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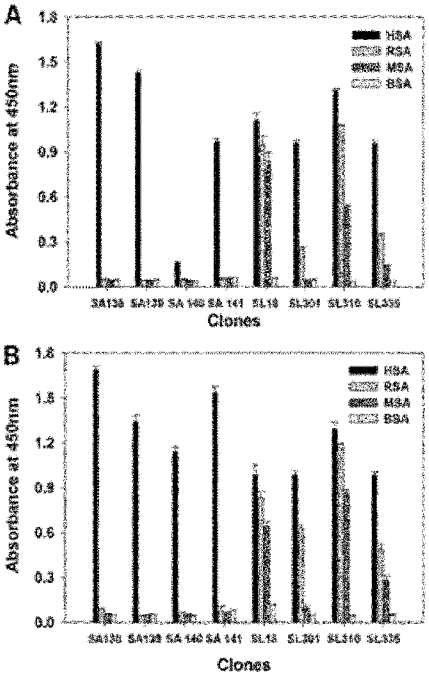
(54) Title: AN ANTI SERUM ALBUMIN FAB-EFFECTOR MOIETY FUSION CONSTRUCT, AND THE PREPARING METHOD THEREOF



(57) Abstract: The present invention relates to antigen-binding fragment(Fab) and a Fab-effector fusion protein or (poly)peptide comprising thereof. The Fab of the present invention specifically binds to serum albumin and thereby has extended *in vivo* half-life. The Fab of the present invention is characterized by not having cysteine residues that are responsible for the interchain disulfide bond in C_{H1} domain and $C_{\kappa L}$ domain as well. The Fab-effector fusion protein or (poly)peptide of the present invention can be produced in periplasm of *E. coli* with high yield, and has increased *in vivo* half-life. Further, the present invention provides *E. coli* strain which produces various kinds of Fab-effector fusion proteins or (poly)peptides, and a pharmaceutical composition comprising the fab-effector fusion proteins or (poly)peptides.

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Description

Title of Invention: AN ANTI SERUM ALBUMIN FAB-EFFECTOR MOIETY FUSION CONSTRUCT, AND THE PREPARING METHOD THEREOF

Technical Field

[1] The present invention relates to antigen-binding fragment(Fab) and a Fab-effector fusion protein comprising thereof.

[2]

Background Art

[3] Antigen-binding fragment (Fab) preparation is one of the most successful monoclonal antibody therapeutic agents. For example, Abciximab(ReoPro®), Ranibizumab(Lucentis®), and Certolizumab pegol(Cimzia ®) etc. had already been approved as drugs in many countries. Furthermore, polyclonal Fab preparations including Abciximab(ReoPro®), Ranibizumab(Lucentis®) and Certolizumab pegol (Clmzia®) are commercially available in EU.

[4] Conjugation of an exogenous effector domain may confer therapeutic effects to Fab fragments, when they form a Fab-effecter fusion format. Therefore, in fact, lots of antibody fragments in clinical development status are conjugated to an exogenous functional moiety. In such a Fab-fusion protein construct (or Fab-effector moieties construct), the antigen binding fragment may provide a target-specific delivery, and the fusion protein or (poly)peptide (effector domain) may provide therapeutic effects. Fusion domains originated from prokaryotic origin may include cytotoxins, for example, deBouganin (a de-immunized plant toxin) (see Entwistle *et al.*, (2012) *Cancer Biother Radiopharm.* 27, 582-92), staphylococcal enterotoxin (SE) (see Ilack *et al.*, (2003) *Toxicology.* 185, 161-174) or a mutant form of *Pseudomonas* exotoxin (see Choe *et al.*, (1994) *Cancer Res.* 54, 3460-3467; see Kreitman *et al.*, (1994) *Int. J. Cancer* 57, 856-864). In addition, fusion domains comprising polypeptides from eukaryotes, such as, scFv (see Lu *et al.*, (2002) *J Immunolog Meth.* 267, 213- 226) or cytokine (see Holzer *et al.*, (1996) *Cytokine.* 8, 214-221; see Sjogaard *et al.*, (1999) *Int J Oncol.* 15, 873-882), may function as therapeutics. Although radioactive isotope is chemically conjugated to Fab or $(Fab')_2$ fragment in general, cytotoxin, cytokine or enzyme is genetically fused to Fab or $(Fab')_2$. It is known that Fab molecules, unlike scFv, Fv or dsFv, can be produced with ease up to 1 - 2 g/L as a soluble form in the periplasm of *E. coli* (see Humphreys et al., *J. Immunol. Methods.* 209, 193-202; Carter et al., *Biotechnology (N Y).* 10, 163167; Venturi et al., J Mol Biol. 315, 1-8; Donzeau et al., *Methods Mol Biol.* 378, 14-31), or even in *Pseudomonas fluorescens* (see

Retallack et al., *Prot Exp Purif.* 81, 157-165). Currently, lots of commercially available biological agents such as rhGH, insulin or various types of cytokines are being produced in *E. coli* (see Graumann and Premstaller, (2006) *Biotechnol J.* 1, 164-186; Chadd and Chamow, (2001) *Curr Opin Biotechnol.* 12, 188-194). In this regard, the genetic linkage of a therapeutic domain to a Fab fragment and other therapeutic agents has great advantage in the development of a new biological medicinal agent, and the improvement of the current biological drugs efficacy as well. Further, a Fab molecule might be fused with other antibody fragments such as scFv, Fv, dsFv or dAb to prepare bi-specific or tri-specific antibody molecule (see Lu *et al.*, (2002) *J Immunolog Meth.* 267, 213-226). However, the expression of Fab-effector fusion proteins of which the effector is of eukaryotic origin in *E. coli* has been hampered because the effector domain could not be biologically functional due to inappropriate folding or the lack of glycosylation process in *E. coli.* Futhermore, the optimal fusion format to produce Fab-effector fusion proteins in *E. coli* periplasm has not yet been throughly studied. Most of serum proteins having molecular weight less than between 50 kDa and 60 kDa, such as, cytokines and growth factors, have a short half-life *in vivo*, for instance, from several minutes to several hours due to renal clearance. Thus, extending the serum half-life of therapeutic polypeptides or proteins is one of the most intensely studied areas in bio-pharmaceutical research (see Kontermann, (2012) Wiley, ISBN: 978-3-527-32849-9). For this purpose, various methods including pegylation, polysialylation, HESylation, glycosylation, or recombinant PEG analogue fused to flexible and hydrophilic amino acid chain (500 to 600 amino acids) have been developed (See Chapman, 2002; *Adv Drug Deliv Rev.* 54. 531~545; Schlapschy *et al.*, (2007) *Prot Eng Des Sel.* 20, 273~283; Contermann (2011) *Curr Op Biotechnol.* 22, 868~876; Jevsevar et al., (2012) *Methods Mol Biol.* 901, 233~246). Furthermore, the FcRn-mediated recycling mechanism has been directly or indirectly employed in order to extend *in vivo* half-life of therapeutic proteins. Among serum proteins, it is known that a human serum albumin (HSA) and an immune globulin (in particular, IgG) have exceptionally a long half-life through the FcRn-mediated recycling mechanism. In a human body, the serum half-life of albumin is 19 days and that of an IgG molecule is between one week and almost 4 weeks depending on the subclass of IgG. Thus, these two molecules have been used as fusion partners to extend half-life of therapeutic proteins and/or (poly)peptides.

