeptide of SEQ ID NO: 2.

In another aspect, the variant comprises an alteration at a position corresponding to position 577. In another aspect, the amino acid at a position corresponding to position 577 is substituted

with Ala, Arg, Asn, Asp, Cys, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, or Val, preferably with Thr or Glu. In another aspect, the variant comprises the substitution A577TE of the mature polypeptide of SEQ ID NO: 2.

In another aspect, the variant comprises an alteration at a position corresponding to position 578. In another aspect, the amino acid at a position corresponding to position 578 is substituted with Ala, Arg, Asn, Asp, Cys, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, or Val, preferably with Arg or Ser. In another aspect, the variant comprises the substitution A578R,S of the mature polypeptide of SEQ ID NO: 2.

In another aspect, the variant comprises an alteration at a position corresponding to position 579. In another aspect, the amino acid at a position corresponding to position 579 is substituted with Ala, Arg, Asn, Asp, Cys, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, or Val, preferably with Cys or Thr. In another aspect, the variant comprises the substitution S579C,T of the mature polypeptide of SEQ ID NO: 2.

In another aspect, the variant comprises an alteration at a position corresponding to position 580. In another aspect, the amino acid at a position corresponding to position 580 is substituted with Ala, Arg, Asn, Asp, Cys, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, or Val, preferably with Lys. In another aspect, the variant comprises the substitution Q580K of the mature polypeptide of SEQ ID NO: 2.

In another aspect, the variant comprises an alteration at a position corresponding to position 581. In another aspect, the amino acid at a position corresponding to position 581 is substituted with Ala, Arg, Asn, Asp, Cys, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, or Val, preferably with Asp. In another aspect, the variant comprises the substitution A581D of the mature polypeptide of SEQ ID NO: 2.

In another aspect, the variant comprises an alteration at a position corresponding to position 582. In another aspect, the amino acid at a position corresponding to position 582 is substituted with Ala, Arg, Asn, Asp, Cys, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, or Val, preferably with Thr. In another aspect, the variant comprises the substitution A582T of the mature polypeptide of SEQ ID NO: 2.

In another aspect, the variant comprises an alteration at a position corresponding to position 584. In another aspect, the amino acid at a position corresponding to position 584 is substituted with Ala, Arg, Asn, Asp, Cys, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, or Val, preferably with Ala. In another aspect, the variant comprises the substitution G584A of the mature polypeptide of SEQ ID NO: 2.

In another aspect, the variant comprises an alteration at positions corresponding to positions 494 and 496 in SEQ ID NO: 2, such as those described above.

In another aspect, the variant comprises alterations at positions corresponding to positions 492 and 493 in SEQ ID NO: 2, such as those described above.

In another aspect, the variant comprises alterations at positions corresponding to positions 494 and 417 in SEQ ID NO: 2, such as those described above.

5 In another aspect, the variant comprises alterations at positions corresponding to positions 492 and 503 in SEQ ID NO: 2, such as those described above.

In another aspect, the variant comprises alterations at positions corresponding to positions 492 and 573 in SEQ ID NO: 2, such as those described above.

10 In another aspect, the variant comprises alterations at positions corresponding to positions 492, 503, and 573 in SEQ ID NO: 2, such as those described above.

In one embodiment the variant albumin or fragments thereof, or fusion polypeptides comprising the variant albumin or fragments thereof according to the invention contains one substitution at a position corresponding to a position in HSA selected from the group consisting of 417, 440, 464, 490, 492, 493, 494, 495, 496, 499, 500, 501, 503, 504, 505, 506, 510, 535, 536,
15 537, 538, 540, 541, 542, 550, 573, 574, 575, 577, 578, 579, 580, 581, 582 and 584 in SEQ ID NO: 2 provided that the variant albumin is not the variant consisting of SEQ ID NO: 2 with the substitution D494N, E501K, K541E, D550G,A, K573E or K574N. The variant albumin, fragment thereof or fusion polypeptides comprising variant albumin or a fragment thereof according to the invention may comprise additional substitutions, insertions or deletions at one or more (several)
20 positions corresponding to other positions in HSA.

In another embodiment the variant albumin or fragments thereof, or fusion polypeptides comprising variant albumin or fragments thereof according to the invention contains two, three, four, five , six, seven, eight, nine, ten, eleven, twelve , thirteen, fourteen fifteen, sixteen, seventeen, eighteen, nineteen twenty or even more substitutions at positions corresponding to positions in
25 HSA selected from the group consisting of 417, 440, 464, 490, 492, 493, 494, 495, 496, 499, 500, 501, 503, 504, 505, 506, 510, 535, 536, 537, 538, 540, 541, 542, 550, 573, 574, 575, 577, 578, 579, 580, 581, 582 and 584 of SEQ ID NO: 2. The variant albumin or fragments thereof, or fusion polypeptides comprising variant albumin or fragments thereof according to the invention may comprise additional substitutions, insertions or deletions at positions corresponding to other
30 positions in HSA.

In a further embodiment the variants of albumin or fragments thereof, or fusion polypeptides comprising variant albumin or a fragment thereof according to the invention have a plasma half-life that is longer than the plasma half-life of the parent albumin fragment thereof or fusion polypeptide comprising the parent albumin or a fragment thereof. Examples according to this embodiment
35 include variants of albumin or fragments thereof, or fusion polypeptides comprising variant albumin

or a fragment thereof comprising a substitution in the position corresponding to 492, 503, 542, 550, 573, 574, 580, 581, 582 or 584 in SEQ ID NO: 2. Preferred substitutions according to this embodiment of the invention include the substitution of the amino acid residue in the position corresponding to 492 in SEQ ID NO: 2 with a G residue, substitution of the amino acid residue in the position corresponding to 503 in SEQ ID NO: 2 with a H or a K residue, substitution of the amino acid residue in the position corresponding to 550 in SEQ ID NO: 2 with an E residue, the substitution of the amino acid residue in a position corresponding to 573 in SEQ ID NO: 2 with an Y, W, P, H, F, V, I, T, N, S, G, M, C, A, E, Q, R, L or a D, the substitution of the amino acid residue in a position corresponding to 574 in SEQ ID NO: 2 with an N residue, or the substitution of the amino acid residue in the position corresponding to 580 in SEQ ID NO: 2 with an K residue. Other preferred variants have a substitution in the position corresponding to 492 in SEQ ID NO: 2 with a G residue and a substitution in the position corresponding to 573 in SEQ ID NO: 2 with an A or a P residue. Other preferred variant has a number of substitutions corresponding to position 492 in SEQ ID NO: 2 with an H residue in position 503 in SEQ ID NO: 2.

Other preferred variants have a substitution in the position corresponding to 492 in SEQ ID NO: 2 with a G residue and a substitution in the position corresponding to position 503 in SEQ ID NO: 2 corresponding to a H or a K and a substitution in position 573 in SEQ ID NO: 2 with an A or a P residue.

In a further embodiment the variants of albumin or fragments thereof, or fusion polypeptides comprising variant albumin or fragments thereof according to the invention have a plasma half-life that is shorter than the plasma half-life of the parent albumin fragment thereof or fusion polypeptide comprising the parent albumin or a fragment thereof. Examples according to this embodiment include variants of albumin or fragments thereof, or fusion polypeptides comprising variant albumin or a fragment thereof comprising a substitution in the position corresponding to 417, 440, 494, 495, 496, 499, 500, 501, 536, 537, 538, 541, 494+496 or 492+493 in SEQ ID NO: 2. Preferred substitutions include the substitutions corresponding to Q417A, H440Q, D494E+Q417H, D494N,Q,A, E495Q,A, T496A, D494N+T496A or, P499A, K500A, E501A , E501Q, K536A, P537A , K538A, K541G, K541A K541D or D550N in SEQ ID NO: 2.

In another embodiment of the invention the variants of albumin or fragments thereof, or fusion polypeptides comprising variant albumin or a fragment thereof according to the invention have lost their ability to bind FcRn. In this connection variants of albumin or fragments thereof, or fusion polypeptides comprising variant albumin or fragments thereof is considered to have lost the ability to bind FcRn if the measured resonance units for the variant in the SPR assay described below is less than 10% of the measured resonance units for the corresponding parent albumin or fragment thereof. Examples according to this embodiment include variants of albumin or fragments

thereof, or fusion polypeptides comprising variant albumin or fragments thereof comprising a substitution at a position corresponding to 464, 500, 510 or 535 in SEQ ID NO: 2. Preferred substitutions include the substitutions corresponding to H464Q, K500A,P,C,S,A,D.G H510Q or H535Q in SEQ ID NO: 2.

5 In addition to the one or more substitutions at one or more positions corresponding to positions 417, 464, 490, 492, 493, 494, 495, 496, 499, 500, 501, 503, 504, 505, 506, 510, 535, 536, 537, 538, 540, 541, 542, 550, 573, 574, 580 581, 582 and 584 in SEQ ID NO: 2 the variant albumin or fragments thereof, or fusion polypeptides comprising variant albumin or fragments thereof according to the invention may contain additional substitutions, deletions or insertions in other
10 positions of the molecules. Such additional substitutions, deletions or insertions may be useful in order to alter other properties of the molecules such as but not limited to altered glycosylation; introduction of reactive groups of the surface such a thiol groups, removing/generating a carbamoylation site; etc.

Residues that might be altered in order to provide reactive residues on the surface and
15 which advantageously could be applied to the present invention has been disclosed in the unpublished patent application WO 2010/092135 (Included by reference). Particular preferred residues include the positions corresponding to positions in SEQ ID NO: 2 .

As examples of alterations that can be made in SEQ ID NO: 2 or in corresponding positions in other albumins in order to provide a reactive thiol group on the surface includes alterations
20 corresponding to following alterations in SEQ ID NO: 2: L585C, D1C, A2C, D562C, A364C, A504C, E505C, T79C, E86C, D129C, D549C, A581C, D121C, E82C, S270C, A578C, L595LC, D1DC, A2AC, D562DC, A364AC, A504AC, E505EC, T79TC, E86EC, D129DC, D549DC, A581AC, A581AC, D121DC, E82EC, S270SC, A579AC, C360*, C316*, C75*, C168*, C558*, C361*, C91*, C124*, C169* and C567*. Alternatively a cysteine residue may be added to the N or C terminal of
25 albumin.

Polynucleotides

The present invention also relates to isolated polynucleotides that encode any of the variants of the present invention.

Nucleic Acid Constructs

The present invention also relates to nucleic acid constructs comprising a polynucleotide encoding a variant of the present invention operably linked to one or more (several) control sequences that direct the expression of the coding sequence in a suitable host cell under
35 conditions compatible with the control sequences.

A polynucleotide may be manipulated in a variety of ways to provide for expression of a variant. Manipulation of the polynucleotide prior to its insertion into a vector may be desirable or necessary depending on the expression vector. The techniques for modifying polynucleotides utilizing recombinant DNA methods are well known in the art.

5 The control sequence may be a promoter sequence, which is recognized by a host cell for expression of the polynucleotide. The promoter sequence contains transcriptional control sequences that mediate the expression of the variant. The promoter may be any nucleic acid sequence that shows transcriptional activity in the host cell including mutant, truncated, and hybrid promoters, and may be obtained from genes encoding extracellular or intracellular polypeptides
10 either homologous or heterologous to the host cell.

In a yeast host, useful promoters are obtained from the genes for *Saccharomyces cerevisiae* enolase (ENO-1), *Saccharomyces cerevisiae* protease A (PRA1), *Saccharomyces cerevisiae* protease B (PRB1), *Saccharomyces cerevisiae* translation elongation factor (TEF1), *Saccharomyces cerevisiae* translation elongation factor (TEF2), *Saccharomyces cerevisiae*
15 galactokinase (GAL1), *Saccharomyces cerevisiae* alcohol dehydrogenase/glyceraldehyde-3-phosphate dehydrogenase (ADH1, ADH2/GAP), *Saccharomyces cerevisiae* triose phosphate isomerase (TPI), *Saccharomyces cerevisiae* metallothionein (CUP1), and *Saccharomyces cerevisiae* 3-phosphoglycerate kinase. Other useful promoters for yeast host cells are described by Romanos *et al.*, 1992, *Yeast* 8: 423-488.

20 The control sequence may also be a suitable transcription terminator sequence, which is recognized by a host cell to terminate transcription. The terminator sequence is operably linked to the 3'-terminus of the polynucleotide encoding the variant. Any terminator that is functional in the host cell may be used.

Preferred terminators for yeast host cells are obtained from the genes for *Saccharomyces cerevisiae* enolase, *Saccharomyces cerevisiae* cytochrome C (CYC1), *Saccharomyces cerevisiae*
25 alcohol dehydrogenase (ADH1) and *Saccharomyces cerevisiae* glyceraldehyde-3-phosphate dehydrogenase. Other useful terminators for yeast host cells are described by Romanos *et al.*, 1992, *supra*.

30 The control sequence may also be a suitable leader sequence, a nontranslated region of an mRNA that is important for translation by the host cell. The leader sequence is operably linked to the 5'-terminus of the polynucleotide encoding the variant. Any leader sequence that is functional in the host cell may be used.

Suitable leaders for yeast host cells are obtained from the genes for *Saccharomyces cerevisiae* enolase (ENO-1), *Saccharomyces cerevisiae* 3-phosphoglycerate kinase,

Saccharomyces cerevisiae alpha-factor, and *Saccharomyces cerevisiae* alcohol dehydrogenase/glyceraldehyde-3-phosphate dehydrogenase (ADH2/GAP).

The control sequence may also be a polyadenylation sequence, a sequence operably linked to the 3'-terminus of the variant-encoding sequence and, when transcribed, is recognized by the host cell as a signal to add polyadenosine residues to transcribed mRNA. Any polyadenylation sequence that is functional in the host cell may be used.

Useful polyadenylation sequences for yeast host cells are described by Guo and Sherman, 1995, *Mol. Cellular Biol.* **15**: 5983-5990.

The control sequence may also be a signal peptide coding region that encodes a signal peptide linked to the N-terminus of a variant and directs the variant into the cell's secretory pathway. The 5'-end of the coding sequence of the polynucleotide may inherently contain a signal peptide coding region naturally linked in translation reading frame with the segment of the coding region that encodes the variant. Alternatively, the 5'-end of the coding sequence may contain a signal peptide coding region that is foreign to the coding sequence. The foreign signal peptide coding region may be required where the coding sequence does not naturally contain a signal peptide coding region. Alternatively, the foreign signal peptide coding region may simply replace the natural signal peptide coding region in order to enhance secretion of the variant. However, any signal peptide coding region that directs the expressed variant into the secretory pathway of a host cell may be used.

Useful signal peptides for yeast host cells are obtained from the genes for *Saccharomyces cerevisiae* alpha-factor and *Saccharomyces cerevisiae* invertase. Other useful signal peptide coding sequences are described by Romanos *et al.*, 1992, *supra*.

Where both signal peptide and propeptide regions are present at the N-terminus of a variant, the propeptide region is positioned next to the N-terminus of the variant and the signal peptide region is positioned next to the N-terminus of the propeptide region.

Methods of Production

The variants of the present invention can be prepared using techniques well known to the skilled person. One convenient way is by cloning nucleic acid encoding the parent albumin or a fragment thereof or fusion polypeptide comprising albumin or a fragment thereof, modifying said nucleic acid to introduce the desired substitution(s) at one or more (several) positions corresponding to positions 417, 464, 490, 492, 493, 494, 495, 496, 499, 500, 501, 503, 504, 505, 506, 510, 535, 536, 537, 538, 540, 541, 542, 550, 573, 574 and 580 in SEQ ID NO: 2, where the variant is not the variant consisting of SEQ ID NO:2 with the substitution D494N, E501K, K541E, D550G,A, K573E or K574N., preparing a suitable genetic construct where the modified nucleic acid

is placed in operative connection with suitable regulatory genetic elements, such as promoter, terminator, activation sites, ribosome binding sites etc., introducing the genetic construct into a suitable host organism, culturing the transformed host organism under conditions leading to expression of the variant and recovering the variant. All these techniques are known in the art and it is within the skills of the average practitioner to design a suitable method for preparing a particular variant according to the invention.

The variant polypeptide of the invention may also be connected to a signal sequence in order to have the variant polypeptide secreted into the growth medium during culturing of the transformed host organism. It is generally advantageous to have the variant polypeptide secreted into the growth medium in order to ease recovery and purification.

Techniques for preparing variant polypeptides have also been disclosed in WO 2009019314 (included by reference) and these techniques may also be applied to the present invention.

Albumins have been successfully expressed as recombinant proteins in a range of hosts including fungi (including but not limited to *Aspergillus* (WO06066595), *Kluyveromyces* (Fleer 1991, *Bio/technology* **9**, 968-975), *Pichia* (Kobayashi 1998 *Therapeutic Apheresis* **2**, 257-262) and *Saccharomyces* (Sleep 1990, *Bio/technology* **8**, 42-46)), bacteria (Pandjaitab 2000, *J. Allergy Clin. Immunol.* **105**, 279-285)), animals (Barash 1993, *Transgenic Research* **2**, 266-276) and plants (including but not limited to potato and tobacco (Sijmons 1990, *Bio/technology* **8**, 217 and Farran 2002, *Transgenic Research* **11**, 337-346)). The variant polypeptide of the invention is preferably produced recombinantly in a suitable host cell. In principle any host cell capable of producing a polypeptide in suitable amounts may be used and it is within the skills of the average practitioner to select a suitable host cell according to the invention. A preferred host organism is yeast, preferably selected among *Saccharomycaceae*, more preferred *Saccharomyces cerevisiae*.

The variant polypeptides of the invention may be recovered and purified from the growth medium using a combination of known separation techniques such as filtration, centrifugation, chromatography, and affinity separation techniques etc. It is within the skills of the average practitioner to purify the variants of the invention using a particular combination of such known separation steps. As an example of purification techniques that may be applied to the variants of the present invention can be mentioned the teaching of WO0044772.

The variant polypeptides of the invention may be used for delivering a therapeutically beneficial compound to an animal or a human individual in need thereof. Such therapeutically beneficial compounds include, but are not limited, to labels and readily detectable compounds for use in diagnostics, such as various imaging techniques; pharmaceutical active compounds such as drugs, or specifically binding moieties such as antibodies. The variants of the invention may even be connected to two or more different therapeutically beneficial compounds, e.g., an antibody and a

drug, which gives the combined molecule the ability to bind specifically to a desired target and thereby provide a high concentration of the connected drug at that particular target.

Fusion polypeptides

5 The variants of albumin or fragments thereof according to the invention may also be fused with a non-albumin polypeptide fusion partner. The fusion partner may in principle be any polypeptide but generally it is preferred that the fusion partner is a polypeptide having therapeutic or diagnostic properties. Fusion polypeptides comprising albumin or fragments thereof are known in the art. It has been found that such fusion polypeptide comprising albumin or a fragment thereof
10 and a fusion partner polypeptide have a longer plasma half-life compared to the unfused fusion partner polypeptide. According to the invention it is possible to alter the plasma half-life of the fusion polypeptides according to the invention compared to the corresponding fusion polypeptides of the prior art.

 One or more therapeutic polypeptides may be fused to the N-terminus, the C-terminus of
15 albumin, inserted into a loop in the albumin structure or any combination thereof. It may or it may not comprise linker sequences separating the various components of the fusion polypeptide.

 Teachings relating to fusions of albumin or a fragment thereof are known in the art and the skilled person will appreciate that such teachings can also be applied to the present invention. WO 2001/79271 A and WO 2003/59934 A also contain examples of therapeutic polypeptides that may
20 be fused to albumin or fragments thereof, and these examples apply also to the present invention.

Conjugates

 The variants of albumin or fragments thereof according to the invention may be conjugated to a second molecule using techniques known within the art. Said second molecule may comprise a
25 diagnostic moiety, and in this embodiment the conjugate may be useful as a diagnostic tool such as in imaging; or the second molecule may be a therapeutic compound and in this embodiment the conjugate may be used for therapeutic purposes where the conjugate will have the therapeutic properties of the therapeutic compound as well as the long plasma half-life of the albumin. Conjugates of albumin and a therapeutic molecule are known in the art and it has been verified that
30 such conjugates have long plasma half-life compared with the non-conjugated, free therapeutic molecule as such. The conjugates may conveniently be linked via a free thio group present on the surface of HSA (amino acid residue 34 of mature HSA) using well known chemistry.

 In one particular preferred aspect the variant albumin or fragment thereof is conjugated to a beneficial therapeutic compound and the conjugate is used for treatment of a condition in a patient
35 in need thereof, which condition is responsive to the particular selected therapeutic compound. Techniques for conjugating such a therapeutically compound to the variant albumin or fragment

thereof are known in the art. WO 2009/019314 discloses examples of techniques suitable for conjugating a therapeutically compound to a polypeptide which techniques can also be applied to the present invention. Further WO 2009/019314 discloses examples of compounds and moieties that may be conjugated to substituted transferrin and these examples may also be applied to the present invention. The teaching of WO 2009/019314 is included herein by reference.

HSA contains in its natural form one free thiol group that conveniently may be used for conjugation. As a particular embodiment within this aspect the variant albumin or fragment thereof may comprise further modifications provided to generate additional free thiol groups on the surface. This has the benefit that the payload of the variant albumin or fragment thereof is increased so that more than one molecule of the therapeutic compound can be conjugated to each molecule of variant albumin or fragment thereof, or two or more different therapeutic compounds may be conjugated to each molecule of variant albumin or fragment thereof, *e.g.*, a compound having targeting properties such as an antibody specific for example a tumour; and a cytotoxic drug conjugated to the variant albumin or fragment thereof thereby creating a highly specific drug against a tumour. Teaching of particular residues that may be modified to provide for further free thiol groups on the surface can be found in copending patent application WO 2010/092135, which is incorporated by reference.

Associates

The variants of albumin or fragments thereof may further be used in form of “associates”. In this connection the term “associate” is intended to mean a compound comprising a variant of albumin or a fragment thereof and another compound bound or associated to the variant albumin or fragment thereof by non-covalent binding. As an example of such an associate can be mentioned an associate consisting variant albumin and a lipid associated to albumin by a hydrophobic interaction. Such associates are known in the art and they may be prepared using well known techniques. As an example of a preferred associate according to the invention can be mentioned an associate comprising variant albumin and paclitaxel.

Other uses

The variant albumin or fragments thereof or fusion polypeptides comprising variant albumin or fragments thereof according to the invention have the benefit that their plasma half-life is altered compared to the parent albumin or fragments thereof or fusion polypeptides comprising parent albumin or fragments thereof. This has the advantage that the plasma half-life of conjugates comprising variant albumin or a fragment thereof or fusion polypeptide comprising variant albumin or a fragment thereof, or an associate comprising variant albumin or a fragment thereof according to the invention can be selected in accordance with the particular therapeutic purpose.

For example for a conjugate, associate or fusion polypeptide used for imaging purposes in animals or human beings, where the imaging moiety has an very short half-life and a conjugate or a fusion polypeptide comprising HSA has a plasma half-life that is far longer than needed for the imaging purposes it would be advantageous to use a variant albumin or fragment thereof of the invention having a shorter plasma half-life than the parent albumin or fragment thereof, to provide
5 conjugates of fusion polypeptides having a plasma half-life that is sufficiently long for the imaging purpose but sufficiently short to be cleared form the body of the particular patient on which it is applied.

In another example for a conjugate, an associate or fusion polypeptide comprising a
10 therapeutic compound effective to treat or alleviate a particular condition in a patient in need for such a treatment it would be advantageous to use the variant albumin or fragment thereof having a longer plasma half-life than the parent albumin or fragment thereof, to provide associates or conjugates or fusion polypeptides having longer plasma half-lives which would have the benefit that the administration of the associate or conjugate or fusion polypeptide of the invention would be
15 needed less frequently or reduced dose with less side affects compared to the situation where the parent albumin or associates thereof or fragment thereof was used.