[5] Recombinant hGH (~ 19kDa) prepared in cytoplasm or the periplasm of *E. coli* has been used in clinics to treat diseases caused by the lack of growth hormones in infants and adults as well, after *in vitro* folding process (see Blethen *et al.*, (1997) *J. Clin. Endocrinol. Metab.* 82, 418-420). One major inconvenience in rhGH administration is the daily injection due to the short period of half-life (< 30 minutes). To extend the serum

half-life of hGH, chemical conjugation of polyethylene glycol (see Clark *et al.*, (1996) *J. Biol. Chem.* 271, 21969-21977; Pradhananga *et al.*, 2002 *J Mol Endocrinol.* 29, 1114; Cho *et al.*, 2011; Sondergaard *et al.*, (2011) *J Clin Endocrinol Metabol.* 96, 681-688), and chemical conjugation of the modified hGH to the arm of Fab of humanized CovX-Body IgG (see Palanki *et al.*, (2013) *Bioorg. Med. Chem. Lett.* 23, 402-406) had been attempted. In addition, the elongation of the half-life of hGH in serum has been successfully achieved by the genetic fusion of human serum albumin (HSA) (Albutropin®) or the polypepeptide sequences comprising hundreds of Pro-Ala-Ser (PAS) residues (PASylation) (see Osborn *et al.*, 2002 *Eur J Pharmacol.* 456, 149-158; Anderson *et al.*, (2011) *J Biol Chem.* 286, 5234-5241; Sleep *et al.*, (2013) *Biochimica et Biophysica Acta.* 1830, 5526-5534; Schlapschy *et al.*, (2013) *Protein Eng Des Sel.* 26, 489~501). The most well studied one in this category is VRS-317, a rGH genetically linked with XTEN amino acid sequences to the N-terminus and the C-terminus, which allows one month dosage regimen (see Schellenberger et al., (2007) *Nat Biotech.* 27, 1186-1190; Cleland et al., (2012) *J Pharm Sci.* 101, 2744-2754; Yuen et al., (2013) *J Clin Endocrinol Metab.* 98, 2595-2603). Also, hGH is associated with vascular disease(See *Thomas J Merimee et. al.*, (1973), *Diabetes*, 22, 813-819) and CRETZFELDT-JAKOB disease(See *John Powell-Jackson et al.*, 1985, *Lancet*, 2, 244-246). In addition, IFN-γ accelerates Graft-Versus-Host-Disease (See *Bruce R.Blazar et.al.*, 2003, *The Journal of Immunology*, 171, 1272-1277) and IFN-α is related with autoimmune disease(See *A Imagawa et al.*, 1995, *The Journal of clinical endocrinology & metabolism*, 80, 922-926). Also, GSCF is related with auto-immune disease (See Anke Franzke et al., 2003, *Blood*, 102, 734-739) and HCV associated with liver disease (See *Van Thiel DH et al.*, 1995, *Hepato-gastroenterology*, 42, 907-912).

[6] A Fab-fusion protein (or polypeptide) has a great potential as a therapeutic agent for treating chronical diseases which require a large dose of drugs for a long period of time, in particular, especially when the Fab-fusion protein can be produced in microorganism expression system with low cost. Despite such possible potent advantages of employing a Fab, however, there has been no attempt applying an anti-serum albumin (SA) Fab antibody in the development of a protein or a (poly)peptide drug having extended *in vivo* half-life. Herein, the inventors have completed the present invention by constructing a novel anti-serum albumin (SA) Fab-effector protein (or (poly)peptide) fusion constructs, and confirming the high-yield production of funtional fusion constructs in the periplasm of *E. coli.*

[7]

Disclosure of Invention

Technical Problem

[8] The technical problem to be solved by the present invention is to provide a novel antigen binding fragment (Fab) having extended *in vivo* serum half-life.

[9] Another technical problem to be solved by the present invention is to provide the Fab-effector moieties fusion construct which enables the optimal production in the periplasm of host cell.

[10] Yet another technical problem to be solved by the present invention is to provide an expression vector and an host cell to produce the Fab-effector constructs in soluble form with high yield.

[11] Yet another technical problem to be solved by the present invention is to provide a pharmaceutical composition comprising the fusion constructs above.

[12]

Solution to Problem

[13] In order to solve the problems above, the present invention provides an optimal Fab-effector fusion construct (or format) for the periplasmic expression in *E. coli*, wherein the Fab has a heavy chain variable domain binding to heavy chain constant 1 domain (C_{H1}), and has a light chain variable domain binding to light chain constant domain (C_L).

[14] In one embodiment of the present invention, a human anti-SA Fab was chosen as an antibody fragment, considering that the fusion of various therapeutic proteins to albumin or to albumin-binding moieties, such as small peptides or domain antibodies (dAb) has been shown to extend the half-lives of therapeutic proteins through the FcRn-mediated recycling mechanism (see Dennis *et al.*, (2002) *Biochimica et Biophysica Acta.* 1830, 5526-5534; Sleep *et al.*, (2013) *Biochimica et Biophysica Acta.* 1830, 5526-5534; Nguyen *et al.*, (2006) *Protein Eng Des Sel.* 19, 291-297; Kontermann, (2011) *Curr Op Biotechnol.* 22, 868~876). According to the prior studies, a Fab fragment has an eleimination half-life of 16-20h in humans(See Ujhelyi and Robert, (1995) *Clin Pharmacokinet.* 28, 483493) and ~ 3 h in rats after intravenous administration (see Nguyen *et al.*, 2006 *Protein Eng Des Sel.* 19, 291 ~ 297). Surprisingly, the half-life of Fab (SL335) in this invention is 37 h in rats which is approximately 12-fold longer than conventional human Fabs, and thus it is reasonable to assume that SL335 might have a half-life of at least 160 200 h (6 - 8 days) in humans. In the meantime, two Vk domains, dAbr3 and dAbr16 possessing 13 nM and 1 mM of binding affinities to RSA, respectively, had been known to have the $t_{1/2}$ values of 53 h (dAbr3) and 43 h (dAbr16) in rats (see Holt *et al.*, (2008) *Protein Eng Des Sel.* 21, 283 - 288). Moreover, the $t_{1/2b}$ of Ab Fab4D5-H with a 92 nM affinity to RSA was 26.9 h (see Nguyen *et al.*, 2006). Therefore, it is implied that the *in vivo* functionality of

SL335 is comparable to that of previously reported dAbs and peptides specific for SA. It is noteworthy that the V_H and the V_L of SL335 shared only a 65 - 67% amino acid homology at the full sequence level, and a ~ 50% amino acid homology at the complementarity determining region (CDR) level with the previously reported albumin-specific dAbs (data not shown). Specifically, the Fab specific for serum albumin (SA) in an embodiment of the present invention comprises a heavy chain variable domain which has an amino acid sequence selected from the group consisting of SEQ ID NO.1 (SA138 VH: QVQLLQSGAE VKKPGASVKV SCKASGYTFT SYGISWVRQA PGQGLEWVGW INTYSGGTKYA QKFQGRVTMT RDTSISTVYM ELS-GLKSDDTAVY YCARLGHCQRGICSDAL DTWGQGTLVT VSS), SEQ ID NO.2 (SA139 VH: EVQLLQSGAE VKEPGASVKV SCKASGYTFS SYGISWVRQA PGQGLEWVGR INTYNGNTGYA QRLQGRVTMT TDTSTSIAYM EVRSLRSDDTAVY YCARLGHCQRGICSDAL DTWGQGTMVT VSS), SEQ ID NO.3 (SA140 VH: QVQLVQSGGG VVQTGGSLRL SCAASGFTFR NY-GIHWVRQA PGKGLEWVAS ISYDGSNKYYA DSVKGRFTIS RDNSRNTVHV QMDSLRGGDTAVY YCARDVHYYGSGSYYNAF DIWGQGTLVT VSS), SEQ ID NO.4 (SA141 VH: QVQLVQSGGG LVQPGGSLRL SCAASGFTFS SYAMSWVRQA PGKGLEWLSV ISHDGGFQYYA DSVKGRFTVS RDNSKNTLYL QMNSLRAEDTAVY YCARAGWLRQYGM DVWGQGTLVT VSS), SEQ ID NO.5 (SL18 VH: EVQLVQSGTE VKKPGESLKI SCKISGYSFT AY-WIAWVRQM PGKGLEWMGM IWPPDADARYS PSFQGQVTFS VDKSISTAYL QWHSLKTSDTAVY YCARLYSGSY SPWGQGTLVT VSS) and SEQ ID NO.6(SL301, SL310 and SL335 VH: QVQLVQSGGG PVKPGGSLRL SCAASGFMFR AYSMNWVRQA PGKGLEWVSS ISSSGRYIHYA DSVKGRFTIS RDNAKNSLYL QMNSLRAEDTAVY YCARETVMAGKAL DYWGQGTLVT VSS); and a light chain variable domain which has an amino acid sequence selected from the group consisting of SEQ ID NO.7 (SA130: ELVLTQSPSS LSASVGDRVT ITCRASQSIS RYLNWYQQKP GKAPKLLIYG ASRLESGVPS RFSGSGSGTD FTLTISSLQP EDFATYYCQQ SDSVPVTFGQ GTRLEIKR), SEQ ID NO.8 (SA139 VL: DIVLTQSPSS LSASVGDRVT ITCRASQSIS SYLNWYQQKP GKAPKLLIYA ASSLQSGVPS RFSGSGSGTD FTLTISSLQP EDFATYYCQQ SYSTPPYTFGQ GTKLEIKR), SEQ ID NO.9 (SL18 VL: ELVLTQSPGT LSLSPGERAT LSCRASQSIF NYVAWYQQKP GQAPRLLIYD ASNRATGIPA RFSGSGSGTD FTLTISSLEP EDFAVYYCQQ RSKWPPTWTFGQ GTRVDIKR), SEQ ID NO.10 (SL301 VL: ELVLTQSPGT LSLSPGERAT LSCRASETVSS RQLAWYQQKP GQAPRLLIYG ASSRATGIPD RFSGSGSGTD FTLTISRLEP EDSAVFYCQQ YGSSPRTFGG GTKLEIKR), SEQ ID NO.11 (SL310 VL: ELVLTQSPGT LSLSPGERAT LSCRASQSVSS SSLAWYQQKP GQAPRLLIYG ASSRATGIPD

RFSGSGSGTD FTLTISSLQP EDAATYYCQK YSSYPLTFGQ GTKLEIKR) and SEQ ID NO.12 (SL335 VL: ELVLTQSPGT LSLSPGETAT LSCRASQSVG SNLAWYQQKP GQAPRLLIYG ASTGATGVPA RFSGSRSGTD FTLTITSLQP EDFATYYCQQ YYSFLAKTFGQ GTQLEIKR). And the V_H domain of the Fab above is bound to the heavy chain constant 1 domain (C_{H1} domain), and VL domain of the Fab is bound to light chain constant domain ($C_{\kappa L}$ domain). Furthermore, the Fab specific for serum albumin (SA) of the present invention comprises the amino acid sequences of SEQ ID NO. 13 (CDR1)(AYSMN), 14 (CDR2) (SISSSGRYIHYADSVKG) and 15 (CDR3) (ETVMAGKALDY) in the VH region of SL335, and the amino acid sequence of SEQ ID NOS. 16 (CDR1)(RASQSVGSNLA), 17 (CDR2)(GASTGAT) and 18 (CDR3)(QQYYSFLAKT) in the VL region of SL335.

[15] In one embodiment, the amino acid of cysteine of C_{H1} domain and $C_{\kappa L}$ domain of the Fab might be deleted or substituted with serine residues. In particular, as for the SL335 above, the amino acid of cysteine of C_{H1} domain is the 233th amino acid starting from the N-terminus of the C_{H1} domain , and the cysteine of $C_{\kappa L}$ domain is the 214th amino acid starting from the N-terminus of the $C_{\kappa L}$ domain are substituted with serine residues. To avoid confusion, the H chains and the L chains that compose the Fab were named as follow: 1) Hcys: the H chain with cysteine at the 233th position, 2) Lcys: the L chain with cysteine at the 214th position, 3) Hser: the H chain with serine at the 233th position, and 4) Lser: the L chain with serine at the 214th position.

[16] In another embodiment of the present invention, the Fab-effector fusion is constructed by linking the effector domain to the N- or C-terminus of either the Fd or light chain of a Fab molecule through genetic fusion. Since the folding and heterodimerization mechanisms of recombinant proteins in the periplasmic environment of *E. coli* are rather complicated and largely unknown, it is unpredictable which Fab-effector fusion format is optimal for a functional expression.

[17] Further, in another embodiment, a fusion construct of an antigen binding fragment(Fab) and effector domain (a bioactive effector moiety) is provided, wherein the amino acid of Cysteine of C_{H1} domain and the amino acid of Cysteine of $C_{\kappa L}$ domain of the Fab are deleted or substituted with serine residues; and wherein the bioactive effector moiety is a protein or a (poly)peptide; and wherein the Fab and the bioactive effector moiety are covalently linked by genetic fusion. The Fab and the bioactive effector moiety may be covalently linked by genetic fusion using a peptide linker of O to 20 amino acids. Among six Fab-effector fusion formats (or constructs) comprising hGH of the present invention, the results clearly demonstrated that HserG/ Lser exhibited the highest expression yield in *E. coli.* That is, in accordance with this embodiment, the removal of both Cys^{233} in the C_{H1} domain and Cys^{214} of in the C_{Lk} either by deletion or substitution with other amino acid residue improves soluble ex-