In a further aspect the invention relates to compositions comprising the variant albumin, associates thereof or fragment thereof, variant albumin fragment or associates thereof or fusion polypeptide comprising variant albumin or fragment thereof according to the invention. The
20 compositions are preferably pharmaceutical compositions. The composition may be prepared using techniques known in the area such as disclosed in recognized handbooks within the pharmaceutical field.

In a particular embodiment the compositions comprise a variant albumin or a fragment thereof according to the invention and a compound comprising a pharmaceutically beneficial moiety
25 and an albumin binding domain (ABD). According to the invention ABD means a site, moiety or domain capable of binding to circulating albumin *in vivo* and thereby conferring transport in the circulation of the ABD and any compound or moiety bound to said ABD. ABD's are known in the art and have been shown to bind very tight to albumin so a compound comprising an ABD bound to albumin will to a certain extent behave as a single molecule. The inventors have realized by using
30 the variant albumin or fragment thereof according to the invention together with a compound comprising a pharmaceutically beneficial moiety and an ABD makes it possible to alter the plasma half-life of the compound comprising a pharmaceutically beneficial moiety and an ABD compared to the situation where said compound were injected as such in a patient having need thereof or administered in a formulation comprising natural albumin or a fragment thereof.

The variant albumin or fragments thereof, conjugates comprising variant albumin or a fragment thereof or fusion polypeptide comprising variant albumin or a fragment thereof, or an associate comprising variant albumin or a fragment thereof according to the invention may also be incorporated into nano- or microparticles using techniques well known within the art. A preferred method for preparing nano- or microparticles that may be applied to the variant albumins or fragments thereof according to the invention is disclosed in WO 2004/071536, which is incorporated herein by reference.

Compositions

The present invention is also directed to the use of a variant of albumin or a fragment thereof or fusion polypeptides comprising variant albumin or fragments thereof, or a conjugate comprising a variant of albumin or a fragment thereof, or an associate comprising a variant of albumin or a fragment thereof for the manufacture of a pharmaceutical composition, where in the variant of albumin or a fragment thereof or fusion polypeptides comprising variant albumin or fragments thereof, or a conjugate comprising a variant of albumin or a fragment thereof, or an associate comprising a variant of albumin or a fragment thereof has an altered plasma half-life compared with HSA or the corresponding fragment thereof or fusion polypeptide comprising HSA or fragment thereof or conjugate comprising HSA.

In this connection the corresponding fragment of HSA is intended to mean a fragment of HSA that aligns with and has same number of amino acids as the fragment of the variant albumin with which it is compared. Similarly the corresponding fusion polypeptide comprising HSA or conjugate comprising HSA is intended to mean molecules having same size and amino acid sequence as the fusion polypeptide of conjugate comprising variant albumin, with which it is compared.

Preferably the variant of albumin or a fragment thereof or fusion polypeptides comprising variant albumin or fragments thereof, fragment thereof, or a conjugate comprising a variant of albumin or a fragment thereof has a plasma half-life that is higher than the plasma half-life of HSA or the corresponding fragment thereof or fusion polypeptide comprising HSA or fragment thereof.

Alternatively, this may be expressed as the variant of albumin or a fragment thereof or fusion polypeptides comprising variant albumin or fragments thereof, fragment thereof, or a conjugate comprising a variant of albumin or a fragment thereof has a KD to FcRn that is lower than the corresponding KD for HSA or the corresponding fragment thereof or fusion polypeptide comprising HSA or fragment thereof. Preferably, is KD for the variant of albumin or a fragment thereof or fusion polypeptides comprising variant albumin or fragments thereof, fragment thereof, or a conjugate comprising a variant of albumin or a fragment thereof less than 0.9X KD for HSA, more

preferred less than 0.5X KD for HSA, more preferred less than 0.1X KD for HSA, even more preferred less than 0.05X KD for HSA, even more preferred less than 0.02X KD for HSA and most preferred less than 0.01X KD for HSA.

The variant of albumin or a fragment thereof or fusion polypeptides comprising variant albumin or fragments thereof, fragment thereof, or a conjugate comprising a variant of albumin or a fragment thereof is preferably the variant of albumin or a fragment thereof or fusion polypeptides comprising variant albumin or fragments thereof, fragment thereof, or a conjugate comprising a variant of albumin or a fragment thereof according to the invention.

The present invention is further described by the following examples that should not be construed as limiting the scope of the invention.

Examples

Materials and Methods

ELISA:

Wells were coated with wild-type HSA or variants diluted in phosphate buffered saline (PBS) to stated concentrations, incubated overnight at 4 C and then blocked with 4% skimmed milk (Acumedia) for 1 hour at room temperature. The wells were then washed four times with PBS/0.005% TWEEN® 20 (PBS/T) pH 6.0 before glutathione-S-transferase (GST) -fused (shFcRn (0.5 µg/ml) as described in *FEBS J.* 2008 Aug;275(16):4097-110. pre-incubated with an horseradish peroxidase (HRP)-conjugated polyclonal anti-GST from goat (1:5000; GE Healthcare), diluted in 4% skimmed milk PBS/0.005% TWEEN® 20 (PBS/T) pH 6.0 was added to each well and incubated for 1.5 h at room temperature followed by washing four times with PBS/T pH 6.0. One hundred µl of the substrate tetramethylbenzidine (TMB) (Calbiochem) was added to each well and incubated for 45 min before 100 µl of 0.25 M HCl was added. The absorbance was measured at 450 nm using a Sunrise TECAN spectrophotometer (TECAN, Maennedorf, Switzerland). The same ELISA was repeated with PBS/T pH 7.4.

Surface Plasmon Resonance (SPR):

SPR experiments were carried out using a Biacore 3000 instrument (GE Healthcare). Flow cells of CM5 sensor chips were coupled with shFcRn-GST (~1400-5000RU) using amine coupling chemistry as described in the protocol provided by the manufacturer. The coupling was performed by injecting 10µg/ml of the protein in 10mM sodium acetate pH 5.0 (GE healthcare). Phosphate buffer (67mM phosphate buffer, 0.15M NaCl, 0.005% TWEEN® 20) at pH 6.0) was used as running buffer and dilution buffer. Regeneration of the surfaces were achieved using injections of HBS-EP buffer (0.01M HEPES, 0.15M NaCl, 3mM EDTA, 0.005% surfactant P20) at pH 7.4 (Biacore AB). For binding to immobilized shFcRn-GST, 1.0-0.5 µM of each HSA variant was injected over the

surface at constant flow rate (40 µl/ml) at 25 C. In all experiments, data was zero adjusted and the reference cell subtracted. Data evaluation was performed using BIAevaluation 4.1 software (BIAcore AB).

The same SPR assay was repeated with HBS-EP buffer pH 7.4.

For the purposes of this patent unless otherwise stated HSA, WT HSA, rHA refer to Recombinant human serum albumin commercially available under the registered tradename RECOMBUMIN (available from Novozymes Biopharma UK Ltd, Nottingham UK) was used for the examples.

Serum albumin from other species: The albumins were recombinant wheres stated, produced using sequences provided from publicly available databases. Or purchased from commercial suppliers.

FcRn Expression and purification of soluble Human (shFcRn) and Mouse (smFcRn) FcRn : Methods for the generation of shFcRn and smFcRn expression plasmids, expression and purification of each heterodimer can be found in Berntzen *et al.* (2005) J. Immunol. Methods 298:93-104). Alternatively shFcRn FcRn heterodimer was produced by GeneArt AG (Germany). Sequences for the two sub units of the heterodimer can be found in SEQ ID NO: 3 and SEQ ID NO: 4. The soluble receptor was expressed in HEK293 cells and purified from culture supernatant using Ni-HiTrap chromatography columns.

Example 1. Preparation of variants

Preparation of specific HSA mutein expression plasmids

Methods for the expression of HSA mutant variants and HSA fusion variants were produced using several techniques. Standard molecular biology techniques were employed throughout such as described in Sambrook, J. and D. W. Russell, 2001. Molecular Cloning: a laboratory manual, 3rd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

Method 1. Amino acid substitutions in HSA detailed in Table1

Synthetic DNA *Nco*I/*Sac*I fragments (859 bp) were generated by gene assembly (GeneArt AG, Germany) containing point mutations within the HSA-encoding gene (SEQ ID NO: 1) to introduce the desired amino acid substitution in the translated protein. Table 2 details the codons used to introduce the amino acid substitutions into the HSA-encoding gene. The nucleotide sequence of the synthetic fragment encoding unchanged amino acids (i.e. wild type) was identical to that in pDB2243 (described in WO 00/44772). The synthetic nucleotide fragments were ligated into *Nco*I/*Sac*I-digested pDB2243 to produce plasmids pDB3876 – pDB3886 (Table 1). For the production of expression plasmids, pDB3876 – pDB3886 (see Table 1) were each digested with

NotI and *PvuI*, the DNA fragments were separated through a 0.7% (w/v) TAE gel, and 2992bp fragments ('*NotI* cassettes' including *PRB1* promoter, DNA encoding the fusion leader (FL) sequence (disclosed in WO 2010/092135), nucleotide sequence encoding HSA and *ADH1* terminator; see Figure 1) were purified from the agarose gel using a Qiagen Gel Extraction Kit

5 following the manufacturer's instructions. '*NotI* cassettes' were ligated into a *NotI*/Shrimp Alkaline Phosphatase (Roche)- treated "disintegration" plasmid pSAC35, disclosed in EP-A-286 424 and described by Sleep, D., *et al.* (1991) *Bio/Technology* **9**, 183 – 187. Ligation mixtures were used to transform chemically-competent *E. coli* DH5 α . Expression plasmids pDB3887 – pDB3897, pSAC35-derivatives containing the "*NotI* cassettes", were identified using standard techniques.

10 Disintegration plasmids pDB3887 – pDB3897 and pDB2244 (For the expression of wild type HSA, described in WO 00/44772) (Table 1) were used to transform *S. cerevisiae* BXP10cir⁰ (as previously described WO/2001/079480 as described below.

Table 1: Plasmid, amino acid substitution introduced into HSA

Plasmid	Construct
pDB2244	HSA
pDB3876	HSA D494N
pDB3877	HSA D494A
pDB3878	HSA E495Q
pDB3879	HSA E495A
pDB3880	HSA D494Q
pDB3881	HSA D494N, T496A
pDB3882	HSA T496A
pDB3883	HSA E492G
pDB3884	HSA E492G, V493P
pDB3885	HSA E492P
pDB3886	HSA E492H
pDB3887	HSA D494N
pDB3888	HSA D494A
pDB3889	HSA E495Q
pDB3890	HSA E495A
pDB3891	HSA D494Q
pDB3892	HSA D494N, T496A
pDB3893	HSA T496A
pDB3894	HSA E492G
pDB3895	HSA E492G, V493P
pDB3896	HSA E492P
pDB3897	HSA E492H

n/a = Not applicable. pDB3876-pDB3886 are sub-cloning plasmids.

Table 2: Codons used to introduce amino acid substitutions into HSA

Amino acid	Codon
Gly	GGT
Glu	GAA
Asp	GAT
Val	GTT
Ala	GCT
Arg	AGA
Lys	AAA
Asn	AAT
Met	ATG
Ile	ATT
Thr	ACT
Trp	TGG
Cys	TGT
Tyr	TAT
Leu	TTG
Phe	TTT
Ser	TCT
Gln	CAA
His	CAT
Pro	CCA
Stop	TAA

5

Method 2. Production of HSA variants D494N+E495Q+T496A and E495Q+T496A

A PCR-based method, using a QuickChange Lightening Kit (Statagene), was employed to introduce point mutations into HSA. Oligonucleotide pairs xAP094 (SEQ ID NO: 5)/xAP095 (SEQ ID NO: 6) and xAP096 (SEQ ID NO: 7)/xAP097 (SEQ ID NO: 8) were used to generate two HSA variants (D494N+E495Q+T496A and E495Q+T496A, respectively). Plasmid pDB3927 (disclosed in WO 2010/092135) was used as template DNA and the methodology recommended by the manufacturer of the kit was followed. The resulting plasmids were named pDB3995 and pDB3996 (contain HSA D494N+E495Q+T496A and E495Q+T496A expression cassettes, respectively). pDB3995 and pDB3996 were digested with *Bst*EII/*Bsr*BI and the linearised DNA molecules were purified using standard techniques. One hundred ng of each *Bst*EII/*Bsr*BI digested DNA, purified using a Qiagen PCR-Purification kit following the manufacturer's instructions, was mixed individually with 100ng *Acc*65I/*Bam*HI-digested pDB3936) (disclosed in WO 2010/092135) and

15

used to directly transform *S. cerevisiae* BXP10cir⁰ using the Sigma Yeast Transformation kit described below.

Method 3. Amino acid substitutions in HSA detailed in Table 3

Plasmid pDB3927 (disclosed in WO 2010/092135) (containing an identical nucleotide sequence encoding HSA as in pDB2243) was manipulated to amino acid substitutions within the mature HSA protein. Synthetic DNA fragments were generated (GeneArt AG, Germany or DNA2.0 Inc, USA) (*NcoI/Bsu36I*, *AvrII/SphI* or *SacI/SphI* fragments), containing point mutations within the HSA-encoding gene to introduce the desired amino acid substitution(s) into the translated protein sequence. Table 2 details the codons used to introduce the amino acid substitutions into the HSA-encoding gene. The nucleotide sequence of the synthetic fragment encoding unchanged amino acids (i.e. wild type) was identical to those in pDB3927. Synthetic DNA fragments were sub-cloned into *NcoI/Bsu36I*, *AvrII/SphI*-, *SacI/SphI* -digested pDB3927 (described in PCT 11527.204-WO) to generate pDB4006-pDB4010, pDB4083-pDB4101 and pDB4103-pDB4111 and pDB4194, pDB4200, pDB4202 (see Table 3).

Similarly, *BamHI/SalI* fragments containing point mutations in the nucleotide sequence encoding HSA were generated by gene assembly (DNA2.0 Inc, USA) and ligated into *BamHI/SalI*-digested pDB3964 (described in WO 2010/092135) to produce plasmids pDB3986-pDB3989 (Table 3).

The C-terminal string of amino acids from position 573-585 (KKLVAAASQAALGL) (SEQ ID NO: 9) in HSA were mutated to those in macaque (PKFVAASQAALA) (SEQ ID NO: 10), mouse (PNLVTRCKDALA) (SEQ ID NO: 11), rabbit (PKLVESSKATLG) (SEQ ID NO: 12) and sheep (PKLVASTQAALA) (SEQ ID NO: 13) serum albumin. The codons used to introduce each amino acid substitution are given in Table 2. Synthetic DNA fragments (*SacI/SphI*) were generated (DNA2.0 Inc, USA) by gene assembly (the nucleotide sequence of the synthetic fragment encoding unchanged amino acids (i.e. wild type) was identical to that in pDB3927) and were sub-cloned into *SacI/SphI*-digested pDB3927 to produce plasmids pDB4114-4117 (Table 3).

Plasmids pDB3883 (Table 1), pDB4094 and pDB4095 (Table 3) were digested with *NcoI/SacI* and 857bp fragments from each digest were purified before being ligated into *NcoI/SacI*-digested pDB4006 or pDB4110 (8.688kb) (Table 3) to produce pDB4156-pDB4161.

Expression plasmids were generated *in vivo* (i.e. via homologous recombination in *S. cerevisiae*; a technique referred to as gap repair or *in vivo* cloning- see Orr-Weaver & Szostak. 1983. *Proc. Natl. Acad. Sci. USA*. 80:4417-4421). Modified plasmids listed in Table 3 were digested with *BstEII/BsrBI* and the linearised DNA molecules were purified using standard techniques. One hundred ng of each *BstEII/BsrBI* digested DNA, purified using a Qiagen PCR-Purification kit

following the manufacturer's instructions, was mixed individually with 100ng *Acc65I/BamHI*-digested pDB3936 (disclosed in WO 2010/092135) and used to directly transform *S. cerevisiae* BXP10cir⁰ using the Sigma Yeast Transformation kit described below.

5 Table 3.

Plasmid	Amino acid substitution in HSA
pDB3986	HSA H440Q
pDB3987	HSA H464Q
pDB3988	HSA H510Q
pDB3989	HSA H535Q
pDB4006	HSA K573A
pDB4007	HSA E492T/N503K/K541A
pDB4008	HSA K541G
pDB4009	HSA K541D
pDB4010	HSA D550N
pDB4083	HSA D494E/Q417H
pDB4084	HSA Q417A
pDB4085	HSA P499A
pDB4086	HSA K500A
pDB4087	HSA K536A
pDB4088	HSA P537A
pDB4089	HSA K538A
pDB4090	HSA E492G/V493P/K538H/K541N/E542D
pDB4091	HSA E492P/N503K/K541G/E542P
pDB4092	HSA N503K
pDB4093	HSA N503H
pDB4094	HSA E492G/N503K
pDB4095	HSA E492G/N503H
pDB4096	HSA E492T
pDB4097	HSA N503D
pDB4098	HSA E492T/N503D
pDB4099	HSA K538H
pDB4100	HSA K541A
pDB4101	HSA K541N
pDB4103	HSA E542D
pDB4104	HSA E542P
pDB4105	HSA D550E
pDB4106	HSA E492H/E501P/N503H/E505D/T506S/T540S/K541E
pDB4107	HSA A490D/E492T/V493L/E501P/N503D/A504E/E505K/T506F/K541D
pDB4108	HSA E501A
pDB4109	HSA E501Q
pDB4110	HSA K573P

pDB4111	HSA E492G/K538H/K541N/E542D
pDB4114	HSA K573P/L575F/G584A
pDB4115	HSA K573P/K574N/A577T/A578R/S579C/Q580K/A581D/G584A
pDB4116	HSA K573P/A577E/A578S/Q580K/A582T
pDB4117	HSA K573P/A578S/S579T/G584A
pDB4156	HSA E492G K573A
pDB4157	HSA E492G N503K K573A
pDB4158	HSA E492G N503H K573A
pDB4159	HSA E492G K573P
pDB4160	HSA E492G N503K K573P
pDB4161	HSA E492G N503H K573P
pDB4194	HSA D550E
pDB4200	HSA K574N
pDB4202	HSA Q580K

Table 4: K500 primers and plasmids

Original primers		CODONS USED	
xAP216	CTTTGGAAGT <u>CGAC</u> GAAACTTACGTTCCA <u>GGT</u> GAATTCAACGCTG (SEQ ID NO: 14)	Gly	GGT
xAP217	CTTTGGAAGT <u>CGAC</u> GAAACTTACGTTCCA <u>GAA</u> GAATTCAACGCTG (SEQ ID NO: 15)	Glu	GAA
xAP218	CTTTGGAAGT <u>CGAC</u> GAAACTTACGTTCCA <u>GAC</u> GAATTCAACGCTG (SEQ ID NO: 16)	Asp	GAC
xAP219	CTTTGGAAGT <u>CGAC</u> GAAACTTACGTTCCA <u>GTT</u> GAATTCAACGCTG (SEQ ID NO: 17)	Val	GTT
xAP220	CTTTGGAAGT <u>CGAC</u> GAAACTTACGTTCCA <u>AGA</u> GAATTCAACGCTG (SEQ ID NO: 18)	Arg	AGA
xAP221	CTTTGGAAGT <u>CGAC</u> GAAACTTACGTTCCA <u>AAC</u> GAATTCAACGCTG (SEQ ID NO: 19)	Asn	AAC
xAP222	CTTTGGAAGT <u>CGAC</u> GAAACTTACGTTCCA <u>ATG</u> GAATTCAACGCTG (SEQ ID NO: 20)	Met	ATG
xAP223	CTTTGGAAGT <u>CGAC</u> GAAACTTACGTTCCA <u>ATT</u> GAATTCAACGCTG (SEQ ID NO: 21)	Ile	ATT
xAP224	CTTTGGAAGT <u>CGAC</u> GAAACTTACGTTCCA <u>ACC</u> GAATTCAACGCTG (SEQ ID NO: 22)	Thr	ACC
xAP225	CTTTGGAAGT <u>CGAC</u> GAAACTTACGTTCCA <u>TGG</u> GAATTCAACGCTG (SEQ ID NO: 23)	Trp	TGG
xAP226	CTTTGGAAGT <u>CGAC</u> GAAACTTACGTTCCA <u>TGT</u> GAATTCAACGCTG (SEQ ID NO: 24)	Cys	TGT
xAP227	CTTTGGAAGT <u>CGAC</u> GAAACTTACGTTCCA <u>TAC</u> GAATTCAACGCTG (SEQ ID NO: 25)	Tyr	TAC
xAP228	CTTTGGAAGT <u>CGAC</u> GAAACTTACGTTCCA <u>TTG</u> GAATTCAACGCTG (SEQ ID NO: 26)	Leu	TTG
xAP229	CTTTGGAAGT <u>CGAC</u> GAAACTTACGTTCCA <u>TTT</u> GAATTCAACGCTG	Phe	TTC

	(SEQ ID NO: 27)		
xAP230	CTTTGGAAGTCGACGAAACTTACGTTCCATCTGAATTCAACGCTG (SEQ ID NO: 28)	Ser	TCT
xAP231	CTTTGGAAGTCGACGAAACTTACGTTCCACAAGAATTCAACGCTG (SEQ ID NO: 29)	Gln	CAA
xAP232	CTTTGGAAGTCGACGAAACTTACGTTCCACAGAATTCAACGCTG (SEQ ID NO: 30)	His	CAC
xAP233	CTTTGGAAGTCGACGAAACTTACGTTCCACCAGAATTCAACGCTG (SEQ ID NO: 31)	Pro	CCA
xAP234	CTTTGGAAGTCGACGAAACTTACGTTCCATAAGAATTCAACGCTG (SEQ ID NO: 32)	STOP	taa
xAP235	GAATT <u>AAGCTT</u> ATTACAAACCCAAAGCAGCTTGGGAAGC (SEQ ID NO: 33)		

Table 5: K573 primers and plasmids

Original primers		CODONS USED	
xAP187	ATAAGCCTAAGGCAGCTTGACTTGCAGCAACAAGTTTACCACCCTCCTCG (SEQ ID NO: 34)	Gly	GGT
xAP188	ATAAGCCTAAGGCAGCTTGACTTGCAGCAACAAGTTTTCCACCCTCCTCG (SEQ ID NO: 35)	Glu	GAA
xAP189	ATAAGCCTAAGGCAGCTTGACTTGCAGCAACAAGTTTATCACCCTCCTCG (SEQ ID NO: 36)	Asp	GAT
xAP190	ATAAGCCTAAGGCAGCTTGACTTGCAGCAACAAGTTTAACACCCTCCTCG (SEQ ID NO: 37)	Val	GTT
xAP191	ATAAGCCTAAGGCAGCTTGACTTGCAGCAACAAGTTTTCTACCCTCCTCG (SEQ ID NO: 38)	Arg	AGA
xAP192	ATAAGCCTAAGGCAGCTTGACTTGCAGCAACAAGTTTATTACCCTCCTCG (SEQ ID NO: 39)	Asn	AAT
xAP193	ATAAGCCTAAGGCAGCTTGACTTGCAGCAACAAGTTTCATACCCTCCTCG (SEQ ID NO: 40)	Met	ATG
xAP194	ATAAGCCTAAGGCAGCTTGACTTGCAGCAACAAGTTTAATACCCTCCTCG (SEQ ID NO: 41)	Ile	ATT
xAP195	ATAAGCCTAAGGCAGCTTGACTTGCAGCAACAAGTTTAGTACCCTCCTCG (SEQ ID NO: 42)	Thr	ACT
xAP196	ATAAGCCTAAGGCAGCTTGACTTGCAGCAACAAGTTTCCAACCCTCCTCG (SEQ ID NO: 43)	Trp	TGG
xAP197	ATAAGCCTAAGGCAGCTTGACTTGCAGCAACAAGTTTACAACCCTCCTCG (SEQ ID NO: 44)	Cys	TGT
xAP198	ATAAGCCTAAGGCAGCTTGACTTGCAGCAACAAGTTTATAACCCTCCTCG (SEQ ID NO: 45)	Tyr	TAT
xAP199	ATAAGCCTAAGGCAGCTTGACTTGCAGCAACAAGTTTCAAACCCTCCTCG (SEQ ID NO: 46)	Leu	TTG
xAP200	ATAAGCCTAAGGCAGCTTGACTTGCAGCAACAAGTTTAAAACCCTCCTCG (SEQ ID NO: 47)	Phe	TTT
xAP201	ATAAGCCTAAGGCAGCTTGACTTGCAGCAACAAGTTTAGAACCTCCTCG (SEQ ID NO: 48)	Ser	TCT

xAP202	ATAAGCCTAAGGCAGCTTGACTTGCAGCAACAAGTTTTGACCCTCCTCG (SEQ ID NO: 49)	Gln	CAA
xAP203	ATAAGCCTAAGGCAGCTTGACTTGCAGCAACAAGTTTATGACCCTCCTCG (SEQ ID NO: 50)	His	CAT
xAP204	ATAAGCCTAAGGCAGCTTGACTTGCAGCAACAAGTTTTAACCCTCCTCG (SEQ ID NO: 51)	STOP	taa
xAP205	AATGCTGCCATGGAGATCTGCTTGAATGTGCTGATG (SEQ ID NO: 52)		

Method 4. HSA K500 and K573 permutation library

PCR was used to produce two permutation libraries in which the codons encoding amino acid 500 or 573 of mature HSA were changed (mutated) to alternative non-wild type amino acids and a termination codons (K5XXSTOP). Mutagenic oligonucleotides (Table 4 and Table 5), were designed to amplify HSA- encoding DNA and incorporate the desired changes. That is, for the changes at position 500, pDB4082 (Figure 1) was used as a template DNA. pDB4082 is a derivative of pDB2305 (disclosed in EP1788084) and was produced as follows. pDB2305 (Figure 2) was digested with *NsiI/Spel* and the yielded 8.779kb *NsiI* fragment was self-ligated to produce pDB4005 (Figure 3). A synthetic DNA fragment (*BsaI/SphI*) was generated by gene assembly (DNA2.0 Inc, USA) (SEQ ID NO: 1) (containing 3' region of the PRB1 promoter, modified fusion leader sequence, nucleotide sequence encoding HSA and 5' region of the modified *ADH1* terminator), and ligated into *HindIII/SphI*-digested pDB4005 (Figure 3) to produce pDB4082. Note. The *HindIII* site in *PRB1* promoter site has been removed and a *SacI* site within the nucleotide sequence encoding HSA has been introduced.