pression of SL335-fusion effector constructs in the culture supernatant. This addresses three important issues. First, the fusion of an effector moiety, for example, hGH to the C-terminus of C_{H1} is preferable to the C-terminus of C_{Lk}. Previously, Lu *et al.* had reported that the genetic linkage of the anti-Flt-1 scFv to the C-terminus of C_{H1} of the anti-KDR Fab produced a five-fold higher yield than linkage to the C-terminal of C_L domain (see Lu *et al.*, (2002) *J Immunolog Meth.* 267, 213 - 226). Although the data were not included, we inventor's western blot analysis using total *E. coli* lysates revealed that the Fd fragments of LcysG/Hcys and LserG/Hcys were almost completely degraded, resulting in no detection of the soluble form of the fusion proteins in the *E. coli* supernatant. Because V_H domains are prone to aggregate in *E. coli* (Dudgeon *et al.*, (2009) *Protein Eng Des Sel.***22**, 217 - 220), it can be speculated that the presence of an effector domain at the C-terminal end of C_L may restrain the interaction of a V_H domain to a V_L domain and a C_{H1} domain to a C_L domain, leading to rapid aggregation and degradation of Fd fragments. Comparing the soluble expression yields between LserG/Hcys and LserG/Hser, the presence of Cys^{233} in the C_{H1} domain seemed to accelerate this process probably due to aberrant disulfide bond formations. After removing Cys^{233} in the C_{H1} domain, the presence of an effector domain at the end of a C_{H1} might have a beneficial effect on reducing V_H domain aggregation by the partial blocking of hydrophobic surfaces on the V_H domain before V_H-V_L pairing. Second, the presence of the Cys^{214} of C_{Lk} further aggravates the soluble production of SL335-hGH fusion protein in an additive manner. Lower yield of HserG/Lcys than that of HserG/Lser could be explained by the tendency of L chains to form homodimers, known as Bence Jones proteins (see Kirsh *et al.*, (2005) *J Immunol Methods.* 301, 173 - 185), in which the Cys^{214} of C_{Lk} may act on stabilization of homodimers, or is involved in forming aberrant disulfide bond(s) with other cysteine residues in the fusion protein. It has been also known that the disulfide bonds between the C-termini of C_{H1} and C_L in a Fab are highly mobile with a considerable degree of flexibility (see Rothlisberger *et al.*, (2005) *J. Mol. Biol.* 347, 773 - 789; Humphreys *et al.*, (2007) *Protein Eng Des Sel.* 20, 227 - 234). In this regard, the present invention provides an antigen-binding fragment (Fab) without the Cys^{233} of heavy chain constant domain 1 (C_{H1}) and the Cys^{214} of light chain constant domain (C_{Lk}). Likewise, HerGF/Lser and HserIFNb/Lser exhibited the highest expression yield in *E. Coli.* In the fusion construct of the present invention, the molar ratio of the bioactive polypeptide (or protein) to the Fab is between 1:1 and 10:1, preferably between 1:1 and 4:1. Third, not only the expression yield but the accessibility of the anti-hGH antibody to the hGH domain is also restrained at some extend by the presence of these two C-terminal cysteine residues in SL335. This could be important for the therapeutic function of an effector domain in a Fab-effector fusion if the interaction between an effector domain and its ligand is also

interfered. We inventors demonstrated that the utilization of $Fab_{\Delta ds}$ as a fusion partner is beneficial not just for hGH, because other effectors such as G-CSF and IFN-b produced identical conclusions.

[18] In another aspect of the present invention, an expression vector and the mutant *E. Coli* SUPEX5 strain (KCTC 12657BP) as a host cell are provided to solve the technical problems. This strain was created by random chemical mutagenesis of MC1061 *E. coli* strain which was chosen because it derives from *E. coli* K12 stain, one of major host strain for producing commercial bio-pharmaceuticals. By comparing with the parental MC1061 strain, utilization of the mutant SUPEX5 *E. coli* strain as an expression host further implemented the beneficial effect on the production of HserG/ Lser. Not only for SL335-hGH fusion, but the combination of Fab_{ds} and SUPEX5 *E. coli* strain is also advantageous in soluble expression of a Fab-effector fusion protein in general, which was clearly demonstrated by the results obtained from SL335-GCSF fusions ($SL335_{wt}$-GCSF *vs.* $SL335_{\Delta ds}$-GCSF), SL335-IFNβ fusions ($SL335_{wt}$-IFNb *vs.* $SL335_{\Delta ds}$-IFNβ) EGL4-hGH fusions ($EGL4_{wt}$-hGH *vs.* $EGL4_{\Delta ds}$-hGH), and 1β28-hGH fusions ($1\beta28_{wt}$-hGH *vs.* $1\beta28_{\Delta ds}$-hGH). Therefore, the results strongly support that the utilization of $Fab_{\Delta ds}$, the mutant form of Fab without the Cys^{233} of C_{H1} and the Cys^{214} of C_{LK}, is beneficial over a conventional Fab in the soluble expression of Fab-effector fusion proteins at least in SUPEX5 *E. coli* strain. The coexpression of chaperone proteins or disulfide isomerase (FkpA, SurA, Skp, Sec A, Sec B, DsbA or Dsb C) would improve the soluble and functional expression of $SL335_{wt}$-GCSF or even $SL335_{\Delta ds}$-GCSF, since these fusions are known to increase the periplasmic production yield of soluble Fab fragments in *E. coli* (see Schlapschy *et al.*, (2006) *Escherichia coli. Protein Eng Des Sel.***19**, 385 - 390). We inventors believe the utilization of Fab_{ds} can be beneficial especially when chaperones and the catalytic machinery for disulfide formation in the endoplasmic reticulum are overloaded because of the high expression of Fab-effector fusion proteins in host cells.

[19] In one embodiment of the present invention, $SL335_{\Delta ds}$-hGH was produced at approximately 10 mg/L concentration using a culture flask, which is higher yield than the previous reports, despite of a 4-fold increase in molecular size in the present invention. According to the prior reports, studies on soluble expression of rhGH in the periplasm of *E. coli* showed that the yield was 0.64 - 2.57 mg/L for pelB-hGH and 0.32 - 2.29 mg/L for ompA-hGH (see Sockolosky and Szoka, (2013) *Protein Exp Purif.* 87, 129 - 135), while the yields of rhGH were largely dependent on the promoters and host *E. coli* strains that were used (see Soares *et al.*, (2003) *Protein Engineering.* 16, 1131 - 1138). Through a simple medium optimization, we inventors routinely obtained the yield of ~ 50 mg/L in the culture supernatant using a culture flask that allows the cell density of OD_{600nm}= ~ 10 - 11 (manuscript in preparation), which can be further

improved enough for an industrial scale through the refined adjustment of medium compositions and a fed-batch culture system.

[20] In another aspect of the present invention, $SL335_{ds}$-effector proteins shows increased affinity to HSA. In one embodiment, $SL335_{ds}$-hGH showed a five to nine-fold increase in response to HSA(Human Serum Albumin) and a 1.3 to 4-fold decrease in response to RSA(Rat Serum Albumin) depending on the pH condition compared to those of parent SL335. Genetic linking of an antibody fragment and an effector domain would affect an antigen-binding affinity of the antibody fragment, and the changes in affinity can be varied at large extent depending on the nature of an antibody fragment, an effector domain and how to link these two functional moieties. It is not clear whether these differences in affinity result from the absence of the interchain disulfide bond or the presence of the hGH fusion domain. Nonetheless, the effect of hGH fusion on the binding affinities of $SL335_{\Delta ds}$ to the antigens seems negligible compared to that of IFN-a2b-DOM7 h-14, whose affinities to human, mouse and rat SA decreased 7.7, 22.3 and 15.8-fold relative to the parent DOM7 h-14 (see Walker *et al.*, (2010) *Protein Eng Des Sel.* 23, 271 - 278). Therefore, Fab might have an advantage over domain Ab in maintaining the affinity and effector folding because the C_{H1} and C_L domains provide space for reducing steric hindrance between an antigen-binding region and an effector domain that binds to the respective ligands.

[21] In another embodiment of the present invention, $SL335_{\Delta ds}$-hGH profoundly extended the serum half-life in that its $t_{1/2}$ (16.6 h in intravenous administration) was similar to that of PEG5-hGH (250 kDa) (see Clark *et al.*, 1996). Interestingly, the $t_{1/2}$ of $SL335_{\Delta ds}$ -hGH was 5.6-fold longer than that of Albutropin® ($t_{1/2}$ = 2.96 h), and the difference in the $t_{1/2}$ between $SL335_{\Delta ds}$-hGH and Albutropin® was further extended in the S.C. (subcutaneous) administration up to 16-fold (97.2 h *vs.* 5.93 h) (see Osborn *et al.*, 2002), although these comparisons are circumstantial unless the experiments are performed under the same settings. Similarly, the $t_{1/2}$ of IFN-a2b-DOM7 h-14 was also approximately 1.5 times longer than that of HSA-IFN-a2b (see Walker *et al.*, 2010). Therefore, it seems likely that the fusion of an albumin-binder provides a longer half-life than the fusion with albumin, and the underlying mechanisms are yet to be determined. It is noteworthy that the serum $t_{1/2}$ of $SL335_{\Delta ds}$-hGH in I.V. administration was similar to that of VRS-317 ($t_{1/2}$ = 15 h) (Cleland *et al.*, (2012) *J Pharm Sci.* 101, 27442754). This may suggest that longer than once-weekly or even once a month dosing could be possible for $SL335_{\Delta ds}$-hGH (termed SAFAtropin®).

[22] In another embodiment of the present invention, the pharmacodynamic effects of $SL335_{\Delta ds}$-hGH seemed far superior to those of Albutropin®, and 7-fold more potent than Growtropin® at molar basis considering the once-weekly dosage regimen. Unfortunately, we had to discontinue a 2-week pharmacodynamic study at Day 11 because

some of the hypophysectormized rats, especially those belonging to the Excipient Only group, died early. It seemed likely that the animals were severely stressed by the long-distance transportation from Japan to South Korea after surgery during August, which manifested by 5% weight loss of those belonging to the Excipient Only group and the bigger standard deviation values than we anticipated. Nonetheless, it seems clear that $SL335_{\Delta ds}$-hGH has a huge potential being developed as a long-acting hGH, and, therefore, we referred it to SAFAtropin® now on.

[23] In another embodiment of the present invention, the bioactive polypeptide fused to the Fab above is anyone selected from the group consisting of hormone, cytokine, enzyme, antibody, growth factor, transcription factor, blood factor, vaccine, structure protein, ligand protein, and receptor.

[24] In yet another embodiment of the present invention, the bioactive polypeptide is anyone selected from the group consisting of human growth hormone, growth hormone releasing hormone (GHRH), growth hormone releasing peptide, interferons, interferon receptors, colony stimulating factors (CSFs), glucagon-like peptides, G-protein-coupled receptor, interleukins, interleukin receptors, enzymes, interleukin binding proteins, cytokine binding proteins, macrophage activating factor, macrophage peptide, B cell factor, T cell factor, protein A, allergy inhibitor, cell necrosis glycoproteins, immunotoxin, lymphotoxin, tumor necrosis factor, tumor suppressors, metastasis growth factor, alpha-1 antitrypsin, albumin, alpha-lactalbumin, apolipoprotein-E, erythropoietin, highly glycosylated erythropoietin, angiopoietins, hemoglobin, thrombin, thrombin receptor activating peptide, thrombomodulin, factor VII, factor VIIa, factor VIII, factor IX, factor XIII, plasminogen activating factor, fibrin-binding peptide, urokinase, streptokinase, hirudin, protein C, C-reactive protein, renin inhibitor, collagenase inhibitor, superoxide dismutase, leptin, platelet-derived growth factor, epithelial growth factor, epidermal growth factor, angiostatin, angiotensin, bone growth factor, bone stimulating protein, calcitonin, insulin, atriopeptin, cartilage inducing factor, elcatonin, connective tissue activating factor, tissue factor pathway inhibitor, follicle stimulating hormone, luteinizing hormone, luteinizing hormone releasing hormone, nerve growth factors, parathyroid hormone, relaxin, secretin, somatomedin, insulin-like growth factor, adrenocortical hormone, glucagon, cholecystokinin, pancreatic polypeptide, gastrin releasing peptide, corticotropin releasing factor, thyroid stimulating hormone, autotaxin, lactoferrin, myostatin, receptors, receptor antagonists, cell surface antigens, virus derived vaccine antigens, monoclonal antibodies, polyclonal antibodies, and antibody fragments.

[25] In another aspect of the present invention, a pharmaceutical composition is provided, wherein the composition comprises the Fab-effector moieties fusion constructs of the present invention and pharmaceutically acceptable excipient, and has increased *in vivo*

sustainability. The pharmaceutical composition of the president invention can be administered into a body through various ways including oral, transcutaneous, subcutaneous, intravenous, or intramuscular administration, and more preferably can be administered as an injection type preparation. Further, the pharmaceutical composition of the present invention can be formulated using the method well known to the skilled in the art to provide rapid, sustained or delayed release of the active ingredient following the administration thereof. The formulations may be in the form of a tablet, pill, powder, sachet, elixir, suspension, emulsion, solution, syrup, aerosol, soft and hard gelatin capsule, sterile injectable solution, sterile packaged powder and the like. Examples of suitable carriers, excipients, and diluents are lactose, dextrose, sucrose, mannitol, xylitol, erythritol, maltitol, starches, gum acacia, alginates, gelatin, calcium phosphate, calcium silicate, cellulose, methyl cellulose, microcrystalline cellulose, polyvinyl pyrrolidone, water, methylhydroxybenzoates, propylhydroxybenzoates, talc, magnesium stearate and mineral oil. Further, the formulations may additionally include fillers, anti-agglutinating agents, lubricating agents, wetting agents, favoring agents, emulsifiers, preservatives and the like.

[26] It should be be understood that the amount of the fusion protein or polypeptide actually administered ought to be determined in light of various relevant factors including the condition to be treated, the selected route of administration, the age, sex and body weight of the individual patient, and the severity of the patients symptom; and the type of bioactive polypeptide of active ingredient. Since the fusion protein of the present invention has very excellent sustainability in blood, the number and frequency of administration of the peptide preparations comprising the fusion protein of the present invention can be reduced significantly.

[27] As used herein, the singular forms "a," "an," and "the" are intended to include the plural forms as well, unless the context clearly indicates otherwise. Furthermore, to the extent that the terms "including," "includes," "having," "has," "with," "such as," or variants thereof, are used in either the specification and/or the claims, such terms are not limiting and are intended to be inclusive in a manner similar to the term "comprising".

[28] In the present invention, the "bioactive polypeptide or protein" is the (poly)peptide or protein representing useful biological activity when it is administered into a mammal including human.