For the permutation library for position 500 of HSA, the nucleotide sequence encoding HSA corresponding to that between the *Sall* / *HindIII* sites (see plasmid map pDB4082, Figure 1) was generated using the New England Biolabs Phusion kit (Table 6) and oligonucleotides listed in Table 4. Table 7 describes the PCR method employed.

The permutation library at amino acid position 573 in HSA was generated using pDB3927 as template DNA and involved amplifying the albumin-encoding DNA corresponding to that between the *NcoI* and *Bsu36I* sites using oligonucleotides detailed in Table 5.

Table 6:. PCR ingredients

	500 library	573 library
20μl Buffer HF(5X)		
2μl dNTP mix (10mM)		
2μl oligonucleotide (10μM)	xAP235	xAP205
2μl oligonucleotide (10μM)	xAP216- xAP234	xAP187 xAP204

1 \square Phusion polymerase		
1 \square Template DNA (~5ng)	pDB4082	pDB3927
72 \square dH ₂ O		

Table 7: PCR conditions:

98°C for 2 min	1 cycle
98°C for 10sec	35 cycles
57°C for 30sec	
72°C for 20sec	
72°C for 5 min	1 cycle

5 For the albumin variants based at positions 500 and 573, each PCR-product was purified using a Qiagen PCR-clean up kit (according to the manufactures instructions), digested with *Sall/HindIII* (position 500 library) or *NcoI/Bsu36I* (position 573 library). The digested DNAs were then purified using a Qiagen PCR-clean up kit and ligated into *Sall/ HindIII* - or *NcoI/Bsu36I* - digested pDB4082 or pDB3927, respectively, replacing the equivalent native sequence. Ligations were transformed into *E. coli* DH5 α , subsequent plasmids isolated from transformants using a Qiagen miniprep kit (according to the manufacturer's instructions) and the correct constructs identified by restriction analysis. This produced a collection of plasmids, pDB4204 – pDB4222 (position 500 library) pDB4173 to pDB4190 (position 573 library), containing albumin genes which differed only in their sequence corresponding to the codon for the amino acid at position 500 or 573 Table 4 and 5, respectively). The specific changes in each plasmid were confirmed by sequencing.

The resultants plasmids were used to generate expression plasmids and albumin fusion producing yeast by *in vivo* cloning as described above. That is, *S. cerevisiae* was transformed using the Sigma Yeast Transformation kit (described below), using a mixture of a 100 ng *BstEII/BsrBI*-digeste HSA variant containing plasmid and 100 ng *Acc65I/BamHI* digested pDB3936.

20 Transformation of *S. cerevisiae*

S. cerevisiae BXP10 cir⁰ (as previously described WO/2001/079480) or Strain A cir⁰ (described in WO/2005/061718) was streaked on to YEPD plates (1% (w/v) yeast extract, 2% (w/v) Bactopeptone, 2% (w/v) glucose), 1.5% agar) and allowed to grow for 4 days at 30°C prior to transformation. One μ g of whole plasmid (i.e. circular plasmids) or, for gap repair, 100 ng *BstEII/BsrBI*- or *NsiI/PvuI*-digested HSA variant or HSA variant fusion containing plasmid and 100 ng *Acc65I/BamHI* digested pDB3936 were used to transform *S. cerevisiae* using a Sigma Yeast Transformation kit using a modified lithium acetate method (Sigma yeast transformation kit, YEAST-1, protocol 2; Ito *et al.* (1983) *J. Bacteriol.*, **153**, 16; Elble, (1992) *Biotechniques*, **13**, 18). The protocol was amended slightly by incubating the transformation at room temperature for 4 h

prior to heat shock. Following heat shock, the cells were briefly centrifuged before being re-suspended in 200µl 1M sorbitol then spread over BMMD agar plates, the composition of BMMD is described by Sleep *et al.*, (2001), *Yeast*, **18**, 403. Plates were incubated at 30°C for 4 days before individual colonies were patched on to fresh BMMD plates. Yeast strain numbers are detailed in Table 1.

Stocks were prepared for each yeast strain as follows: BMMD broth was inoculated with a heavy loop of each yeast patch and grown for 24h at 30°C with orbital shaking at 200rpm. Cells were harvested by centrifugation at 1900 × g for 5 min in a Sorval RT600 centrifuge, 15mL supernatant was removed and replaced by trehalose 40% (w/v). The cells were resuspended and transferred to cyrovials (1mL) for storage at -80°C.

Shake flask growth of *S. cerevisiae*

BMMD (recipe 0.17% (w/v) yeast nitrogen base without amino acid and ammonium sulphate (Difco), 37.8mM ammonium sulphate, 29mM citric acid, 142mM disodium hydrogen orthophosphate dehydrate pH6.5, 2% (w/v) glucose) media (10mL) was inoculated with each yeast strain and grown for 12h at 30°C with orbital shaking at 200rpm. An aliquot of each starter culture (4mL) was used to inoculate 2 × 200mL BMMD media and grown for 36h at 30°C with orbital shaking at 200rpm. Cells were harvested by filtration through 0.2µm vacuum filter membranes (Stericup, Millipore) including a GF-D prefilter (Whatman) and the supernatant retained for purification.

Primary concentration

Retained culture supernatant was concentrated using Tangential Flow Filtration using a Pall Filtron LV system fitted with a Omega 10KD (0.093sq.m2) filter (LV Centramate™ cassette, Pall Filtron) with a transmembrane pressure of 20psi and a recirculation rate of 180mL.min⁻¹.

Fermentation

Fed-batch fermentations were carried out in a 10 L Sartorius Biostat C fermenter at 30°C; pH was monitored and adjusted by the addition of ammonia or sulphuric acid as appropriate. The ammonia also provided the nitrogen source for the cultures. The level of dissolved oxygen was **monitored** and linked to the stirrer speed, to maintain the level at >20% of saturation. Inocula were grown in shake flasks in buffered minimal media (recipe). For the batch-phase the cultures was inoculated into fermenter media (approximately 50% of the fermenter volume) containing 2% (w/v) sucrose. The feed stage was automatically triggered by a sharp rise in the level of dissolved oxygen. Sucrose was kept at growth-limiting concentrations by controlling the rate of feed to a set nominal growth rate. The feed consisted of fermentation media containing 50% (w/v) sucrose, all essentially as described by Collins. (Collins, S.H., (1990) Production of secreted proteins in yeast, in: T.J.R. Harris (Ed.) Protein production by biotechnology, Elsevier, London, pp. 61-77).

GP-HPLC quantitation

Purified albumin variants, fusions and conjugates were analysed by GP-HPLC and quantification as follows. Injections of 25µL were made onto a 7.8mm id x 300mm length TSK G3000SWXL column (Tosoh Bioscience), with a 6.0mm id x 40mm length TSK SW guard column (Tosoh Bioscience). Samples were chromatographed in 25mM sodium phosphate, 100mM sodium sulphate, 0.05% (w/v) sodium azide, pH 7.0 at 1mL/min, Samples were quantified by UV detection at 280nm, by peak area, relative to a recombinant human albumin standard of known concentration (10mg/mL) and corrected for their relative extinction coefficients.

Purification of albumin variants from shake flask

Albumin variants were purified from shake flask (either culture supernatant or concentrated culture supernatant) using a single chromatographic step using an albumin affinity matrix (AlbuPure™ - ProMetic BioSciences, Inc.). Chromatography was performed at a constant linear velocity of 240cm/h throughout. Culture supernatant was applied to a 6cm bed height, 2.0mL packed bed pre-equilibrated with 50mM sodium acetate pH5.3. Following load the column was washed with 10 column volume (CV) of equilibration buffer, then 50mM ammonium acetate pH8.0 (10CV). Product was eluted with either 50mM ammonium acetate 10mM octanoate pH8.0, 50mM Ammonium Acetate 30mM Sodium Octanoate 200mM Sodium Chloride pH7.0 or 200mM Potassium thiocyanate. The column was cleaned with 0.5M NaOH (3cv) and 20mM NaOH (3.5cv). Eluate fraction from each albumin variant were concentrated and diafiltered against 10 volumes of 50mM sodium chloride (Vivaspin20 10,000 MWCO PES with optional diafiltration cups, Sartorius). Purified albumin variants were quantified by GP-HPLC as described above.

Purification of albumin-fusion variants from shake flask

Albumin-fusion variants were purified from shake flask culture supernatant using a single chromatographic step using an albumin affinity matrix (AlbuPure™ - ProMetic BioSciences, Inc.). Chromatography was performed at a constant linear velocity of 240cm/h throughout. Culture supernatant or concentrated culture supernatant was applied to a 6cm bed height, 2.0mL packed bed pre-equilibrated with 50mM sodium acetate pH5.3. Following load the column was washed with 10 column volume (cv) equilibration buffer then 50mM ammonium acetate pH8.0 (10cv). Product was eluted with either 50mM ammonium acetate 10mM octanoate pH8.0, 50mM Ammonium Acetate 30mM Sodium Octanoate 200mM Sodium Chloride pH7.0, 50mM Ammonium Acetate 100mM Sodium Octanoate pH9.0 or 200mM Potassium thiocyanate. The column was cleaned with 0.5M NaOH (3cv) and 20mM NaOH (3.5cv). Eluate fraction from each albumin variant-fusion were concentrated and diafiltered against 10 volumes of 25 mM Tris, 150 mM NaCl, 2 mM KCl, pH 7.4 (Vivaspin20 10,000 MWCO PES with optional diafiltration cups, Sartorius). Purified albumin-fusion variants were quantified by GP-HPLC as described above.

Purification of albumin variants from fermentation

Albumin variants were purified from high cell density fed batch fermentation supernatants after separation by centrifugation, using a Sorvall RC 3C centrifuge (DuPont). Culture supernatant was chromatographed through an 11cm bed height column 8.6mL packed bed packed with a custom synthesised albumin affinity matrix (AlbuPure™ - ProMetic BioSciences, Inc.) as described above. Product was eluted using elution buffers describe above at a flow rate of 120cm/h. The eluate fraction(s) was analysed by GP-HPLC. (above) .and reducing SDS-PAGE for purity and if required concentrated (Vivaspin20 10,000 MWCO PES) and applied to a 2.4x96cm column packed with Superdex 75 run at a flow rate of 39cm/h in 25 mM Tris, 150 mM NaCl, 2 mM KCl, pH 7.4 . The peak was fractionated, assayed by GP-HPLC and pooled in order to generate the monomeric protein of interest. Pooled fractions were concentrated (Vivaspin20 10,000 MWCO PES, Sartorius).

All proteins to be assayed for receptor (FcRn) binding properties and or other analysis were quantified by GP-HPLC as described above corrected for their relative extinction coefficients.

15 **Example 2. Determination of receptor (shFcRn) binding properties of blood derived HSA and recombinant human albumin**

Essentially fatty acid-free HSA (Sigma-Aldrich) was further purified by size exclusion chromatography as described in Andersen *et al* (2010). J.Biol.Chem. 285, (7),4826–4836. Ten µM of monomeric HSA and rHA were analysed using SPR as described above and the data presented in Figure 4.

Direct comparison of HSA (blood derived) with recombinant human albumin (Recombunin) at the same concentration (10µM) (Figure 4A and 4B) shows for both samples binding to immobilized shFcRn (pH6.0, pH7.4 respectively) was reversible and pH dependent. In addition, comparison of HSA vs recombinant human albumin by Bosse *et al* (2005).. J. Clin. Pharmacol. 45; 57-67, demonstrated equivalent half life *in vivo* human study

Example 3. Determination of receptor (shFcRn) binding properties of albumin variants

Two established FcRn binding assays were used, ELISA and SPR. There are major differences between the assays: In the ELISA system HSA is coated directly in wells and shFcRn-GST is added in solution whereas in the SPR assay shFcRn-GST is immobilized to a CM5 chip and HSA injected in solution. The pH can be varied in both systems.

The variants were analysed using ELISA at pH 6.0 and pH 7.4. Results are disclosed in Figure 5. The ELISA values represent the mean of duplicates.

The variants were analysed using SPR analysis at pH 6.0 and pH 7.4. Results are disclosed for a representative number of variants in Figure 6 using a concentration of the variants of 0.2 μ M and in Figure 7 using a concentration of the variants of 1 μ M.

The SPR data disclosed in Figures 6 and 7 were normalized and the relative binding of variants at each concentration is shown in Figure 8 A and B respectively.

The conclusions of the analysis are that all tested variants have the characteristic binding to the receptor at pH 6.0 but no binding at pH 7.4. The variants D494N,Q,A, E495Q,A, T496A, and D494N+T496A show reduced binding to the receptor compared to HSA.

Example 4. Determination of receptor (shFcRn/smFcRn) binding properties of albumin variants

Using the SPR analysis method below the association constant K_a , the dissociation constant K_d and the binding constant K_D calculated for HSA and mouse serum albumin (MSA) binding to human and mouse FcRn (Table 8).

SPR analyses- SPR analyses were performed on a BIAcore 3000 instrument (GE Healthcare) using CM5 chips and immobilization of smFcRn-GST and shFcRn-GST variants or smFcRn was performed using the amine coupling kit (GE Healthcare). Protein samples (10 μ g/ml) were injected in 10 mM sodium acetate at pH 4.5 (GE Healthcare), all as described by the manufacturer. Unreacted moieties on the surface were blocked with 1 M ethanolamine. For all experiments, phosphate buffer (67 mM phosphate buffer, 0.15 M NaCl, 0.005% TWEEN® 20) at pH 6.0 or pH 7.4, or HBS-P buffer (0.01 M HEPES, 0.15 M NaCl, 0.005% surfactant P20) at pH 7.4 were used as running buffer or dilution buffer. Kinetic measurements were performed using a low density immobilized surface (100-200 resonance units (RU)). Serial dilutions of hIgG1 (2000.0-31.2 nM), mIgG1 (1000.0-15.6 nM), MSA (20.0-0.3 μ M) and HSA (200.0-3.1 μ M) were injected at pH 6.0 or pH 7.4, at a flow rate 50 μ l/minute at 25°C. Additive binding was recorded by injecting HSA (10 μ M), MSA (5 μ M), hIgG1 (100 nM) or mIgG1 (100 nM) alone or two at a time at 25°C at 20 μ l/minute at pH 6.0 over immobilized shFcRn (~600 RU) or smFcRn (~600 RU). Competitive binding was measured by injecting shFcRn (50 nM) or smFcRn (100 nM) alone or together with different amounts of HSA or MSA (10.0-0.05 μ M) over immobilized HSA (~2600 RU) or MSA (~2000 RU). In all cases, to correct for nonspecific binding and bulk buffer effects, responses obtained from the control surfaces and blank injections were subtracted from each interaction curve. Kinetic rate values were calculated using predefined models (Langmuir 1:1 ligand model, heterogeneous ligand model and steady state affinity model) provided by the BIAevaluation 4.1 software. The closeness of the fit, described by the statistical value χ^2 that represents the mean square, was lower than 2.0 in all affinity estimations.

Table 8: Binding constants of HSA and MSA shFcRn and smFcRn.

Albumin Species	FcRn Species	Ka (10 ³ /Ms)	Kd (10 ³ /s)	KD (μM)	KD Req. (μM)
MSA	Mouse	4.2±0.5	39.4±3.1	9.3±0.4	ND ^d
MSA	Human	3.8±0.0	3.1±0.1	0.8±0.2	ND
HSA	Mouse	NA	NA	NA	86.2±4.1
HSA	Human	2.7±1.3	12.2±5.9	4.5±0.1	4.6 ±0.5

The KD's were generated using the BIAevaluation 4.1 software) A Langmuir 1:1 ligand model was used throughout. The kinetic values represent the average of triplicates. ND means:

5 Not determined. NA means: Not acquired

Example 5. Binding of albumins from other species to human FcRn

10 Commercially available animal albumin (either Sigma-Aldrich or Calbiochem) were further purified as described in Andersen *et al* (2010). J.Biol.Chem. 285, (7),4826–4836. The binding of donkey serum albumin, bovine serum albumin, goat serum albumin, sheep serum albumin, rabbit serum albumin, dog serum albumin, hamster serum albumin, guinea pig albumin, rat serum albumin and chicken serum albumin to shFcRn was determined using the techniques described in Materials and Methods.. The ELISA results are disclosed in Figure 9 A-D and the relative bindings
15 summarized in Figure 9 E.

The SPR results are shown in Figure 10, where the binding at pH 6.0 and pH 7.4 for each albumin species are shown. Table 10 shows an overview of the relative binding responses measured using ELISA and SPR:

20 Table 10: Cross-species albumin-FcRn binding

Albumin specie	shFcRn			
	ELISA		SPR	
	pH6.0	pH7.4	pH6.0	pH7.4
Human	++(+)	-	++(+)	-
Donkey	+++	-	++	- 25
Cow	++	-	++	-
Sheep	+/-	-	-	-
Goat	+/-	-	-	-
Rabbit	++++	-	+++	-
Dog	ND ^a	ND	+++	- 30
G. pig	++++	+	++++	+
Hamster	+++	-	+++	-
Rat	+++	-	+++	-
Mouse	+++	-	+++	- 35
Chicken	-	-	-	-

Relative binding responses are categorized from strongest (++++), to weakest (+) and no binding (-).

a: Not determined (ND).

A hierarchy ranging from strongest to weakest binding is as follows; guinea pig => rabbit > hamster/dog > rat/mouse > donkey > human > bovine > goat/sheep > chicken. This data shows that animal albumins have different affinities for shFcRn.

Example 6. Kinetics of the HSA variant for shFcRn

The binding constants for variants according to the invention were determined according to the methods described in Materials and Methods.

Table 11: Binding constants of HSA variants for shFcRn

Albumin Variant	Ka (10 ³ /Ms)	kd (10 ⁻³ /s)	KD (μM)	KD Req (μM)
WT	3.2±0.2	15.5±2.5	4.8	5.4
D494N	1.7±0.0	18.6±0.0	10.9	11.8
D494A	2.3±0.1	53.4±0.3	23.2	17.0
D494Q	2.1±0.0	58.2±3.8	27.7	ND
E495Q	2.5±0.0	24.1±0.2	9.6	10.9
E495A	2.1±0.0	14.0±0.0	7.0	8.6
D494N+T496A	2.5±0.0	11.0±0.0	4.4	5.5
T496A	2.3±0.0	11.7±0.5	5.1	7.1
E492G	4.1±0.0	11.0±0.0	2.7	ND

The KD's were generated using the BIAevaluation 4.1 software) A Langmuir 1:1 ligand model was used throughout. The kinetic values represent the average of triplicates. ND means: Not determined.

The results correspond with the conclusions made in Example 3 based on SPR and ELISA data but in addition shows that E492G has increased affinity to its receptor,

Example 7. Competitive analysis of the HSA variants

Competitive analysis of the HSA variants prepared in example 1 and WT HSA was performed using the methods described in example 4. Results are shown in Figure 15.

The results show that the variant E492G, unlike E492H E492P and E492G+V493P, has stronger binding to shFcRn than HSA. .

Example 8. Analysis of Q417 substitutions

Using the method of Example 1 variants of HSA having the substitutions Q417A and D494E+Q417H were constructed. The kinetic properties of these variants were tested using the methods in Materials and Methods and are shown in Table 12.

Table 12: Binding constants of HSA variants for shFcRn

Albumin variant ^a	k_a ($10^3/\text{Ms}$)	k_d ($10^{-3}/\text{s}$)	KD^b (μM)	$KD \text{ Req}^c$ (μM)
WT	3.2±0.2	15.5±2.5	4.8	5.4
Q417A	3.2±0.1	26.0±0.0	8.1	ND
D494E+Q417H	3.1±0.1	20.5±0.5	6.6	ND

a: Dilutions of HSA variants were injected over immobilized shFcRn (~1500 RU).

b: The kinetic rate constants were obtained using a simple first-order (1:1) bimolecular interaction model.

c: The steady state affinity constant was obtained using an equilibrium (Req) binding model supplied by the BIAevaluation 4.1 software. The kinetic values represent the average of triplicates.

d: Not determined (ND).

The data show that variants Q417A and D494E+Q417H bind weaker to the receptor than the wild-type HSA.

Example 9. Analysis of HSA variants in position 499, 500, 536, 537, 538 and 573

Using the method of Example 1 variants of HSA having the substitutions P499A, K500A, K536A, P537A, K538A and K573A were constructed. The receptor binding properties of these variants were tested as described in Materials and Methods. Results are shown in Figure 11.

The data demonstrated that variants P499A, K536A, P537A and K538A had a reduced binding affinity to shFcRn relative to HSA. Variant K500A had almost completely lost its ability to bind to shFcRn and K573A had an increased binding affinity to shFcRn both relative to HSA.

Example 10. Analysis of variants in position 501 of HSA

Using the method of Example 1 variants of HSA having the substitutions E501A and E501Q were constructed. The kinetic properties of these variants were tested as described in Materials and Methods.

Table 13 Binding constants of HSA variants for shFcR

Albumin variant ^a	k_a ($10^3/\text{Ms}$)	k_d ($10^{-3}/\text{s}$)	KD^b (μM)	$KD \text{ Req}^c$ (μM)
WT	3.2±0.2	15.5±2.5	4.8	5.4
E501A	3.3±0.0	26.0±0.0	7.8	ND
E501Q	2.7±0.1	15.5±0.5	5.7	ND

a: Dilutions of HSA variants were injected over immobilized shFcRn (~1500 RU).

b: The kinetic rate constants were obtained using a simple first-order (1:1) bimolecular interaction model.

c: The steady state affinity constant was obtained using an equilibrium (Req) binding model supplied by the BIAevaluation 4.1 software. The kinetic values represent the average of triplicates

d: Not determined (ND).