[29] In the present invention, the "Fab-effector moietie(s) fusion construct(or format)" is the construct wherein a bioactive (poly)peptide or protein covalently bonded to the Fab. Further, "Fag-effector moietie(s) fusion construct (or format)" is understood to include Fab-fusion protein, Fab-fusion (poly)peptide, fusion constructs, and fusion formats.

[30] In this regard, the present invention is described in detail in examples. It should be noted that the description of the examples does not limit the scope of the invention as described in the preceding disclosure.

[31]

Advantageous Effects of Invention

[32] In the present invention, an anti-Serum Albumin $Fab_{\Delta ds}$-Associated (SAFA) technology is provided as a novel platform technology for developing long-acting bio-therapeutics. In this regard, the present invention has advantages over other conventional technologies including PEGylation, Fc-fusion, AlbudAb technology and albumin-fusions in terms of long acting in vivo, maintaining the conformation of an effector domain, binding affinities, and simple production and procedures with low costs.

[33]

Brief Description of Drawings

[34] Figure 1 shows the results of monoclonal phage ELISA to determine the binding specificity of anti-SA Fab phage antibodies.

[35] Figure 2 shows the determination of the antigen-binding specificity of the human Fab clones by ELISA.

[36] Figure 3 represents *in vivo* pharmacokinetics of SL335.

[37] Figure 4 is a diagram depicting six SL335-hGH fusion formats constructed in this study.

[38] Figure 5 shows the results of ELISA to determine the yields and the binding reactivity of soluble SL335-hGH fusions in *E. coli* culture supernatant. The binding signals were visualized using TMB substrate, and the absorbance at 450 nm was measured using an ELISA reader. The data represent the average ± SD of three experiments.

[39] Figure 6 represents the ELISA to determine host *E. coli*- and temperature-dependent expression of SL335 and SL335-hGH variants(20℃, A; 25℃, B; or 30℃, C).

[40] Figure 7 represents the ELISA to to determine the yields of soluble SL335-GCSF and SL335-IFNβ fusion constructs in the *E. coli* culture supernatant.

[41] Figure 8 represents the ELISA to determine the yields of soluble EGL4-hGH (A), and 1β28-hGH fusions (B) in *E. coli* culture supernatant.

[42] Figure 9 represents the Analyses of $SL335_{wt}$-hGH and $SL335_{ds}$-hGH by SDS-PAGE and western blot.

[43] Figure 10 represents the analyses of HcycG/Lcys and HserG/Lser by Chip-based capillary electrophoresis.

[44] Figure 11 represents the analysis of HcycG/Lcys and HserG/Lser by MALDI-TOF

mass spectrometry.

[45] Figure 12 represents the purification of HserG/Lser *via* gel filtration using FPLC.

[46] Figure 13 shows the determination of the *in vitro* hGH bioactivity of $SL335_{ds}$-hGH by the Nb2-11 cell proliferation assay.

[47] Figure 14 shows the Determination of serum stability of $SL335_{ds}$-hGH by ELISA and *in vitro* Nb2-11 cell proliferation assay.

[48] Figure 15 is the pharmacokinetic analysis of Growtropin or $SL335_{ds}$-hGH in rats.

[49] Figure 16 shows the dose-dependent weight gain in hypophysectomized rats treated with Growtropin® or $SL335_{\Delta ds}$-hGH. N= 3 rats per treatment group, one daily weight measurement per rat.

[50] Figure 17 shows the dose-dependent increase in tibia length with treated Growtropin® or $SL335_{\Delta ds}$-hGH. N=3-4 rats per treatment group, one tibia measurement per rat.

[51] Figure 18 depicts the pHEKA vector of the present invention.

[52] Figure 19 shows the nucleic acid sequence of the pHEKA vector of the present invention.

[53] Figure 20 shows the Deduced amino acid sequence of the VH and the VLgenes utilized by the anti-SA Fab clones of the present invention.

[54] Figure 21 shows the DNA sequence of the VH(A)and the VLgenes(B)utilized by the anti-SA Fab clones of the present invention.

[55] Fig. 22 shows the sequence informaion of the Fab-effector fusion constructs of the present invention. The linker and the effector domains were underlined and CDRs were written in bold.

[56]

Mode for the Invention

[57] **1. Materials and Analysis**

[58] *1-(1) Cloning and Strains*

[59] All of the DNA cloning experiments were performed according to standard procedure (See Sambrook *et al.*, (1989) Molecular cloning: A laboratory manula, 2nd ed., (New Youk, USA: Cold Spring Harbor Laboratory Press)). The oligonucleotides of sequencing grade and the codon-optimized genes for constructing SL335-effector fusion constructs were synthesized by Bioneer, Daejeon, South Korea. PCR amplification was performed using Pyrobest or Ex-*Taq* DNA polymerase (Takara, tsu, Japan) under the condition of 25 cycles at 94℃ for 1 min, 58℃ for 1 min and 72℃ for 1 min, followed by 72℃ for 10 min unless otherwise noted. The restriction endonucleases, shrimp alkaline phosphatase (SIP) and T4 DNA ligase were also purchased from Takara. The *E. coli* MC1061 strain [*ara*D139 Del(*ara*A-*leu*)7697 Del(

lac)X74 *gal*K16 *gal*E15(GalS) lambda- e14- *mcr*A0 *rel*A1 *rps*L150(*str*R) *spo*T1 *mcr* B1 *hsd*R2] (ATCC, Manassas, USA) was used for cloning and the *E. coli* SUPEX5 strain was used for recombinant protein expression. The *E. coli* TG1 strain {F' [*tra*D36 *pro*AB^{+}*lac*I^{q}*lac*ZΔM15]*sup*E thi-1 Δ(*lac-pro*AB) Δ(*mcr*B-*hsd*SM)5,($r_K^-m_K^-$)}(Agilent Technologies, Palo Alto, USA) was used for recombinant phage preparations.

[60]

[61] *1-(2) Biopanning of the HuDVFab-8L antibody library*

[62] An enrichment of recombinant phages bound to target antigens was performed as previously described (see Joo *et al.*, (2008) *J. Immunol. Methods*. 333, 24-37; Hur *et al* ., (2010) *Immunol Lett.* 132, 24-30). Briefly, tosylated magnetic beads conjugated with human, rat or mouse serum albumin (HSA, RSA or MSA, respectively) (Sigma-Aldrich, St. Louis, MO, USA) were mixed with 10^{10} phages from the HuDVFab-8L antibody library (AprilBio, Chuncheon, South Korea) for 4 h at 4℃, and washed three times with phosphate-buffered saline containing 0.02% Tween (PBST). The phage antibodies that were bound to the beads were eluted with elution buffer (0.1 M glycine, pH 2). Fresh TG1 cells carrying the corresponding light (L) (V_L+C_{Lk}) chains were infected with eluted phages, and grown in 2 YT medium containing 25 μg/ml ampicillin, 10 μg/ml carbenicillin and 10 μg/ml tetracycline (2 × YT /ACT). The recombinant phages were then amplified using Ex-12 helper phage (AprilBio) for subsequent panning. After the final panning, a monoclonal phage ELISA was performed to identify the positive clones. The Fd (V_H+C_{H1}) genes from the positive clones were subcloned into the pHg3A-3 vector (AprilBio, Chuncheon, South Korea), and L chain optimization was performed using 1.410^8 humannave kL chain repertoire in pLf1T-3 phagemid vector(AprilBio).