The data shows that variants E501A and E501Q have a slightly decreased binding affinity to shFcRn relative to HSA.

Example 11. Analysis of HSA variants in position 573

Using the method of Example 1 variants of HSA having a substitution at position 573 were constructed. All variants at position 573 were generated and the receptor binding properties of these variants were tested as described in Materials and Methods but with SPR analysis performed at pH5.5. Results are shown in the table 14 below and Figure 12 and 13.

Table 14 : Kinetics of HSA K573 single point mutants.

Albumin variant ^a	k_a ($10^3/\text{Ms}$)	k_d ($10^{-3}/\text{s}$)	KD^b (nM)
WT	9.0±0.0	6.9±0.1	766
K573A	7.4±0.0	2.2±0.0	297
K573C	4.2±0.0	1.1±0.2	262
K573D	7.9±0.2	4.1±0.3	518
K573E	9.0±0.0	2.9±0.0	322
K573F	7.8±0.1	0.5±0.1	74
K573G	8.5±0.0	1.8±0.1	212
K573H	12.0±0.2	0.8±0.0	68
K573I	8.6±0.0	0.8±0.2	99
K573L	5.1±0.2	2.3±0.1	451
K573M	8.6±0.0	1.9±0.0	221
K573N	7.3±0.2	1.1±0.3	151
K573P	9.8±0.0	0.6±0.1	61
K573Q	7.7±0.2	2.6±0.0	338
K573R	8.5±0.0	3.0±0.2	353
K573S	7.9±0.2	1.2±0.2	152
K573T	8.7±0.2	1.1±0.1	126
K573V	8.1±0.0	0.6±0.2	80
K573W	15.0±0.2	0.4±0.3	29
K573Y	22.0±0.1	0.5±0.1	23
K573STOP	ND	ND	141000

a: Dilutions of HSA variants were injected over immobilized shFcRn (~1500 RU).

b: The kinetic rate constants were obtained using a simple first-order (1:1) bimolecular interaction model.

c: The steady state affinity constant was obtained using an equilibrium (Req) binding model supplied by the BIAevaluation 4.1 software. The kinetic values represent the average of duplicates.

d: Not determined (ND).

The results show that all variants having substitution in position 573 have improved binding to shFcRn compared with WT HSA. In particular the variants K573F, K573H, K573P, K573W and K573Y have more than 10 fold lower KD to shFcRn than the parent HSA. The variant K573STOP is a truncated albumin having a stop codon in position 573. The sensorgram for the K573STOP variant show significantly reduced binding compare to the WT HSA and generated a high KD. The increased affinity that we have shown for the variant K573E, a natural variant characterized by Otagiri (2009). Biol.Pharm. Bull. **32**(4) 527-534, is predicted to have increased half-life *in vivo*.

Example 12. Analysis of further HSA variants

Using the method of Example 1 variants of HSA having the substitutions E492G, E492G+N503H, N503H, D550E, E492G+N503K, E542P, H440Q, K541G, K541D, D550N E492G+K538H+K541N+E542D, E492T+N503K+K541A, E492P+N503K+K541G+E542P, E492H+E501P+N503H+E505D+T506S+T540S+K541E, A490D+E492T+V493L+E501P +N503D+A504E+E505K+T506F+K541D, E492G+V493P+K538H+K541N+E542D were constructed. The receptor binding properties of these variants were tested as described in Materials and Methods, and the results are shown in Table 15 and Figure 14.

Table 15: Binding constants of HSA variants for shFcR

Albumin variant ^a	K _a (10 ³ /Ms)	k _d (10 ⁻³ /s)	KD ^b (μM)	KD Req ^c (μM)
WT	3.2±0.2	15.5±2.5	4.8	5.4
E492G	4.1±0.0	11.0±0.0	2.7	ND
E492G/N503H	6.9±0.1	14.5±0.5	2.1	ND
N503H	5.4±0.0	24.0±0.1	4.4	ND
D550E	3.2±0.4	11.8±0.0	3.6	ND
E492G/N503K	5.9±0.1	16.0±0.0	2.7	ND
E542P	3.4±0.0	15.7±0.2	4.7	ND
H440Q	3.2±0.1	20.8±0.0	6.5	ND
K541G	3.2±0.0	23.0±0.0	7.1	ND
K541D	2.6±0.0	24.0±0.0	9.2	ND
D550N	2.5±0.0	30.0±0.0	12.0	ND

a: Dilutions of HSA variants were injected over immobilized shFcRn (~1500 RU).

b: The kinetic rate constants were obtained using a simple first-order (1:1) bimolecular interaction model.

c: The steady state affinity constant was obtained using an equilibrium (Req) binding model supplied by the BIAevaluation 4.1 software. The kinetic values represent the average of triplicates.

d: Not determined (ND).

The results show that for position 550, a substitution to E results in an increased affinity whilst a substitution to N resulted in reduced affinity for shFcRn at pH6.0. When this analysis was repeated for the D550E substitution at pH5.5 however no observable increase in affinity was seen.

The substituted for an acid amino acid (E) maintains and improves the binding. However the substitution for an uncharged amide amino acid reduces binding at pH6.0. Based on this observation, we would predict for this position that substitutions to basic amino acids (H, K and R) would result in further reductions in binding.

Example 13. Mutations in His residues

The following variants were generated using the methods described in Example 1: H440Q, H464Q, H510Q and H535Q.. Figure 15 shows SPR sensorgrams of these variants interacting with shFcRn as described in Materials and Methods.

It was found that the variant H440Q bound with comparable affinity as HSA. In contrast H464Q, H510Q and H535Q had significantly reduced affinity to shFcRn. This supports the previously published observations that mutagenesis of these Histidine residues significantly reduced HSA binding to shFcRn (Wu *et al* (2010). PEDS,23(10)789–798). Wu *et al* show a reduced half-life for a diabody fusion proteins (scFv-DIII)₂ in mice with an order of removal from slowest to fastest: Db-DIII WT>H535A>H510A>H464A>Db. Based on affinity to shFcRn and when compared to smFcRn (example 5) we would predict the clearance order in humans to be (for glutamine (Q) substitutions) WT> H440Q>H510Q>H464Q>H535Q.

Example 14. Further variants

The following variants were generated using the methods described in Example 1: K574N and Q580K in HSA. Binding of the variants to FcRn was tested using the SPR assay as described in Materials and Methods and the results are shown in Table 16.

The results show that variants K574N and Q580K bound stronger to shFcRn.

Table16: Following kinetic data was found for these variants:

Albumin variant	k_a ($10^3/\text{Ms}$)	k_d ($10^{-3}/\text{s}$)	KD (μM)
WT	9.7±0.0	30.0±0.1	3.1
K574N	4.9±01	8.4±0.1	1.7
Q580K	6.0±0.0	9.3±0.0	1.5

Example 15. Analysis of HSA variants in position 500

Using the method of Example 1 variants of HSA having a substitution at position 500 were constructed. All variants at position 500 were generated and the receptor binding properties of these variants were tested. Biacore X, Biacore X100 and Sensor Chip CM5 were used for all analyses, both supplied by G E Healthcare. shFcRn produced by GeneArt AG (Germany) (diluted to 10µg/mL in 10mM sodium acetate pH5.0 (G E Healthcare)) was immobilised on flow cell 2 (FC2) to levels between 1600 – 2200 response units (RU) via standard amine coupling as per manufacturers instructions (G E Healthcare). A blank immobilisation was performed on flow cell 1 (FC1) for it to serve as a reference cell. To stabilise the assay, 3-5 start up cycles were run first, with running buffer (67mM phosphate buffer, 0.15M NaCl, 0.005% Tween 20 at pH5.75 ±0.25) only, followed by regeneration. WT rHA and K500 library variants were injected at various concentrations (1µM – 150µM) for 90s at a constant flow rate of (30µl/min) at 25 °C followed by regeneration of the surface using HBS-EP buffer pH7.4 (G E Healthcare) until approximate initial baseline RU was restored (usually 12s pulse would suffice).

Results are shown in the Table 17 and Figure 16

Table 17: Kinetics of HSA K500 single point mutants.

Albumin variant	ka (10 ³ /Ms)	kd (10 ⁻³ /s)	KD ^b (µM)	KD Req ^c (µM)
K500R	4.42	7.21	1.63	
K500I	5.18	10.9	2.1	
WT	4.24	9.2	2.2 ^a	
K500L	3.73	11.9	3.2	
K500Q	1.07	3.4	3.2	
K500V	3.29	11.0	3.3	
K500Y	3.97	14.6	3.7	
K500M	2.48	21.5	8.7	
K500T	1.2	13.4	11.2	
K500W	0.5	5.4	11.7	
K500N	1.3	18.2	14	
K500F	5.17	73.7	14.3	
K500H	4	63.8	16	
K500P	ND	ND	ND	51 [*]
K500C	2.38	124	52	
K500S	ND	ND -	ND	70.2 [*]
K500A	2.61	208	79.9	
K500D	ND	ND	ND	83.3 [*]
K500G	ND	ND	ND	95.4 [*]
K500E	KD not calculable see Figure 16			
K500 STOP	Null binder			

a: Mean of 4 values.

b: The kinetic rate constants were obtained using a simple first-order (1:1) bimolecular interaction model.

c: The steady state affinity constant was obtained using an equilibrium (Req) binding model supplied by the BIAevaluation 4.1 software.

The results show for variants K500R and K500I have increased and comparable affinity for shFcRn compared to WT HSA respectively. Variant K500E bound tightly to immobilised shFcRn but still demonstrated the characteristic pH-dependency of the FcRn interaction. This complex was very stable, such that kinetic analysis was not possible (Figure 16). All other variants have reduced binding to shFcRn than wt rHA.

All variants bound to shFcRn (to some extent) at pH5.5. No binding of K500 library variants to shFcRn was detectable at pH7.4.

Example 16. Fusion polypeptides

The generation of albumin fusions containing albumin muteins

Plasmids containing expression cassettes for the production of scFv (vHvL) genetically-fused to HSA, at either the N- or C-terminus or both, (described in, Evans *et al.*, 2010. Protein Expression and Purification. 73,113–124) were modified to allow the production of albumin fusions using *in vivo* cloning (describe above). That is, pDB3017 (Figure 17), pDB3021 (Figure 18), pDB3056 (Figure 19) were digested with *NsiI/Spel* and *NsiI* fragments corresponding 9.511kb, 9.569kb and 8.795kb, respectively, were purified using standard techniques. Purified *NsiI* fragments were self-ligated and used to transform chemically competent *E. coli* DH5 α to produce pDB4168, pDB4169 and pDB4170, respectively (Table 18).

Similarly, pDB3165 (containing the bivalent fusion) (Figure 20) was digested with *NotI* and the expression cassette (4.506kb fragment) was purified before being ligated into *NotI*-digested pDB3927 to produce pDB4172 (Figure 21, Table 18).

Synthetic *Sall/Bsu36I* DNA fragments (269bp), which contain point mutations within the albumin encoding nucleotide sequence to introduce amino acid substitutions corresponding to K500A, or D550N or K573P into the translated albumin protein sequence, were generated by gene assembly (GeneArt AG, Germany). The *Sall/Bsu36I* fragments were individually ligated into *Sall/Bsu36I*-digested pDB4168-pDB4170 and pDB4172 and used to transform chemically competent *E. coli* DH5 α using standard techniques to generate plasmids pDB4265 - pDB4276 (Table 18).

Table 18: Albumin variant fusions

Plasmid	Construct
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pDB3017	scFv (anti-FITC) - HSA – FLAG
pDB3021	HSA - GS linker - scFv (anti-FITC) – FLAG
pDB3056	HSA – FLAG
pDB3165	scFv (anti-FITC) - HSA - GS linker - scFv (anti-FITC) – FLAG
pDB4168	scFv (anti-FITC) - HSA – FLAG
pDB4169	HSA - GS linker - scFv (anti-FITC) – FLAG
pDB4170	HSA – FLAG
pDB4172	scFv (anti-FITC) - HSA - GS linker - scFv (anti-FITC) – FLAG
pDB4265	scFv (anti-FITC) – HSA K500A – FLAG
pDB4266	scFv (anti-FITC) – HSA D550N – FLAG
pDB4267	scFv (anti-FITC) – HSA K573P – FLAG
pDB4268	HSA K500A - GS linker - scFv (anti-FITC) – FLAG
pDB4269	HSA D550N - GS linker - scFv (anti-FITC) – FLAG
pDB4270	HSA K573P - GS linker - scFv (anti-FITC) – FLAG
pDB4271	HSA K500A – FLAG
pDB4272	HSA D550N – FLAG
pDB4273	HSA K573P – FLAG
pDB4274	scFv (anti-FITC) - HSA K500A - GS linker - scFv (anti-FITC) - FLAG
pDB4275	scFv (anti-FITC) - HSA D550N - GS linker - scFv (anti-FITC) - FLAG
pDB4276	scFv (anti-FITC) - HSA K573P - GS linker - scFv (anti-FITC) - FLAG
pDB4277	scFv (anti-FITC) - HSA K573A – FLAG
pDB4278	HSA K573A - GS linker - scFv (anti-FITC) – FLAG
pDB4279	HSA K573A – FLAG
pDB4280	scFv (anti-FITC) - HSA K573A - GS linker - scFv (anti-FITC) - FLAG
pDB4281	HSA K500A - GS linker - scFv (anti-FITC)
pDB4282	HSA D550N - GS linker - scFv (anti-FITC)
pDB4283	HSA K573P - GS linker - scFv (anti-FITC)
pDB4284	HSA - GS linker - scFv (anti-FITC)
pDB2613	HSA- GS linker -IL1RA (N84Q)
pDB4285	HSA K573A- GS linker -IL1RA (N84Q)
pDB4286	HSA D550N- GS linker -IL1RA (N84Q)
pDB4287	HSA K500A- GS linker -IL1RA (N84Q)
pDB4288	HSA K573P- GS linker -IL1RA (N84Q)

Similarly, a DNA fragment was generated by PCR (using standard techniques), to introduce a K573A substitution in the translated albumin protein sequence. PCR was performed using the New England Biolabs Phusion kit using pDB4267 (Figure 22) as template DNA and oligonucleotides xAP238 (SEQ ID NO: 53) and xAP239 (SEQ ID NO: 54):

Table 19 describes PCR cycling.

Table 19: PCR cycling

98°C for 2 min	1 cycle
98°C for 10sec	35 cycles
57°C for 30sec	
72°C for 10sec	
72°C for 5 min	1 cycle

The PCR-product was purified, digested with *Sall/Bsu36I*, and the fragment (269bp) isolated was ligated into *Sall/Bsu36I*-digested pDB4168-pDB4170 and pDB4172 and used to transform chemically competent *E. coli* DH5 α . Resulting plasmids (pDB4277 - pDB4280) are listed in Table 18.

The nucleotide sequence encoding the FLAG tag was removed from plasmids pDB4168 and pDB4268-4270 (plasmids for the expression of scFv N-terminally fused to HSA and HSA mutants K500A, D550N and K573P, respectively. pDB4168 and pDB4268-4270 (Table 18) were digested with *Bsu36I/SphI* to remove a 231bp product comprising 3' region of HSA-encoding gene, nucleotide sequence encoding FLAG tag and 5' region of ADH1 terminator. A *Bsu36I/SphI* fragment (207bp), comprising 3' region of HSA-encoding gene and 5' region of mADH1 terminator (SEQ ID1) from pDB4181 was ligated into *Bsu36I/SphI*-digested pDB4168 and pDB4268-pDB4270 using standard techniques. Ligation mixtures were used to transform chemically competent *E. coli* DH5 α using standard techniques to generate plasmids pDB4281-pDB4284 (Table 18)

pDB4265-pDB4284 were digested with *BstEII/BsrBI* and the linearised DNA molecules were purified using standard techniques. One hundred ng *BstEII/BsrBI* DNA samples were mixed with 100ng *Acc65I/BamHI*-digested pDB3936 and used to transform *S. cerevisiae* BXP10cir⁰ using the Sigma Yeast Transformation kit described below. In each case the expression plasmid was generated in the yeast by homologous recombination (*in vivo* cloning) between the albumin-fusion containing plasmid (pDB4265-pDB4280) (Table 18) and pDB3936.

Plasmids pDB3017, pDB3021, pDB3056 and pDB3165 (wild type HSA fusions, described by Evans *et al.*, 2010. Protein Expression and Purification. **73**,113–124) were used to transform *S. cerevisiae* Strain Acir⁰ (described in WO/2005/061718) using the Sigma Yeast Transformation kit described below.

The nucleotide sequence encoding human IL-1RA (interleukin-1 receptor antagonist) (accession number: CAA59087) could be synthetically generated by gene assembly. The nucleotide sequence of the 708bp synthetic fragment (*Bsu36I/SphI* fragment) is given in SEQ ID NO: 55 and includes the 3' region of the gene encoding HSA, the nucleotide sequence encoding a GS linker, the nucleotide sequence encoding human IL-1RA (N84Q to abolish the N-linked glycosylation motif) and the 5' region of the ADH1 terminator. The synthetic DNA fragment could be ligated into *Bsu36I/SphI*-digested pDB3927 to produce pDB2588.

Plasmids containing the expression cassettes for the production of IL-1RA genetically fused to the C-terminus of HSA and the HSA variants K500A, D550N, K573A and K573P were prepared as follows. pDB2588 was digested with Bsu36I/SphI and a 705bp fragment containing the '3 region of the HSA encoding gene, nucleotide sequence encoding a GS linker, nucleotide sequence encoding human IL1-RA (N84Q) and the 5' region of a modified *S. cerevisiae* ADH1 terminator (SEQ ID3) was purified using standard techniques then ligated into Bsu36I/SphI-digested pDB4006 (containing HSA K573A expression cassette), pDB4010 (containing HSA D550N expression cassette), pDB4086 (containing HSA K500A expression cassette), pDB4110 (containing HSA K573P expression cassette) to generate pDB4287, pDB4286, pDB4285 and pDB4288, respectively (for an example, see Figure 23). pDB4285-pDB4288 were digested with NsiI/PvuI and the linearised DNA molecules were purified using standard techniques. One hundred ng NsiI/PvuI-digested DNA samples were mixed with 100ng Acc65I/BamHI-digested pDB3936 (9721bp) (i.e. in vivo cloning) and used to transform *S. cerevisiae* (i.e. by in vivo cloning) using the Sigma Yeast Transformation kit described below.

Preparation of an *S. cerevisiae* strain expressing wild type HSA genetically fused to a GS linker and IL1-RA (N84Q) (see Table 18) could also be generated following the methods described above.

The fusion polypeptides were analysed for their binding to FcRn using the SPR method described above and following results were obtained:

Table 20: Kinetics of HSA fusion variants.

Albumin variant ^a	k_a ($10^3/\text{Ms}$)	k_d ($10^{-3}/\text{s}$)	KD^b (μM)
HSAWT	9.7±0.0	30.0±0.1	3.1
K574N	4.9±0.1	8.4±0.1	1.7
Q580K	6.0±0.0	9.3±0.0	1.5
K573P	2.8±0.0	0.4±0.0	0.1
HSA-WT-FLAG	8.2±0.2	24.0±0.2	2.9
HSA-D550N-FLAG	5.9±0.0	49.0±0.1	8.3
HSA-K500A-FLAG	ND ^c	ND	ND
HSA-K573A-FLAG	6.1±0.1	7.1±0.1	1.1
HSA-K573P-FLAG	6.2±0.1	1.2±0.1	0.2
HSA-WT-IL1RA	6.2±0.0	25.0±0.2	4.0
HSA-K500A-IL1RA	ND	ND	ND
HSA-D550N-IL1RA	7.3±0.2	38.0±0.0	5.2
HSA-K573A-IL1RA	6.1±0.0	7.1±0.1	1.1
HSA-K573P-IL1RA	6.2±0.1	1.3±0.1	0.2
scFv-HSA-K500A-FLAG	ND	ND	ND

scFv-HSA-D550N-FLAG	6.2±0.0	18.0±0.0	2.9
scFv-HSA-K573A-FLAG	6.4±0.1	5.7±0.2	0.9
scFv-HSA-K573P-FLAG	5.8±0.0	1.1±0.1	0.2
scFv-HSA-WT-scFv-FLAG	7.5±0.0	15.0±0.2	2.0
scFv-HSA-K500A-scFv-FLAG	ND	ND	ND
scFv-HSA-D550N-scFv-FLAG	4.1±0.1	27.0±0.2	6.6
scFv-HSA-K573P-scFv-FLAG	6.0±0.2	0.7±0.1	0.1
HSA-K500A-scFv-FLAG	ND	ND	ND
HSA-D550N-scFv-FLAG	7.3±0.1	42.0±0.3	5.8
HSA-K573A-scFv-FLAG	6.4±0.1	5.7±0.1	0.9
HSA-K573P-scFv-FLAG	4.7±0.1	0.7±0.1	0.1
scFv-HSA-K500A	ND	ND	ND
scFv-HSA-D550N	7.5±0.1	19.0±0.2	2.5
scFv-HSA-K573P	7.4±0.1	0.8±0.1	0.1

a: Dilutions of HSA variants were injected over immobilized shFcRn (~1500 RU).

b: The kinetic rate constants were obtained using a simple first-order (1:1) bimolecular interaction model. The kinetic values represent the average of duplicates.

5 c: Not determined due to weak binding (ND).

In example 8 it was shown that the K500A variant did not significantly bind shFcRn, in Example 10 it was shown that the K573P and K573A variants bind shFcRn stronger than HSA and in Example 11 it was shown that the D550N variant binds FcRn weaker than HSA.

In the present example it is shown that these observed difference in binding properties also are reflected in fusion polypeptides in different configurations: C-terminal fusions with a small moiety (HSA-FLAG), C-terminal fusions with a larger polypeptide (HSA-IL1RA); N-terminal fusions with polypeptide (scFv-HSA); N- and C-terminal fusions (scFv-HSA-FLAG and scFv-HSA-scFv-FLAG).

Example 17. Conjugation of Horseradish peroxidase protein to Albumin and the K573P variant.

For conjugation analysis, commercially available recombinant albumin (Recombunin™) was used as a control molecule. For this example, a final 200mg/mL albumin K573P variant of the invention was purified from a fed batch fermentation by means described in Material and Methods. A two step purification was carried out;

The first step used a column (bed volume approximately 400mL, bed height 11cm) packed with AlbuPure™ matrix (ProMetic). This was equilibrated with 50mM sodium acetate, pH 5.3 and loaded with neat culture supernatant, at approximately pH 5.5-6.5, to approximately 20 mg/mL matrix. The column was then washed with approximately 5 column volumes each of 50mM sodium acetate, pH 5.3, 50mM sodium phosphate, pH 6.0, 50mM sodium phosphate, pH 7.0 and 50mM ammonium acetate, pH 8.0, respectively. Bound protein was eluted using approximately two column volumes of 50mM ammonium acetate, 10mM octanoate, pH 7.0. The flow rate for the entire purification was 154mL/min.

For the second step, the eluate from the first step was diluted approximately two fold with water to give a conductivity of 2.5 ± 0.5 mS/cm after adjustment to pH 5.5 ± 0.3 with acetic acid. This was loaded onto a DEAE-Sepharose Fast Flow (GE Healthcare) column (bed volume approximately 400mL, bed height 11cm), equilibrated with 80mM sodium acetate, 5mM octanoate, pH 5.5. Loading was approximately 30mg protein/mL matrix. The column was washed with approximately 5 column volumes of 80mM sodium acetate, 5mM octanoate, pH 5.5. Followed by approximately 10 column volumes of 15.7mM potassium tetraborate, pH 9.2. The bound protein was eluted using two column volumes of 110mM potassium tetraborate, 200mM sodium chloride, approximately pH 9.0. The flow rate was 183mL/min during the load and wash steps, and 169mL/min during the elution step.

The eluate was concentrated and diafiltered against 145mM NaCl, using a Pall Centrimate Omega 10,000 Nominal MWCO membrane, to give a final protein concentration of approximately 200mg/mL.

Both 200mg/mL stock solutions of the rHA and K573P variant albumin were diluted down to 5mg/mL, using phosphate buffer saline (PBS), pH adjusted to pH 6.5-6.7. This ensured a favourable pH environment for the maleimide reactive group of the EZ-Link® Maleimide Activated Horseradish Peroxidase (Thermo Scientific) to react with the free sulphydryl, to form a stable thioester bond. 2mg of the EZ-Link® Maleimide Activated Horseradish Peroxidase (HRP) was mixed with either 1mL of the 5mg/mL rHA or K573P variant albumin. This mixture ensured an approximate 2 fold molar excess of the albumin, or K573P variant albumin. This mixture was minimally incubated at 4 °C, for 24 hours. The reaction mixtures were then checked for conjugation, using GP-HPLC.

To separate unconjugated species (rHA, or Albumin variant K573P and unreacted HRP) from the corresponding conjugated species the samples were first concentrated (Vivaspin20, 10,000 MWCO PES, Sartorius), and then individually applied to a Tricorn Superdex™ 200, 10/300 GL column (GE Healthcare), run at a flow rate of 45cm/hr in PBS. The elution peak was fractionated and GP-HPLC analysed. Fractions containing the conjugated species were pooled,

concentrated and diafiltered against 50mM NaCl and analysed by GP-HPLC to demonstrate (Figure 24)

These samples were then assayed using the Biacore method described herein (Table 21). This example demonstrates that the K573P maintains its increased affinity for shFcRn compared the the WT HSA.

Example 18. Conjugation of Fluorescein to Albumin and the K573P variant.

The two same albumin samples used in Example 17, were also the start materials for this example. I.e. Approximately 200mg/mL rHA or the K573P albumin variant.

Fluorescein-5-Maleimide, Thermo Scientific (F5M) was dissolved in dimethylformamide, to give a final concentration of 25mg/mL. This was then further diluted into 18mls of PBS, pH adjusted to approximately pH 6.5. To this solution either 1ml of 200mg/mL rHA or 1mL of 200mg/mL K573P variant was added. This gave an approximate 20 fold final molar excess of F5M. These samples were incubated and allowed to conjugate overnight at 4°C, in the dark, to allow the maleimide groups on the F5M to react with predominantly the free sulfhydryl, present in both albumin species.

Following overnight incubation aliquots of the reaction mixtures were extensively diafiltered against 50mM NaCl to remove unconjugated F5M, (Vivaspin20, 10,000 MWCO PES, Sartorius). Conjugation was confirmed by ultraviolet visualization of conjugated Fluorescein::Albumins Following standard SDS-PAGE (Figure 25).

These diafiltered samples were then assayed using the Biacore method described herein (Table 21). This example demonstrates that the conjugation of a small molecule to either rHA or a variant, e.g. K573P does not affect the trend in binding affinities to shFcRn.

Table 21: Representative Biacore assay KD values of conjugated rHA or a variant (K573P) when binding to immobilized shFcRn.

Analyte	KD (μ M)
rHA::HRP	3.6
K573P::HRP	0.02
rHA::F5M	7.3
K573P::F5M	2.5

Example 19. Further albumin variants.

The following variants were generated using the methods described in Example 1 E492T, N503D, E492T+N503D, K538H, E542D, D494N+E495Q+T496A, E495Q+T496A, N403K, K541A and K541N. SPR analysis was carried out as described in Example 15 and the results presented in Figure 26 and figure 27.

Figure 30A and 30B shows the effect on shFcRn binding for the albumin variants.

Substitutions N503D, D494N+E495Q+T496A E492T+N503D, E495Q+T496A within HSA had a negative impact on binding to shFcRn at pH5.5.

Example 20. Variants of albumin at the C-termini.

The following variants were generated using the methods described in Example 1. Binding to the shFcRn was determined as described in Materials and Methods and the results are presented in Table 22.

Table 22: Kinetics of the HSA C-terminal swapped variant interactions with shFcRn.

Albumin variant ^a	k_a ($10^3/\text{Ms}$)	k_d ($10^{-3}/\text{s}$)	KD^b (μM)
HSA	4.4±0.0	24.0±0.1	5.4
MacSA	3.1±0.1	8.6±0.1	2.7
HSA-MacC	4.1±0.1	5.6±0.0	1.3
MouseSA ^c	3.8±0.0	3.1±0.1	0.8
HSA-MouseC	3.7±0.1	1.3±0.0	0.3
RabbitSA ^d	1.9±0.3	1.7±0.1	0.9
HSA-RabC	3.5±0.0	1.6±0.0	0.4
SheepSA	ND	ND	ND
HSA-SheepC	3.3±0.0	2.1±0.0	0.6

a: Dilutions of HSA variants were injected over immobilized shFcRn (~1500 RU).

b: The kinetic rate constants were obtained using a simple first-order (1:1) bimolecular interaction model.

c: Data from Table 2

d: Data from Table 3

Not determined due to weak binding (ND)

This example demonstrates that for all C-terminal swaps to human albumin tested an increase in binding over the donor albumin was observed. All donor sequences contain the K573P substitution shown to significantly increase binding but less than the K573P alone (Table 20).

5 **Example 21. Competitive binding analysis of variant albumin fusions**

Competitive binding studies, using variant albumin fusions and a selection of variant albumins prepared as described in Example 1, were performed as described in Example 4. Results are presented in Figures 28-31.

10 The competitive binding hierarchy was identical for the variants fusions of HSA-FLAG and , N+C-terminal scFv HSA-FLAG to the hierarchy of the individual HSA variants (unfused and fused) affinity data. For the IL1Ra variants K573P, K573A, and the K500A were as predicted, however the D550N appears to inhibit more efficiently than the WT fusion.

Example 22. Further HSA variants

15 The following variants were generated using methods described in Example 1: HSA E492G+K573A, HSA E492G+ N503K+ K573A, HSA E492G+ N503H + K573A, HSA E492G + K573P, HSA E492G + N503K + K573P, HSA E492G + N503H + K573P. SPR analysis was performed as described in Materials and Methods. Results (Figure 32) showed that all HSA variants bound more strongly to shFcRn compared to wild type HSA at pH 5.5. No binding was
20 observed at pH 7.4.

HSA E492G+K573A, HSA E492G+ N503K+ K573A, unlike HSA E492G+ N503H + K573A, had marginally improved binding beyond that of HSA K573A. The combination variants containing K573P did not show improved binding over the K573P single variant.

CLAIMS

1. A Method for preparing a variant of albumin, fragments thereof or fusion polypeptide comprising said variant albumin or fragment thereof, comprising following steps:

a. Providing a nucleic acid encoding a parent albumin having at least 80% sequence identity to SEQ ID NO: 2;

b. Modifying the sequence of step a., to encode a variant albumin, fragments thereof or fusion polypeptide comprising said variant albumin or fragment thereof having one or more substitutions corresponding to the substitutions in SEQ ID NO: 2 selected among:

Q417A,C,D,E,F,G,H,I,K,L,M,N,P, R,S,T,V,W,Y;

H440A,C,D,E,F,G, I,K,L,M,N,P,Q,R,S,T,V,W,Y;

A490C,D,E, F,G,H,I,K,L,M,N,P, R,S,T,V,W,Y

E492A,C,D,F,G,H,I,K,L,M,N,P,Q,R,S,T,V,W,Y;

V493A,C,D,E,F,G,H,I,K,L,M,N,P,Q,R,S,T,W,Y;

D494A,C,E,F,G,H,I,K,L,M,N,P,Q,R,S,T,V,W,Y;

E495A,C,D,F,G,H,I,K,L,M,N,P,Q,R,S,T,V,W,Y;

T496A,C,D,E,F,G,H,I,K,L,M,N,P,Q,R,S,V,W,Y;

P499A,C,D,E,F,G,H,I,K,L,M,N, Q,R,S,T,V,W,Y;

K500A,C,D,E,F,G,H,I, ,L,M,N, Q,R,S,T,V,W,Y;

E501A,C,D,F,G,H,I,K,L,M,N,P,Q,R,S,T,V,W,Y;

N503A,C,D,E,F,G,H,I,K,L,M, P,Q,R,S,T,V,W,Y;

A504C,D,F,G,H,I,K,L,M,N,P,Q,R,S,T,V,W,Y;

E505A,C,D,F,G,H,I,K,L,M,N,P,Q,R,S,T,V,W,Y;

T506A,C,D,F,G,H,I,K,L,M,N,P,Q,R,S, V,W,Y;

H510A,C,D,F,G, I,K,L,M,N,P,Q,R,S,T,V,W,Y;

H535A,C,D,F,G, I,K,L,M,N,P,Q,R,S,T,V,W,Y;

K536A,C,D,E,F,G,H,I, L,M,N,P,Q,R,S,T,V,W,Y;

P537A,C,D,E,F,G,H,I,K,L,M,N, Q,R,S,T,V,W,Y;

K538A,C,D,E,F,G,H,I, L,M,N,P,Q,R,S,T,V,W,Y;

T540A,C,D,E,F,G,H,I, L,M,N,P,Q,R,S, ,V,W,Y;

K541A,C,D,E,F,G,H,I, L,M,N,P,Q,R,S,T,V,W,Y;

E542A,C,D,F,G,H,I,K,L,M,N,P,Q,R,S,T,V,W,Y;

D550A,C,E,F,G,H,I,K,L,M,N,P,Q,R,S,T,V,W,Y;
 K573A,C,D,E,F,G,H,I, L,M,N,P,Q,R,S,T,V,W,Y;
 K574A,C,D,E,F,G,H,I, L,M,N,P,Q,R,S,T,V,W,Y;
 Q580A,C,D,E,F,G,H,I, K, L,M,N,P,R,S,T,V,W,Y;
 A581C,D,E,F,G,H,I,K,L,M,N,P,Q,R,S,T,V,W,Y;
 A582C,D,E,F,G,H,I,K,L,M,N,P,Q,R,S,T,V,W,Y; or
 G584A,C,D,E,F,H,I,K,L,M,N,P,Q,R,S,T,V,W,Y;

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- c. Introducing the modified sequence of step b., in a suitable host cell;
- d. Growing the cells in a suitable growth medium under condition leading to expression of the variant of albumin, fragments thereof or fusion polypeptide comprising said variant albumin or fragment thereof; and
- e. Recovering the variant of albumin, fragments thereof or fusion polypeptide comprising said variant albumin or fragment thereof from the growth medium;

wherein the variant of albumin, fragments thereof or fusion polypeptide comprising said variant albumin or fragment thereof, has an altered plasma half-life compared with the parent albumin, fragments thereof or fusion polypeptide comprising said variant albumin or fragment thereof.

2. A variant of albumin, fragments thereof or fusion polypeptides comprising said variant albumin or a fragment thereof having altered plasma half-life compared with the parent albumin, fragment thereof or fusion polypeptide comprising said parent albumin or fragment thereof, comprising one or more substitutions in positions corresponding to the positions in SEQ ID NO:2 selected among: 573, 500, 550, 492, 580, 574, 417, 440, 464, 490, 493, 494, 495, 496, 499, 501, 503, 504, 505, 506, 510, 535, 536, 537, 538, 540, 541, 542, 575, 577, 578, 579, 581, 582 and 584, where the variant is not the variant consisting of SEQ ID NO: 2 with the substitution D494N, E501K, K541E, D550G,A, K573E or K574N..

3. The variant of albumin, fragments thereof or fusion polypeptide comprising said variant albumin or fragment thereof according to claim 2, having a longer plasma half-life than the parent albumin.

4. The variant of albumin, fragments thereof or fusion polypeptide comprising said variant albumin or fragment thereof according to claim 3, comprising one or more substitutions corresponding to the following substitutions in SEQ ID NO: 2: K573Y,W,P,H,F,V,I,T,N,S,G,M,C,A,E,Q,R,L,D.

K573P+K574N+A577T+A578R+S579C+Q580K+A581D+G584A.

K573P+A577E+A578S+Q580K+A582T, K573P+A578S+S579T+G584A,

K573P+L575F+G584A, E492G, N503K, K500R N503H, D550E, , K574N, Q580K and
 E492G+N503K, E492G+N503H. E492H+E501P+N503H+E505D+T506S+T540S+K541E,
 E492G+K573A, E492G+N503K+K573A, E492G+N503H, E492G+K573P,
 E492G+N503K+K573P, E492G+N503H+K573P.

5. The variant of albumin, fragments thereof or fusion polypeptide comprising said variant albumin or fragment thereof according to claim 2, having a shorter plasma half-life than the parent albumin

6. The variant of albumin, fragments thereof or fusion polypeptide comprising said variant albumin or fragment thereof according to claim 5, comprising one or more substitution(s) corresponding to the substitutions in SEQ ID NO: 2 selected among:
 K500E,G,D,A,S,C,P,H,F,N,W,T,M,Y,V,Q,L,I,R. D550N, H464Q, H510Q, H535Q,
 D494N,Q,A. E495Q,A, T496A, E492G+V493P, K541G,D, D494N+E495Q+T496A,
 E495Q+T496A, E492G+V493P+K538H+K541N+E542D, N503K,D P499A, K541A,N,
 D494E + Q 4 1 7 H , Q 4 1 7 A , E 4 9 2 T + N 5 0 3 D ,
 A490D+E492T+V493L+E501P+N503D+A504E+E505K+T506F+K541D, K536A, P537A,
 E501A,Q, E492G+K538H+K541N+E542D.

7. The variant of albumin, fragments thereof or fusion polypeptide comprising said variant albumin or fragment thereof according to any of the previous claims, comprising one or more further alteration that generates thio group on the surface.

8. The variant of albumin, fragments thereof or fusion polypeptide comprising said variant albumin or fragment thereof according to the previous claims, wherein the sequence identity of the variant of albumin, fragments thereof or fusion polypeptide comprising said variant albumin or fragment thereof to SEQ ID NO: 2 is more than 80%, preferably more than 90%, more preferred more than 95%, more preferred more than 96%, even more preferred more than 97%, more preferred more than 98% and most preferred more than 99%.The variant of albumin, fragments thereof or fusion polypeptide comprising said variant albumin or fragment thereof according to the previous claims being a variant fragment of albumin or a fusion polypeptide comprising a variant fragment of albumin wherein the fragment is at least 20 amino acids, preferably at least 50 amino acids, preferably at least 100 amino acids,

more preferred at least 200 amino acids, more preferred at least 300 amino acids, more preferred at least 400 amino acids and most preferred at least 500 amino acids.

9. A nucleic acid encoding the variant of albumin, fragments thereof or fusion polypeptide comprising said variant albumin or fragment thereof of claim 2.
10. A conjugate comprising the variant albumin or fragment thereof according to claim 2 and a beneficial therapeutic moiety.
11. An Associate comprising the variant albumin or fragment thereof according to claim 2 and a beneficial therapeutic moiety.
12. A fusion polypeptide comprising a variant albumin or fragment thereof according to claim 2 and a fusion partner polypeptide.
13. A composition comprising a variant of albumin, fragments thereof or fusion polypeptide comprising said variant albumin or fragment thereof or a conjugate comprising said variant albumin, wherein the binding to FcRn is stronger than for the corresponding albumin or fragment thereof or fusion polypeptide comprising said albumin or fragment thereof or a conjugate comprising said albumin.
14. The composition of claim 13 where the binding to FcRn is stronger than the binding of HSA to FcRn.
15. The composition of claim 14, wherein the binding coefficient of the variant of albumin, fragments thereof or fusion polypeptide comprising said variant albumin or fragment thereof or a conjugate comprising said variant albumin to FcRn is less than 0.9X KD for HSA, more preferred less than 0.5X KD for HSA, more preferred less than 0.1X KD for HSA, even more preferred less than 0.05X KD for HSA, even more preferred less than 0.02X KD for HSA and most preferred less than 0.01X KD for HSA.
16. The composition according to any of claims 12-15, comprising a variant albumin or a fragment thereof according to claim 1, a conjugate of claim 10, an associate of claim 11 or a fusion polypeptide of claim 12.
17. The composition according to any of claims 13-16, comprising a variant albumin or a fragment thereof according to claim 1, further comprising a compound comprising a ABD and a pharmaceutically beneficial moiety.