[63]

[64] *1-(3)- DNA sequencing analysis*

[65] The pHf1g3A-2 (AprilBio) phagemid and pLf1A-3 plasmid (AprilBio) were isolated from *E. coli* cells producing anti-SA Fab molecules using the Wizard Plasmid Miniprep Kit (Promega, Medison, WI, USA). Two different sequencing primers (5'-gtgccgttctatagccatagcac-3' (SEQ ID NO:19) and 5'-ggcactggctggtttcgctaccgtg-3'(SEQ ID NO:20)) that were complementary to pHf1g3A-2 or pLT-2 were used to read the V_H and V_L genes, respectively. The DNA sequencing was performed by SolGent, Daejeon, South Korea.

[66]

[67] *1-(4) Construction of the pHEKA expression vector*

[68] The DNA fragment #1 containing a *Bgl* II restriction site + *trc* promoter + g10 translation enhancer-ribosome binding site (RBS) was obtained by PCR amplification from the pTrcHis-B vector (Invitrogen, Carlsbad, CA, USA) using Pyrobest DNA

polymerase and a set of the PCR primer #1 (5'-gggagatcttgaaatgagctgttgacaattaatcatccg-3' (SEQ ID NO: 21)) and #2 (5'-cctctttaatttttaataataaagttaatcgataattcc-3' (SEQ ID NO: 22)). The DNA fragment #2 containing a g10 translation enhancer + RBS + *Bam*H I + multi-cloning site (MCS) + transcription terminator was obtained by PCR amplification from the same template as above using the PCR primer #3 (5'-ggaattatcgattaactttattattaaaaattaaagaggtatatattaggatccgagctcgagttctgca-3' (SEQ ID NO: 23)) and #4 (5'-gggcactacgtgcgaaaggcccagtctttcgact-3' (SEQ ID NO: 24)). A linking PCR was performed to assemble these two DNA fragments using Ex-*Taq* DNA polymerase and a set of the PCR #1 and #4 primers. The resulting ~520 bp DNA fragment was isolated through agarose gel electrophoresis. Thereafter, the linking PCR product and the pET28a (Invitrogen) plasmid were restricted with *Bgl* II and *Dra* III and ligated together using T4 DNA ligase 2 h at RT. After transforming MC1061 electrocompetent cells with 3 ml of the ligation reaction, the *E. coli* transformants were selected on 2 YT plates containing 50 μg/ml of kanamycin (Sigma-Aldrich). For subcloning Fab genes into the pHEKA vector, the Fd (V_H+C_{H1}) chain genes were PCR amplified from the pHf1g3A-2 phagemid vector using a set of PCR primer #5 (5'-ggccgcagatctgttaattaaggaggaatttaaagaattcatgaaaaaactgctgttcgcgattccgct-3' (SEQ ID NO: 25)) and #6 (5'-gggaagcttattaacaagatttgggctcaactctcttgtcc-3' (SEQ ID NO: 26)), and the L chain genes were PCR amplified from the pLT-2 plasmid vector using a set of PCR primer #7 (5'-gggggatccatgaaaaagacagctatcgcgattgcagtg-3' (SEQ ID NO: 27)) and #8 (5'-attcctccttaattaacagatctgcggccgcactcgagattaacactctcccctgttgaagctctttgt-3' (SEQ ID NO: 28)). The resulting Fd and L chain gene fragments were assembled through linking PCR using the PCR #6 and #7 primers, and the resulting PCR product of ~1.4 kbp in size was excised from the agarose gel. Thereafter, the PCR product and the pHEKA plasmid were restricted with *Bam*H I and *Hind* III, ligated together using T4 DNA ligase for 2 h at RT, and electroporated into *E. coli* MC1061 or SUPEX5 electrocompetent cells. The PCR primers used in preparing pHEKA expression vector is shown in Table 1 below. And Fig.18 shows a diagram of pHEKA expression vector.

[69]

[70] Table 1

[Table 1]

PCR primers preparing pHEKA expression vector

Constructs	Primers	Oligonucleotide sequence
pHEKA	Primer 1	5'- gggagatcttgaaatgagctgttgacaattaatcatccg-3' (SEQ ID No:21)
	Primer 2	5' - cctctttaatttttaataataaagttaatcgataattcc-3' (SEQ ID No:22)
	Primer 3	5' - ggaattatcgattaactttattattaaaaattaaagaggtatatattaggatccgagctcgagttctgca-3' (SEQ ID No:23)
	Primer 4	5' - gggcactacgtgcgaaaggcccagtctttcgact-3' (SEQ ID No:24)
	Primer 5	5' - ggccgcagatctgttaattaaggaggaatttaaagaattcatgaaaaaact-gctgttcgcgattccgct-3' (SEQ ID No:25)
	Primer 6	5' - gggaagcttattaacaagatttgggctcaactctcttgtcc-3' (SEQ ID No:26)
	Primer 7	5' - gggggatccatgaaaaagacagctatcgcgattgcagtg-3' (SEQ ID No:27)
	Primer 8	5' - attcctccttaattaacagatctgcggccgcactcgagattaacactctccc-ctgttgaagctctttgt-3' (SEQ ID No:28)

[71]

[72] *1-(5)- Establishment of the mutant E. Coli SUPEX5 strain*

[73] Chemical mutagenesis was carried out essentially as described in previous work. Briefly, *E. coli* MC1061 cells expressing the anti-human branched chain keto acid de-hydrogenase complex-E2(BCKD-E2) scFv fused with alkaline phosphatse(AP) were grown in Luria Broth (LB) medium containing 50 μg/ml of ampicilin to an OD_{600} of ~ 0.3. The cells contained in 5 ml of culture were collected by centrifugation at 3,000 g for 10 min, washed twice with cold 0.1 M sodium citrate buffer (pH 5.5). The cells were then resuspended in 1.9 ml of the same buffer, and treated with 50 μg/ml of N-methyl-N'-nitro-N-nitrosoguanidine(MNNG) (Sigma-Adrich,St. Louis, MO, USA) at 37℃ for 15, 30 and 45 min. After MNNG treatment, the cells were mixed, washed twice and resuspended in 2 ml of LB medium. Colony lift assay with a two-membrane system was then performed as described. Briefly, LB agar plates containing 50 μg/ml ampicillin and 10 μg/ml carbenicillin were covered with the first nylon membranes (0.45 m Nytran N Nylon blotting membrane) (GE Healthcare Life Science,