18. The composition according to any of the claims 13-17, being a pharmaceutical composition.

Figure 1

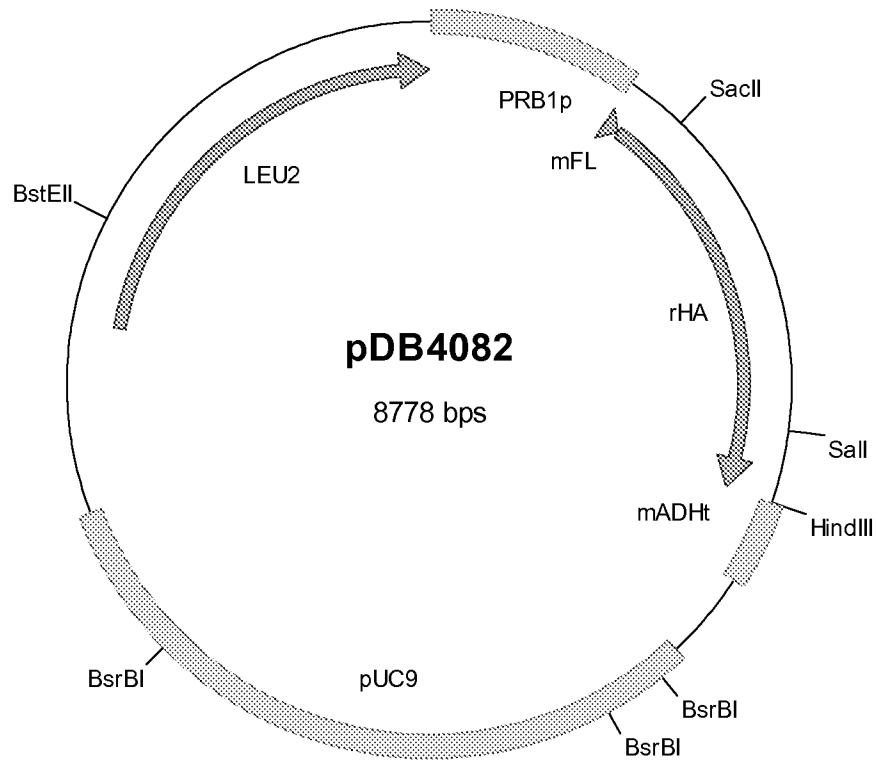


Figure 2

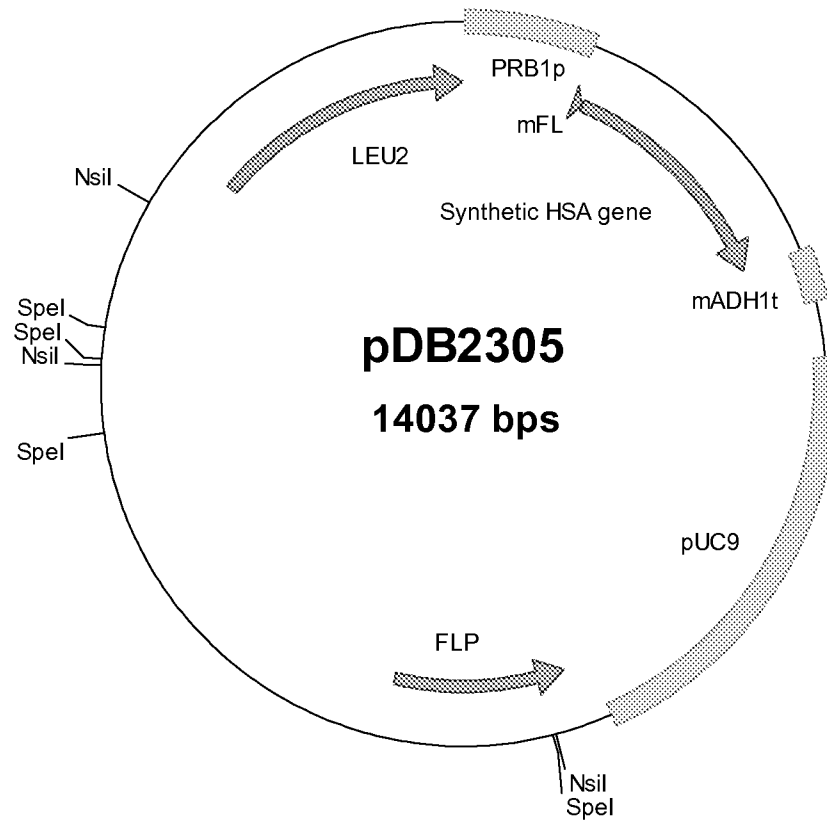


Figure 3

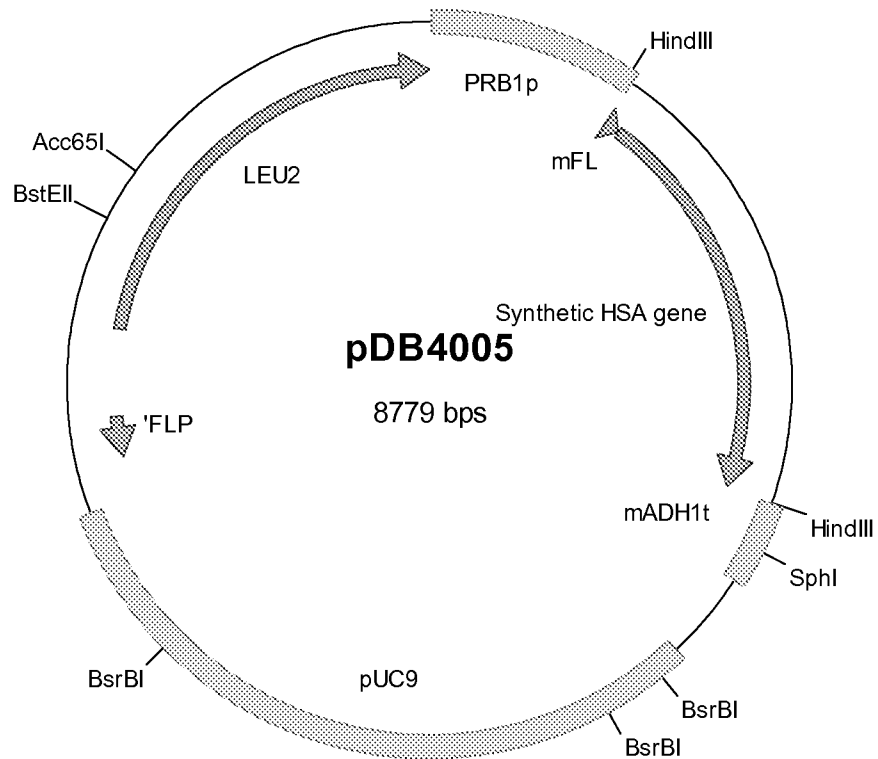


Figure 4

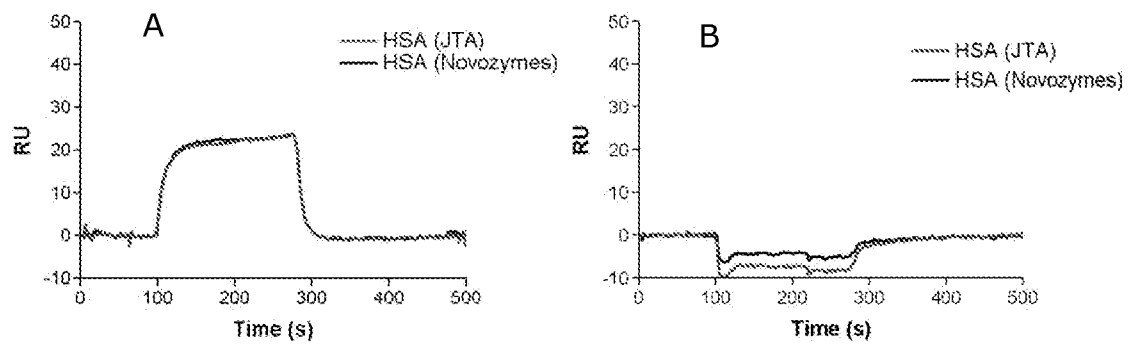


Figure 5

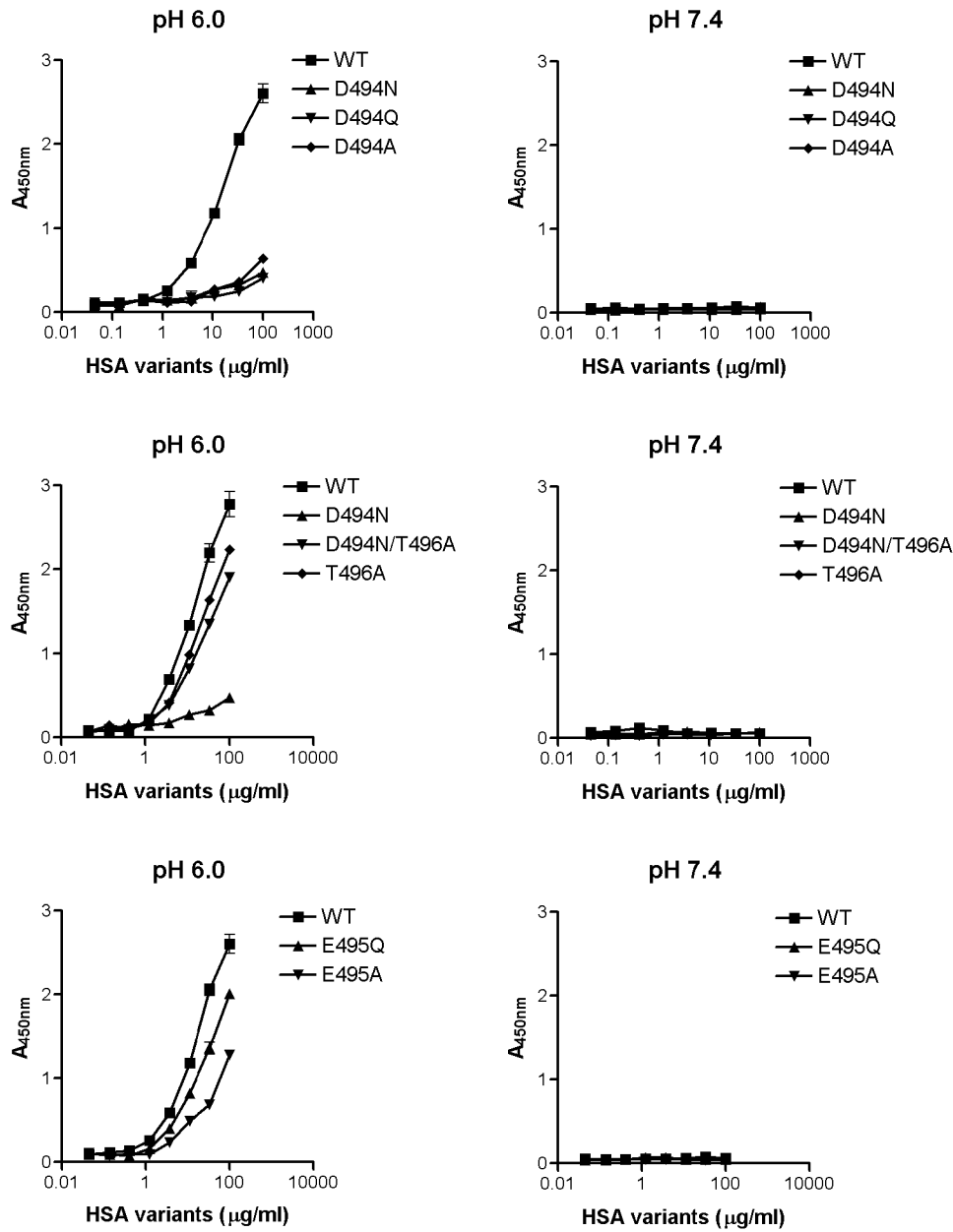


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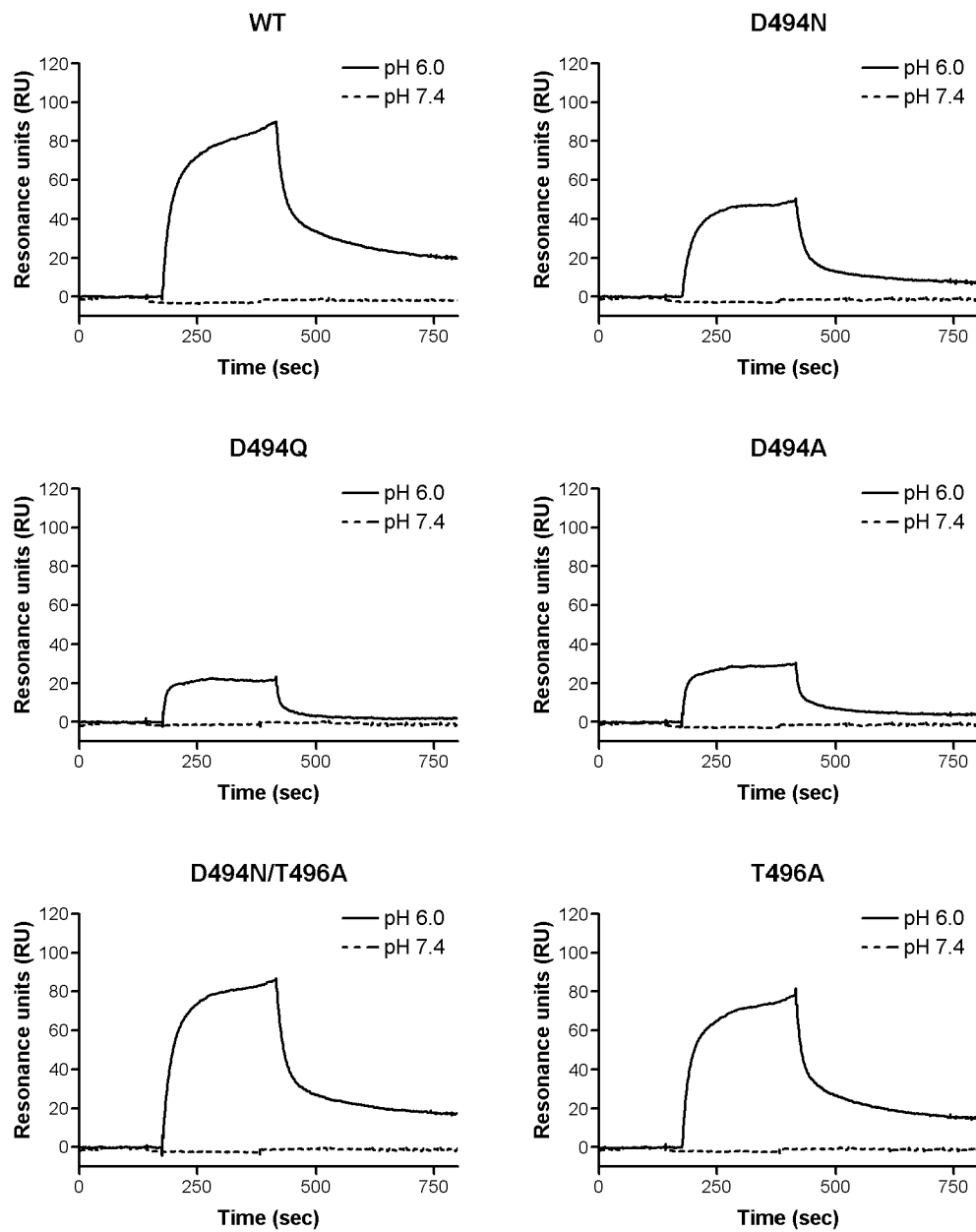


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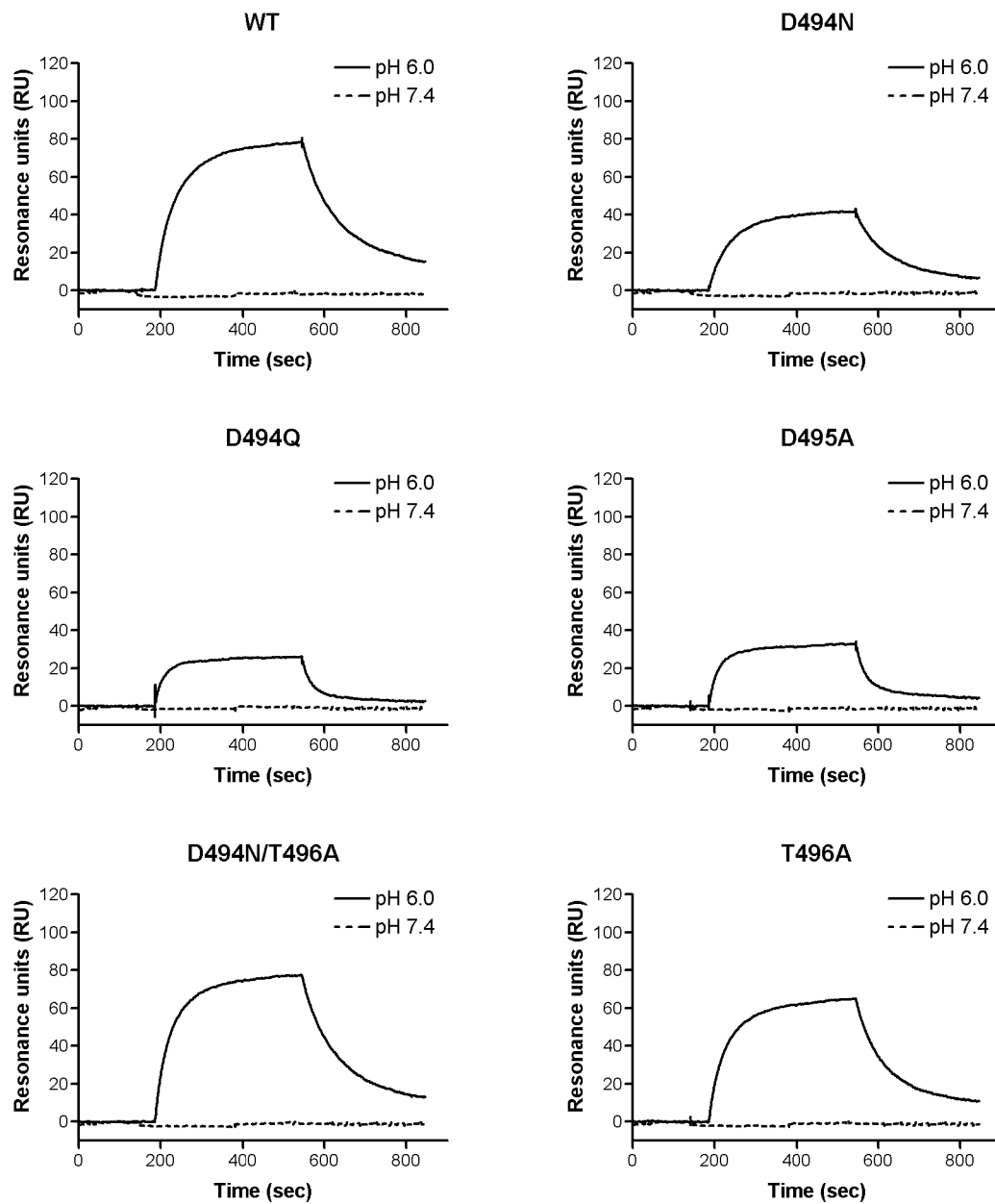
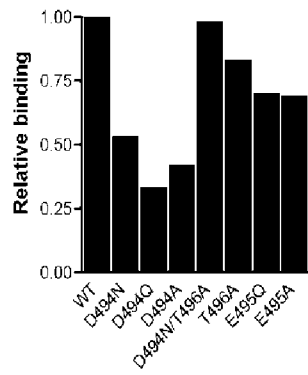


Figure 8

A.



B.

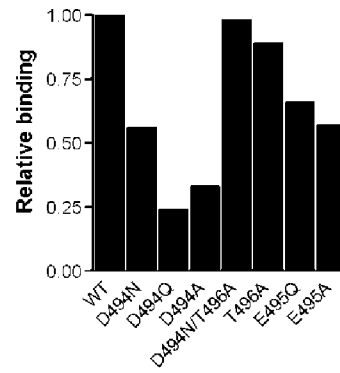


Figure 9

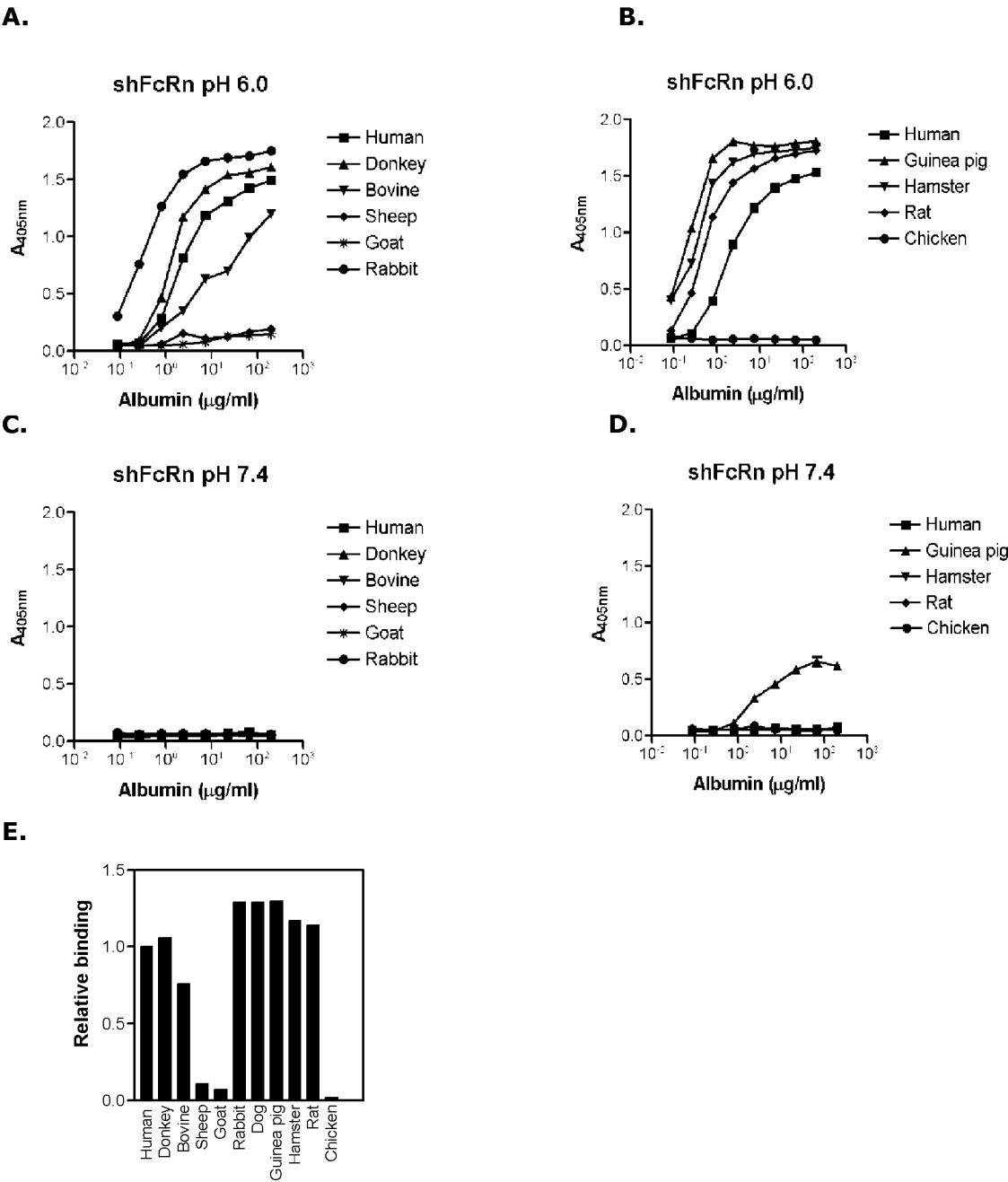


Figure 10

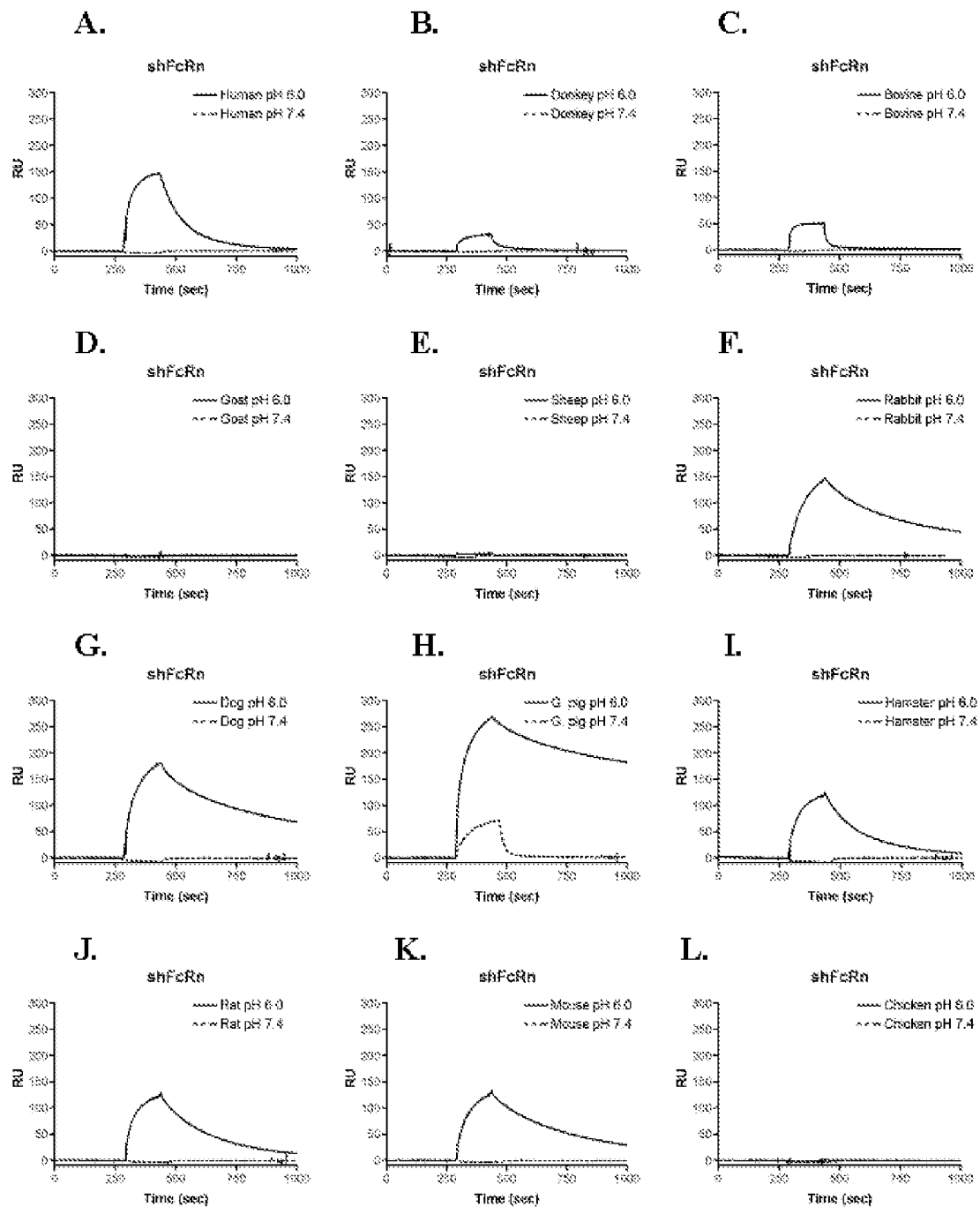


Figure 11

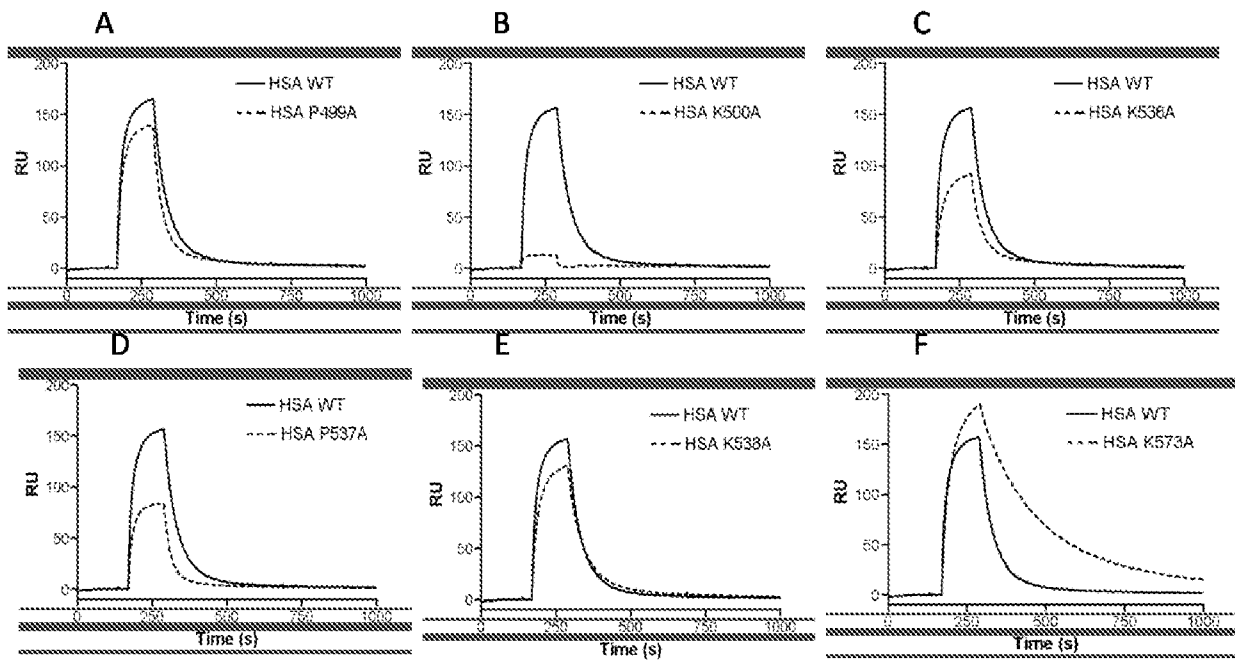


Figure 12

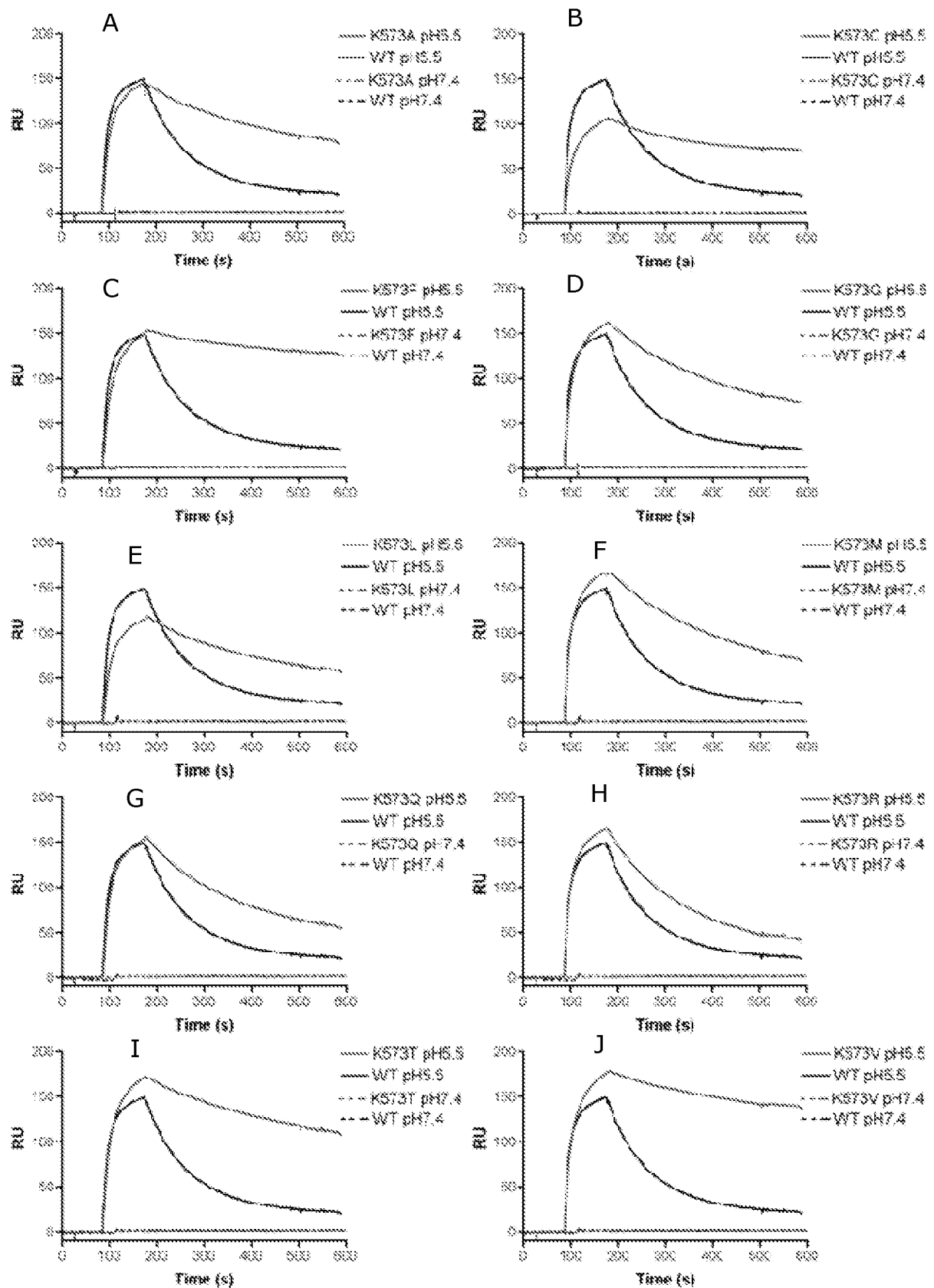


Figure 30

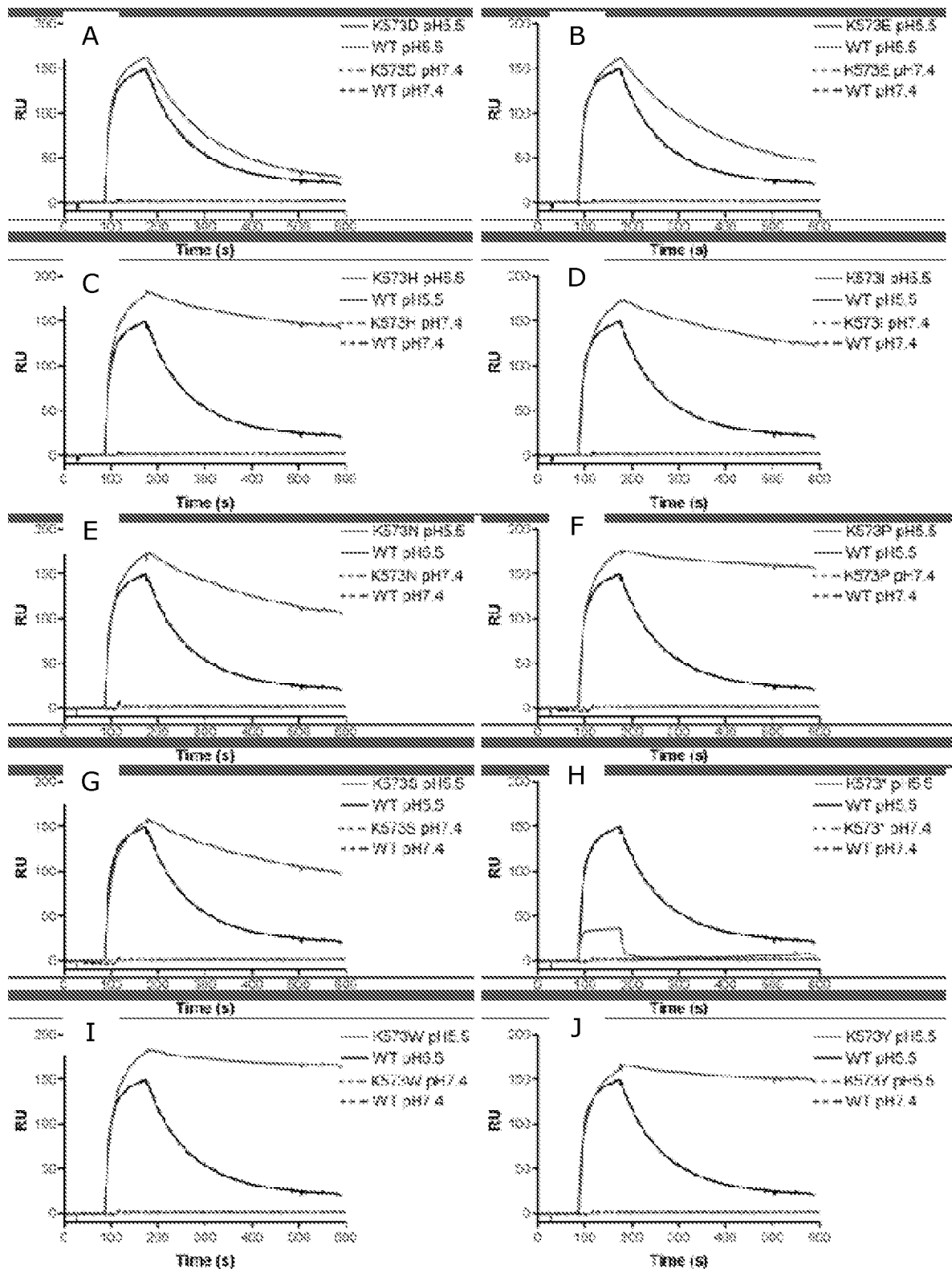
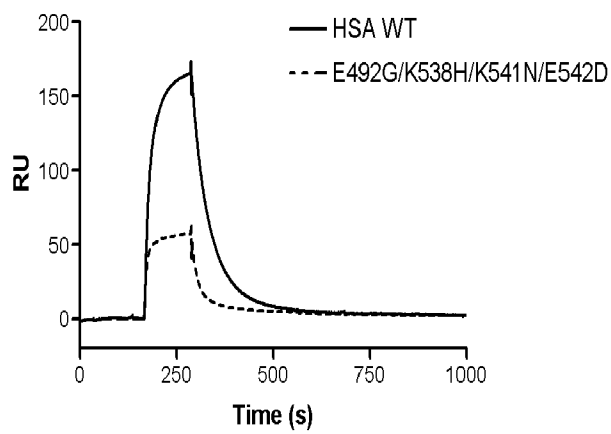
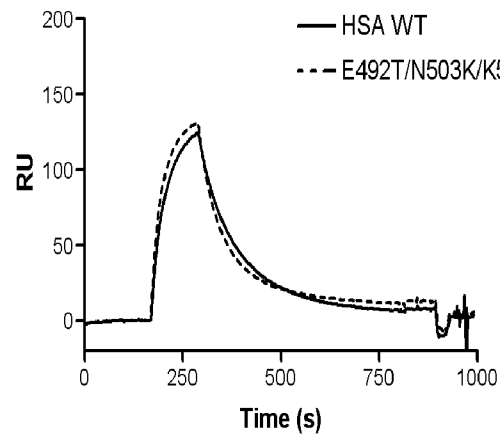


Figure 14

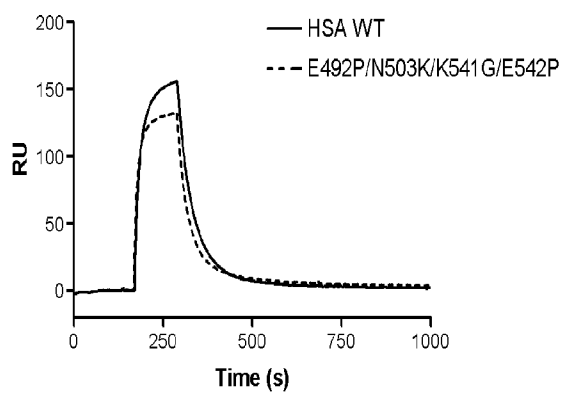
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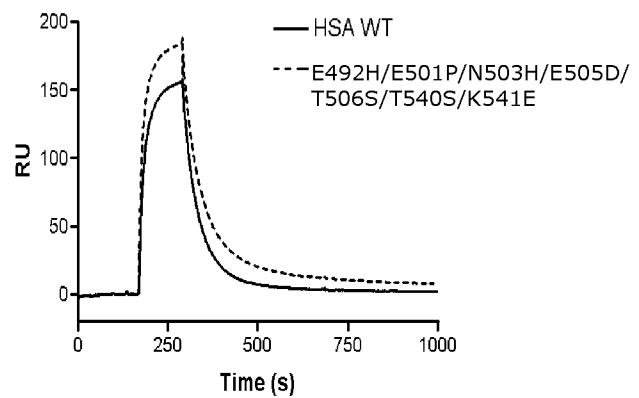
B.



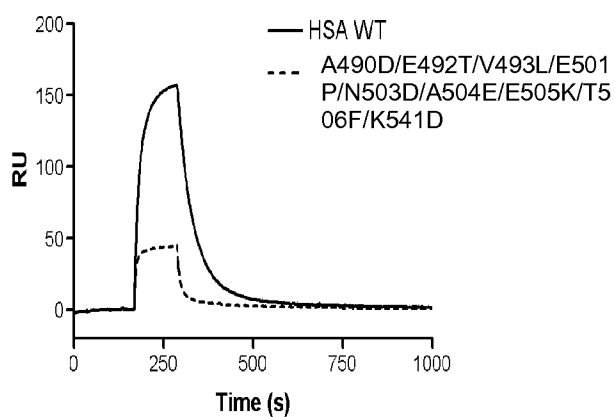
C.



D.



E.



F.

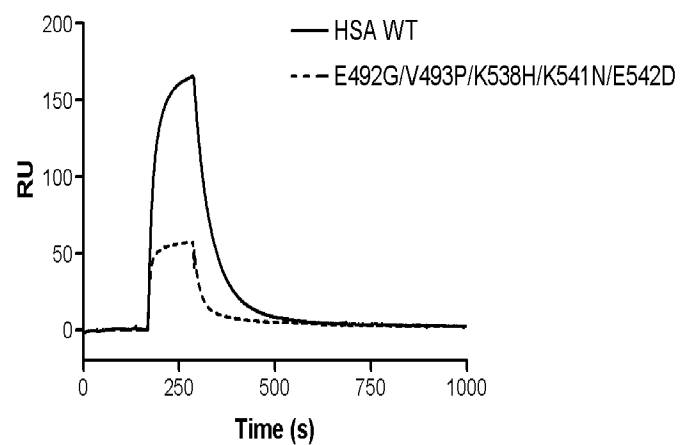


Figure 15

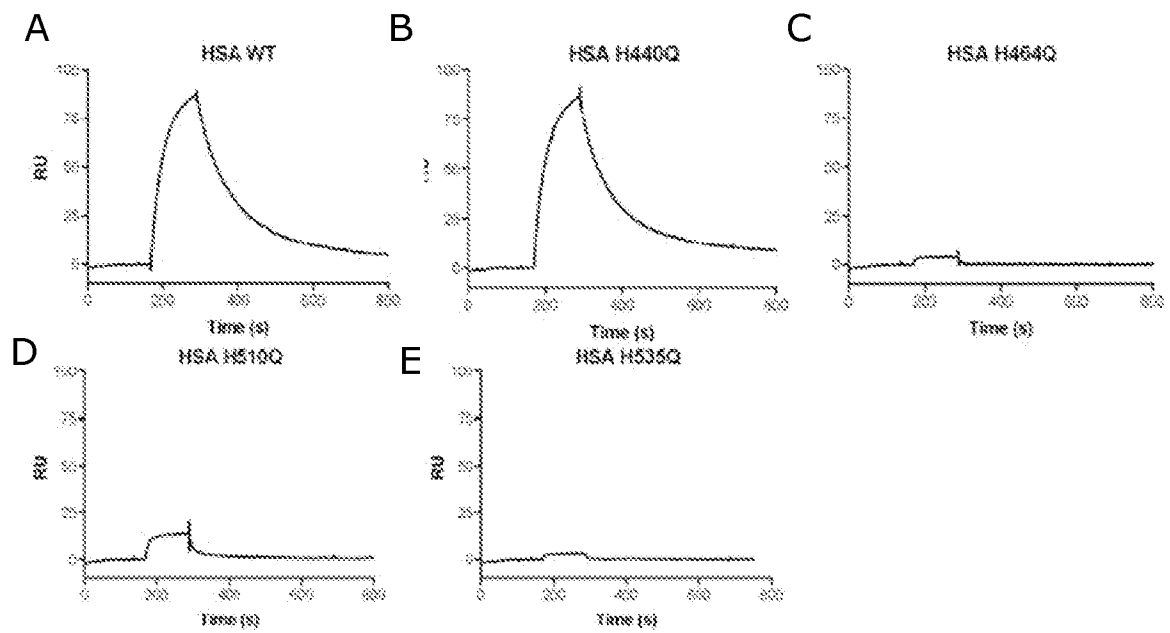


Figure 16

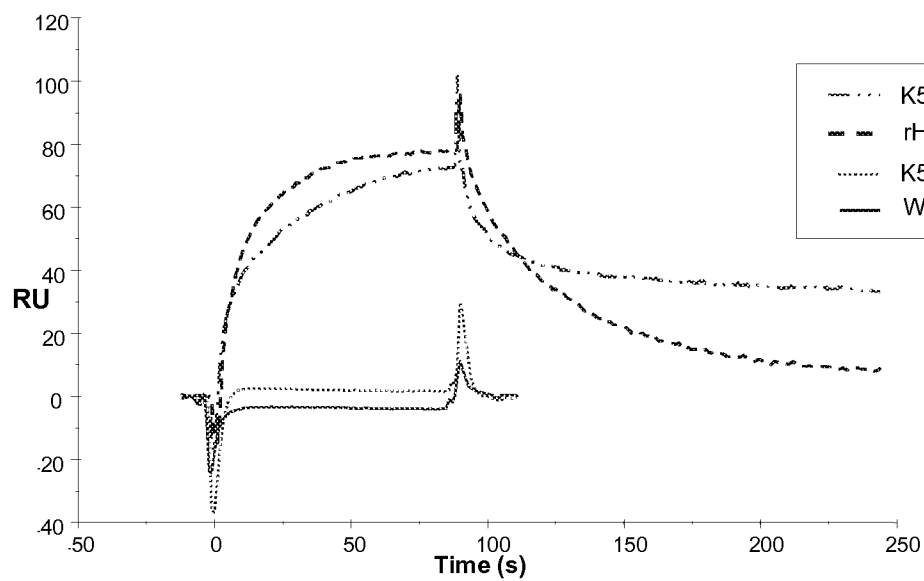


Figure 17

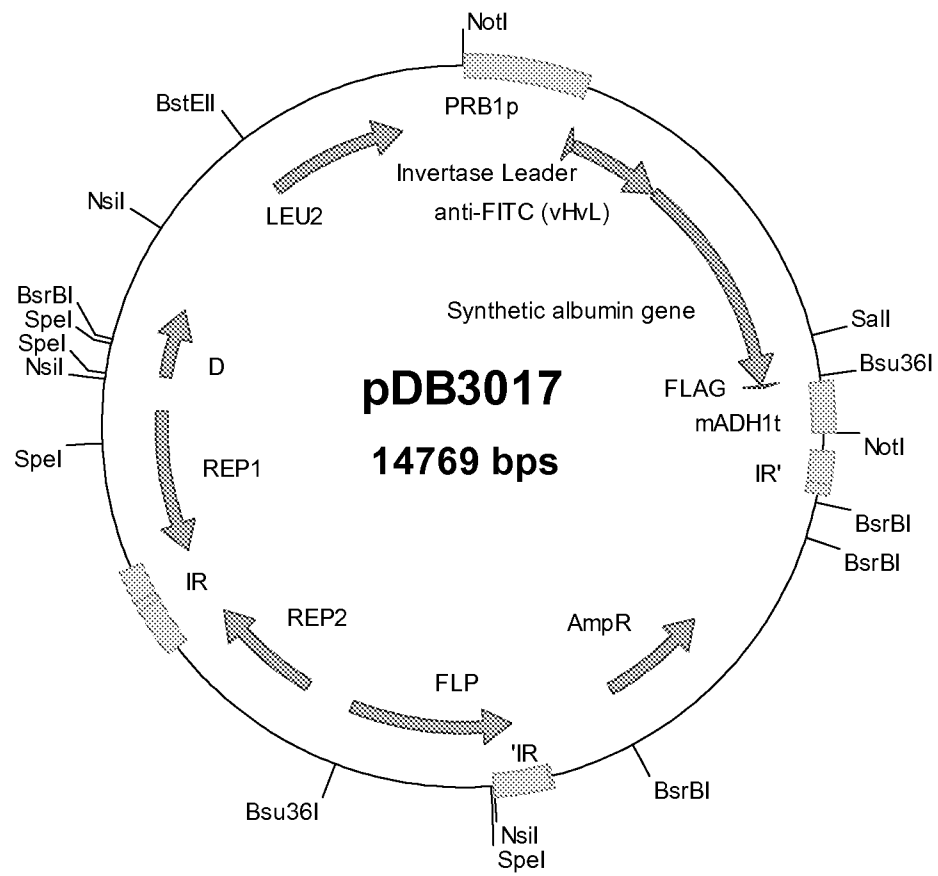


Figure 18

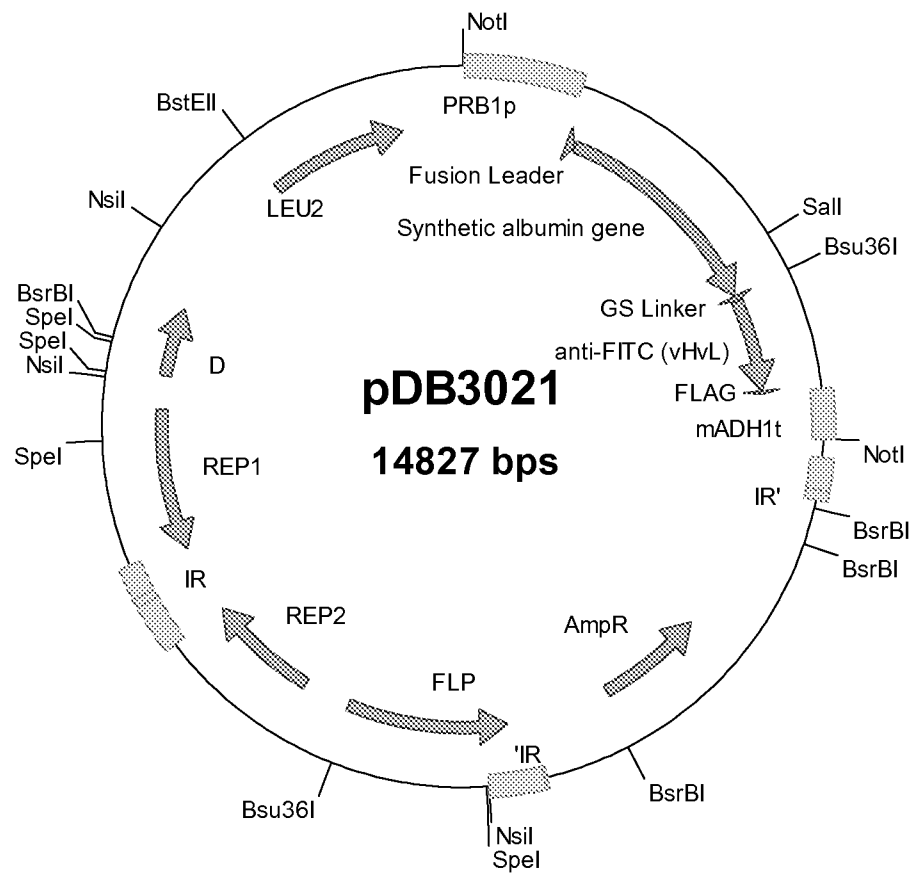


Figure 19

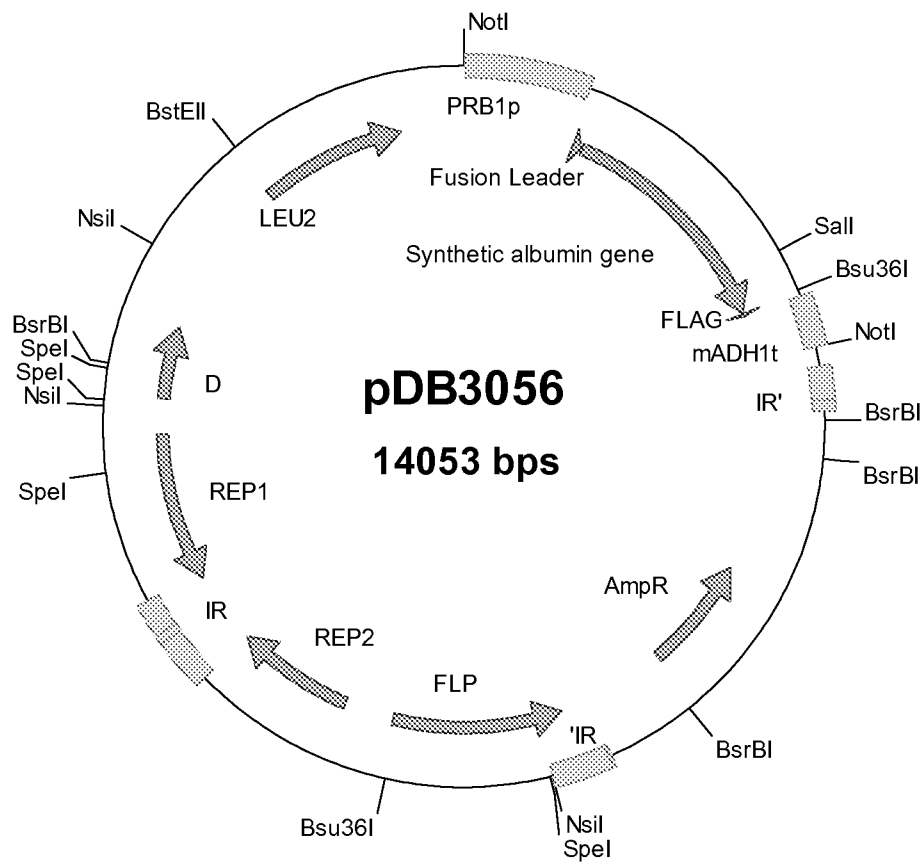


Figure 20

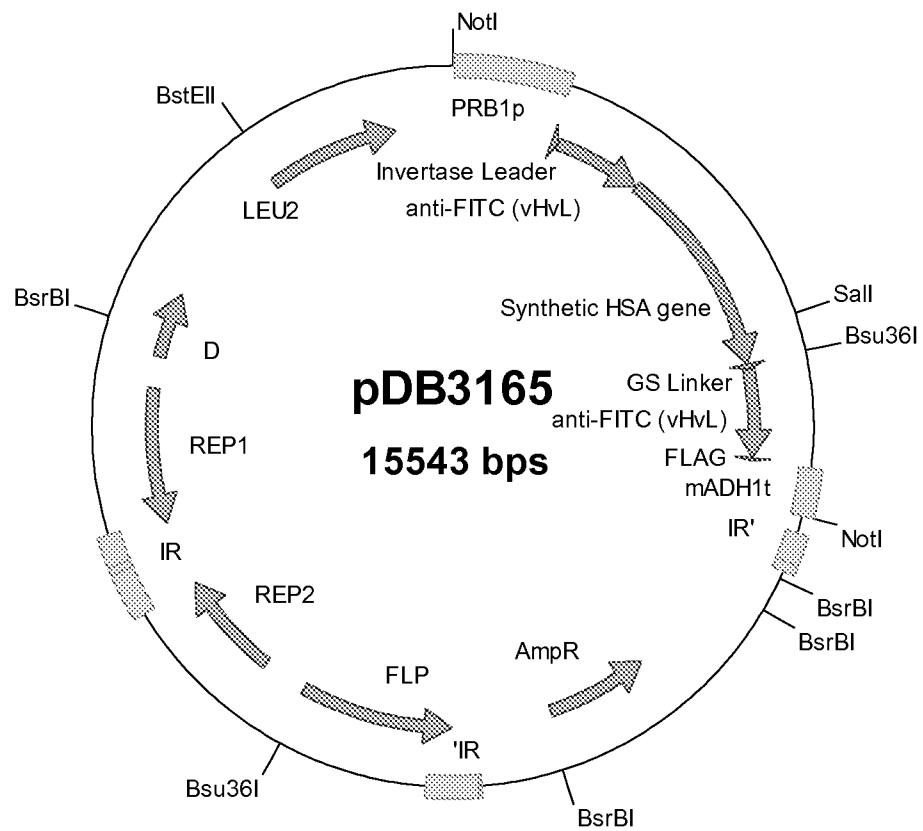


Figure 21

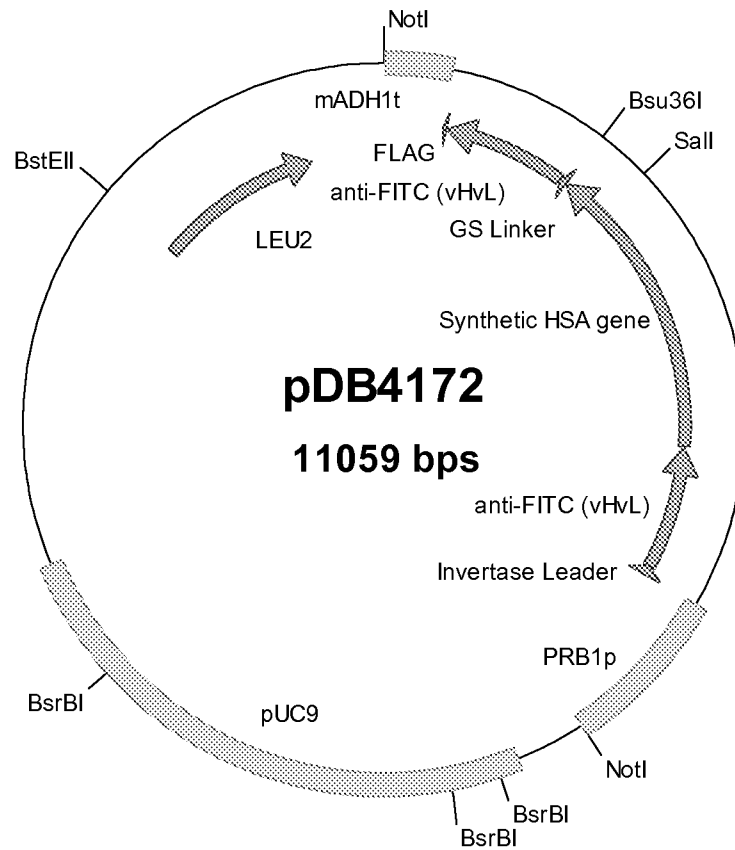


Figure 22

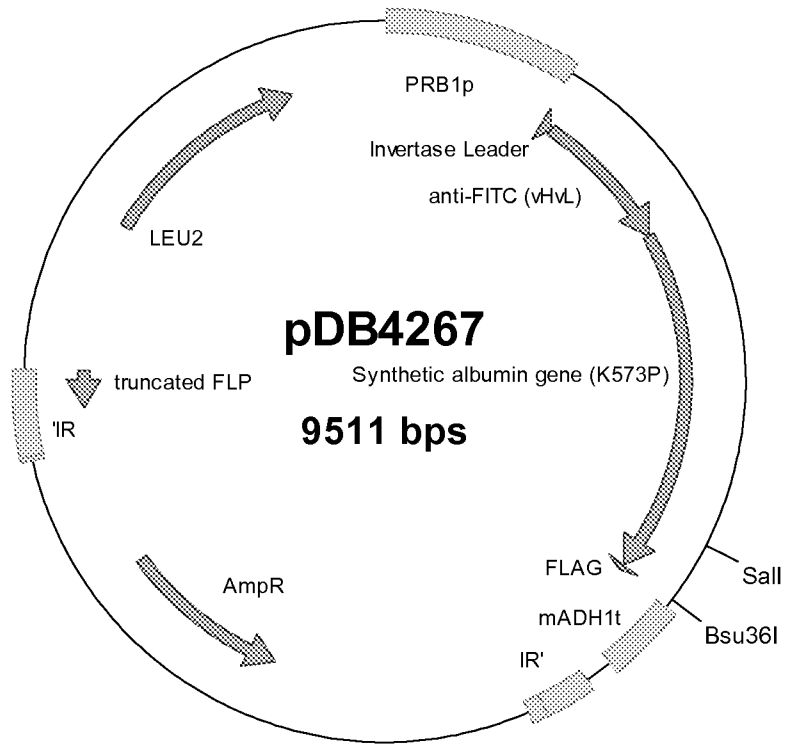


Figure 23

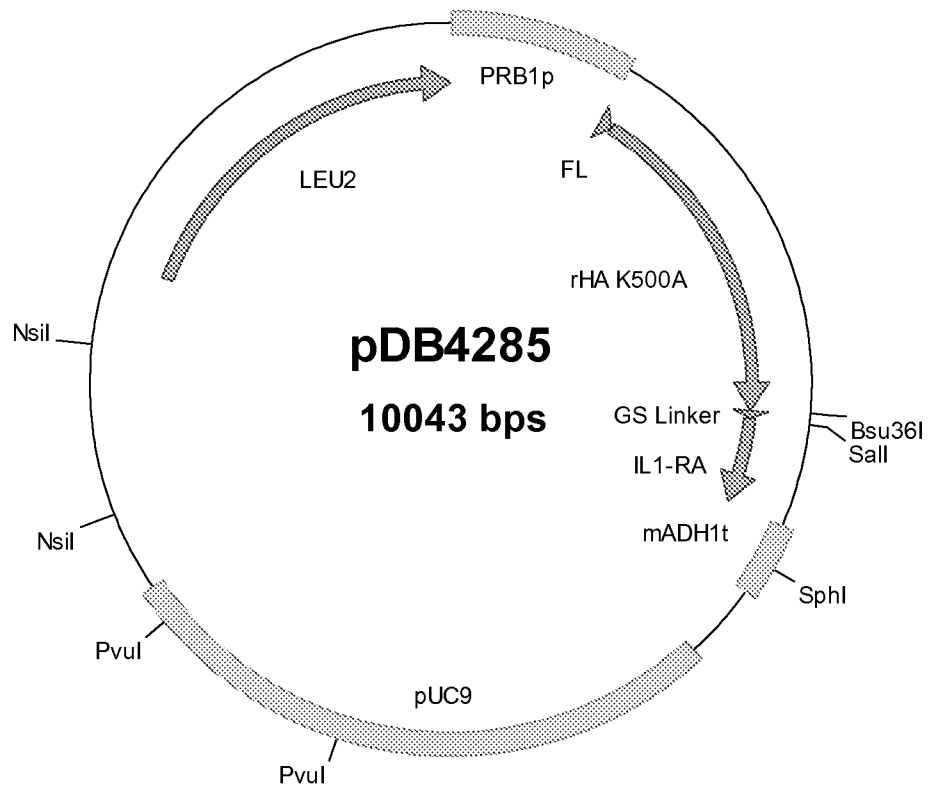


Figure 24

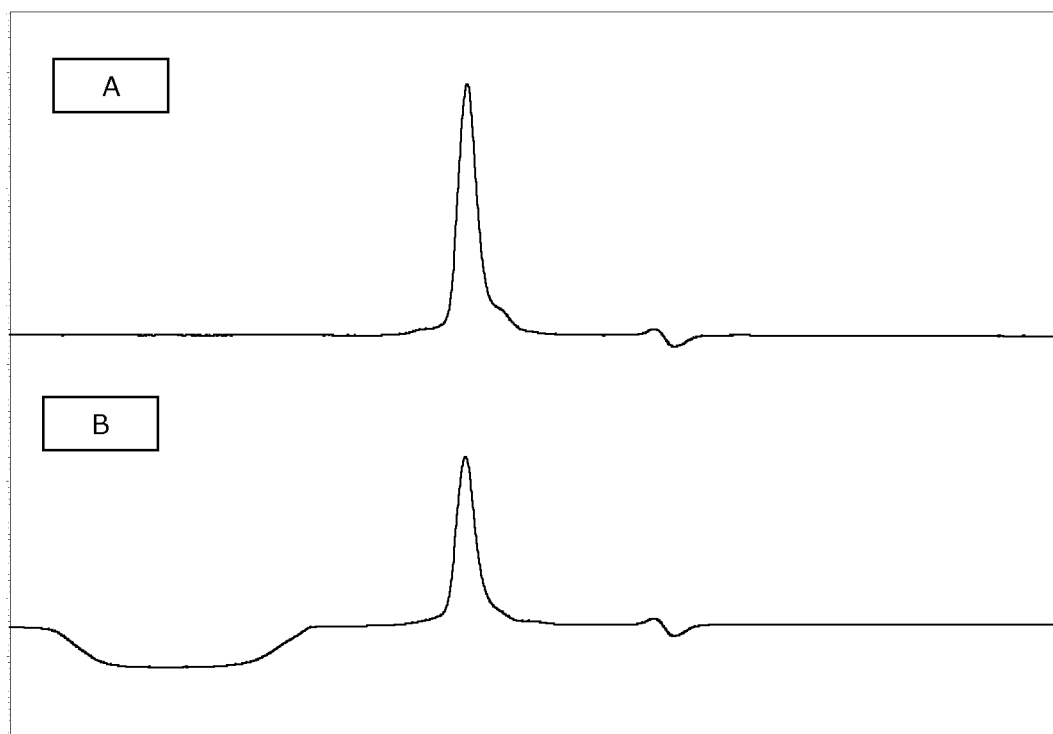


Figure 25

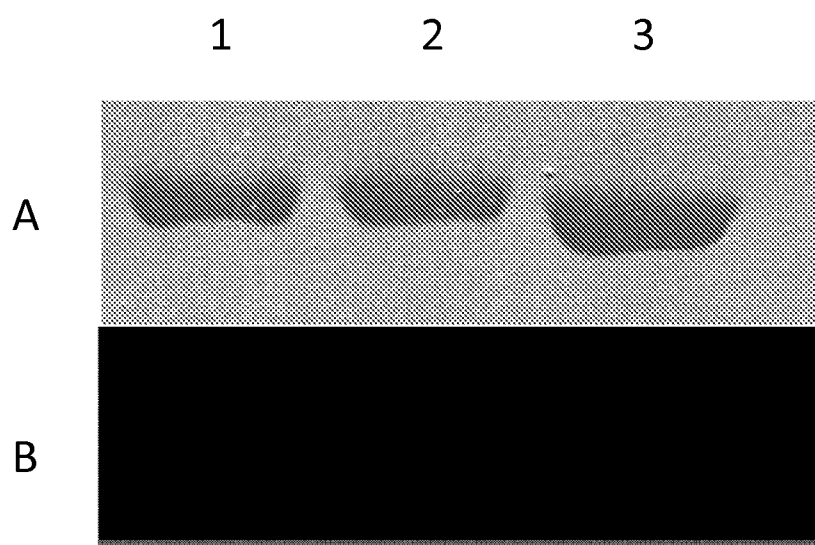


Figure 26

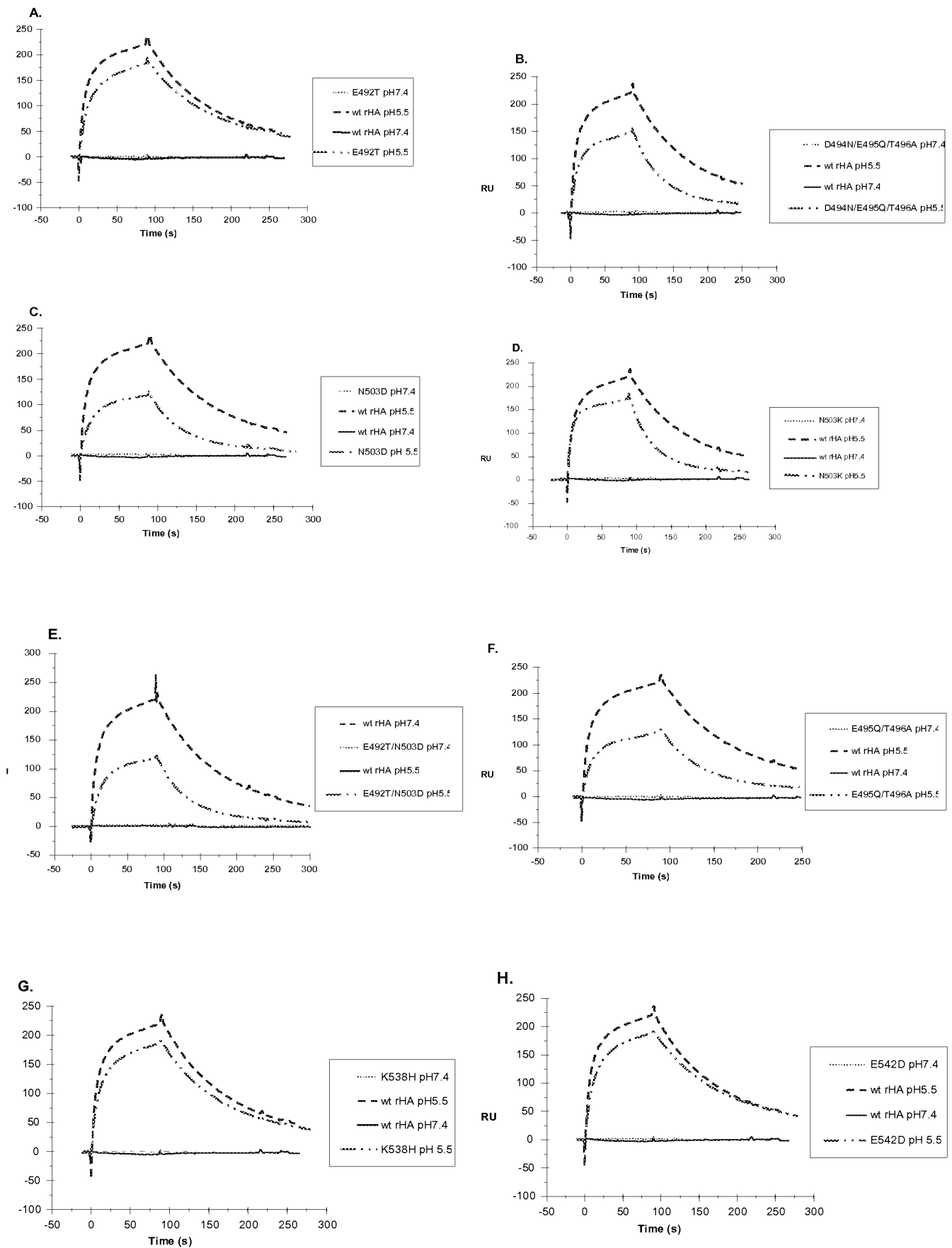


Figure 27

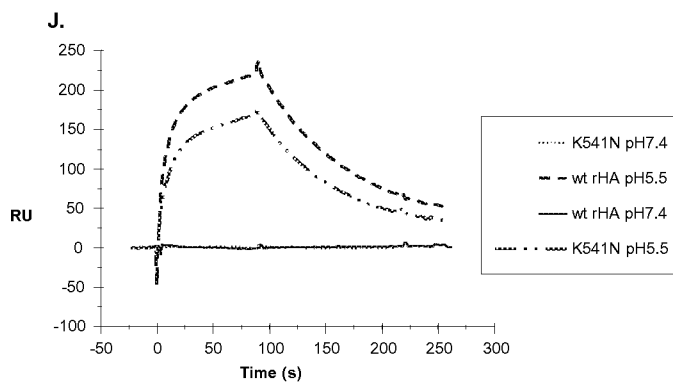
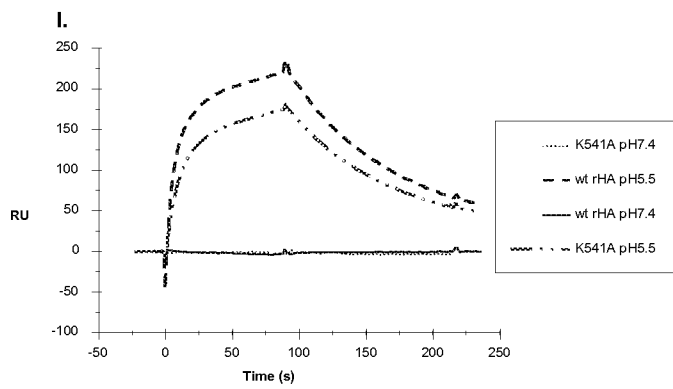


Figure 28

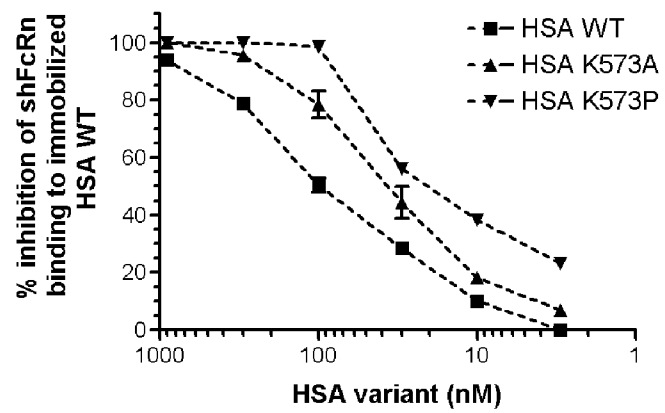


Figure 29

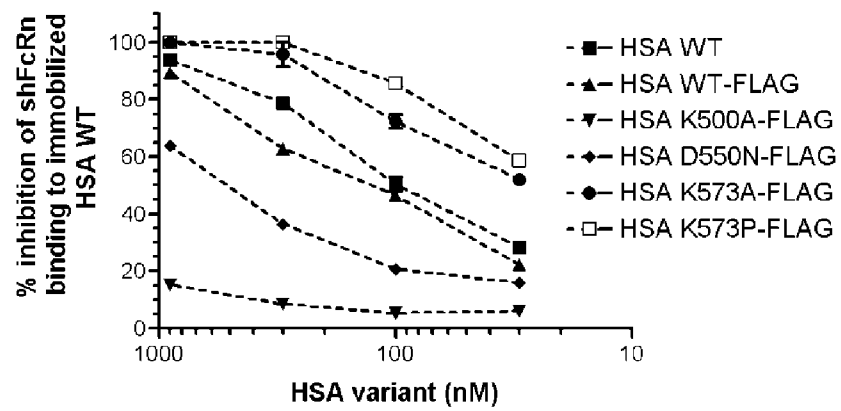


Figure 30

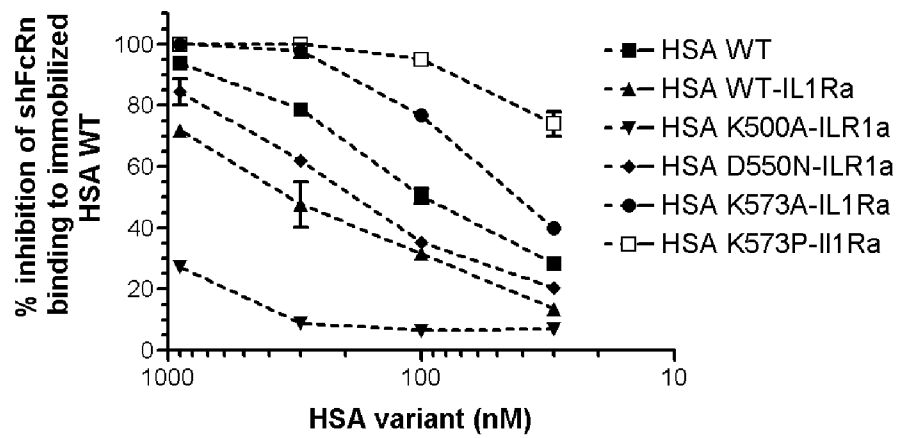


Figure 31

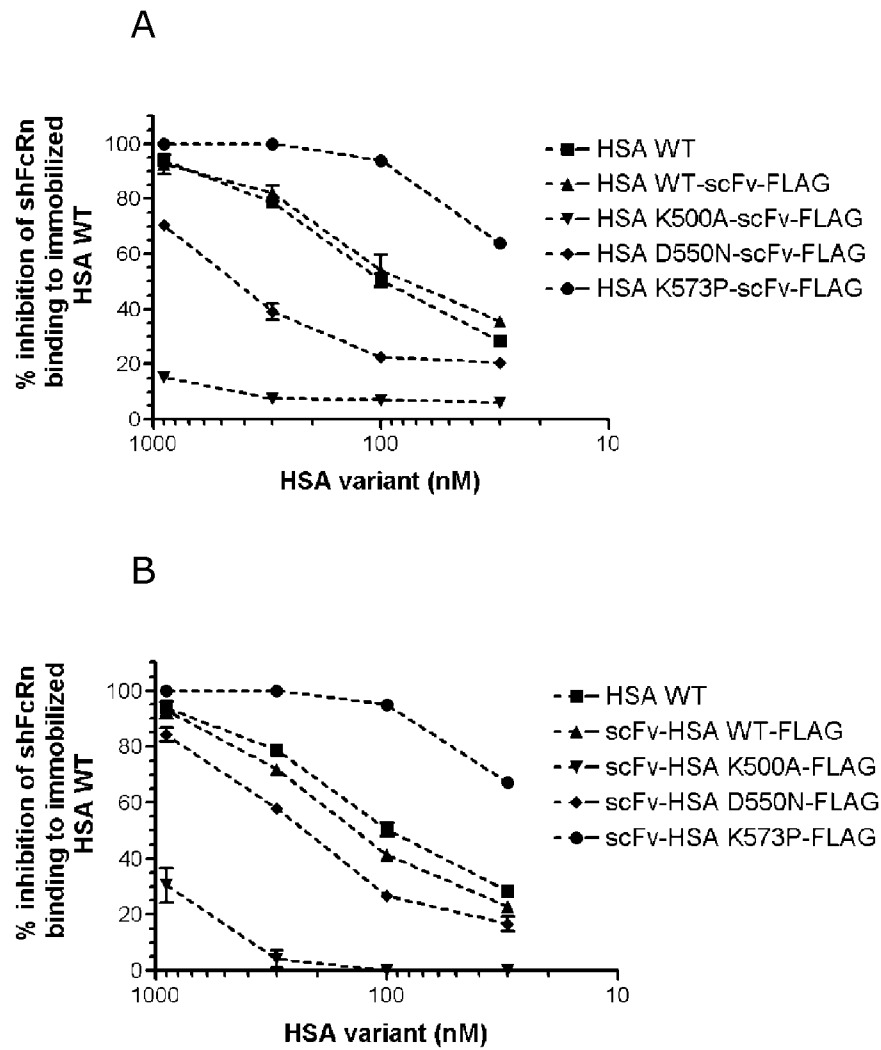


Figure 32