Wauwatosa, WI, USA) of low protein binding capacity. The mutated bacteria were spread on the membranes at the density of a 10^6 cells/plate and grown for 8 h at 37°C. Meanwhile, the second nitrocellulose membranes (Bio-Trace™ NT Nitrocellulose Transfer Membrane) (PALL, Port Washington, NY, USA) were laid over fresh LB agar plates containing 50 μg/ml ampicillin, 10 μg/ml carbenicillin and 1 mM isopropyl--D-1-thiogalactopyranoside (IPTG) (Sigma-Aldrich). The first nylon membranes were removed from the LB agar plated and placed on top of the second membranes, followed by incubation 37°C for 5 h. After incubation, the first membrane (with colonies) was removed, placed onto fresh LB agar plates containing 50 μg/ml ampicillin and 10 μg/ml carbenicillin, and stored at 4°C for later recovery of the bacteria. The second membranes were washed three times for 10 min in fresh phosphate-buffered saline containing 0.1% v/v Tween 20 (PBS/Tween), and immersed into the nitro blue tetrazolium chloride (NBT)/5-bromo-4-chloro-3-indolyl phosphate (BCIP) substrate (Duchefa, Haarelem, Netherlands) to visualize the AP of *E. coli* colonies. The *E. coli* colonies showing a distinctive AP activity were picked from the corresponding first filters, pooled together, and the second round of mutagenesis and colony lift assay were performed. After the second round of colony lift assay, the tentative positive *E. coli* clones were selected, and grown in 10 ml 2 YT medium containing 50 μg/ml ampicillin and 10 μg/ml carbenicillin until OD_{600} reaches 0.5. IPTG was added into the culture at 0.1 mM final concentration, and the cells were grown over night at 27°C. The culture supernatant was then harvested by centrifugation at 3,300 *g* for 20 min. For preparing periplasmic extracts, the cell pellet was re-suspended in the periplasmic extraction buffer (2 stock; 200 mM Tris-HCl, 20 mM EDTA, 2 M NaCl, pH 7.4), frozen and thawed three times, and centrifuged at 10,000 *g* for 20 min at 4°C. The periplasmic extract containing soluble anti-BCKD-AP fusion was finally obtained by harvesting the supernatant. Serial dilutions of the culture supernatant and the periplasmic extract were prepared by using PBS containing 1% bovine serum albumin (BSA) (Sigma-Aldrich), and 50 ml of the culture supernatant or the periplasmic extract samples were mixed with 100 ml of a p-nitrophenyl phosphate (pNPP) substrate (Roche, South Sna Francisco, CA, USA) in a 96-well microtiter plate (SPL, South Korea). After 5 - 10 min, 25 μℓ of 3 M NaOH was added into each well the stop the reaction, and the absorbance at 415 nm was measured suing an ELISA reader (Bio-Rad, Hercules, CA, USA). Four mutant *E. coli* strains (M#5, M#7, M#54 and M#69) showing the enhanced expression of the anti-BCKD-AP fusion were grown in 2 YT medium without antibiotics at 37°C overnight. The cells were then spread onto LB agar plates at a ~ 10^3 cells/plate density, and grown at 37°C overnight. The resulting colonies were replicated onto LB agar plates with or without 50 μg/ml ampicillin. The *E. coli* colonies grown in the LB agar plates without antibiotics but failed to grow in

the LB agar plates with antibiotics were selected, and grown in 2 YT medium without antibiotics until OD_{600} reaches ~1.0. The cell stocks were prepared by adding glycerol (20% v/v), and stored at 80℃. Forbeing used for cloning, the electro competent cells were prepared from the mutant strains according to a standard protocol,and stored at 80℃. M#5, one of the mutant *E. coli* strains, was named as SUPEX5 (KCTC 12657BP), and used for expressing Fab and Fab-effector fusion proteins.

[74]

[75] *1-(6)-Enzyme-linked immunosorbent assay(ELISA)*

[76] For the monoclonal phage ELISA, the recombinant phage was obtained from positive *E. coli* clones by phage rescue, and ~ 10^8 CFU/well were added to MaxiSorb ELISA plates (Nunc, Roskilde, Denmark) that were coated with 5 ㎍/ml HSA, RSA, MSA or BSA. The phage was allowed to bind to the antigens either at pH 6 or at pH 7.4 for 1 h at 37℃. A goat anti-human kappa L Ab-conjugated with HRPO (Sigma-Aldrich) was used as a secondary antibody. The binding signals were visualized with a TMB substrate (BD Science, San Jose, CA, USA), and the absorbance at 450 nm was measured using an ELISA reader (Bio-Rad, Hercules, CA, USA). The data represent the average of three experiments standard deviation. For the conventional ELISA, the various antigens [human SA, rat SA, mouse SA, monkey SA (Alpha diagnositic Intl., San Antonio, TX, USA), canine SA (CUSABIO, Wuhan, Hubei, China). rabbit SA (Sigma-Aldrich), epidermal growth factor receptor (EGFR) (R&D systems, Minneapolis, MN, USA), epithelial cell adhesion molecule (EpCAM) (R&D systems), IL-15 receptor α (IL-15Rα) (R&D systems), IL-1β (eBioscience, San Diego, CA, USA), CD16a (R&D systems), c-MET (Sinobiological, Beijing, China)] at 5 ㎍/ml concentrations were immobilized on the microtiter plates, and the Fab molecules were allowed to bind to the antigens, and detected as above. To determine the concentration of soluble Fab or Fab-hGH fusion proteins, a sandwich ELISA was performed using a mouse anti-human IgG Fd mAb (AprilBio) as a capturing Ab and the goat anti-human kappa L chain pAb-HRPO conjugated (Sigma-Aldrich) as a detecting antibody. The human Fab fragment (Bethyl, Montgomery, TX, USA) with a known concentration was used to draw the standard curve. For detecting the hGH domain, T-20, a goat pAb specific for the C-terminus of the hGH (Santacruz Biotechnology, Dallas, Tx, USA) and NYThGH, a mouse mAb specific for full-length hGH (Prospec, East Brunswick, NJ, USA) were used followed by a rabbit anti-goat IgG pAb-HRPO conjugated (Sigma-Aldrich) or a goat anti-mouse IgG pAb-HRPO conjugated (Sigma-Aldrich), respectively as a secondary antibody. A goat anti-human GCSF pAb (R&D Systems) was used to detecting the G-CSF domain, and a rabbit anti-human IFN-β pAb (PEPROTECH, Rocky Hill, USA) was used to detect the IFN-β domain.

[77]

[78] *1-(7)-Preparation of soluble Fab and Fab-effector fusion proteins*

[79] Soluble Fab and Fab-hGH fusion proteins were produced by growing *E. coli* SUPEX5 cells in 10 ml or 1 L of 2 YT medium containing 50 ㎍/ml kanamycin at 37℃ until an OD_{600nm} = 0.5 followed by the addition of 0.05 mM IPTG. After 20 h of incubation at 20℃ with vigorous shaking, the culture supernatant and cell pellet were separated by centrifugation at 3,300 *g* for 20 min. The periplasmic extracts were obtained as described earlier. For purification, the culture supernatant and/or the periplasmic extracts were then passed through Sepharose 4B resins that were immobilized with HSA (AprilBio). After extensive washing the Fab molecules bound to the resin were eluted with elution buffer (0.1 M glycine, 10% glycerol, pH 3) followed by immediate neutralization with Tris buffer (0.5 M Tris HCl, 2 M NaCl, pH 9.0). Gel filtration of HserG/Lser was also performed after affinity purification using AKTA FPLC (GE Healthcare, Wauwatosa, WI, USA). Briefly, Hiprep™16/60 Sephacryl™ S-200HRP repacked Column was equilibrated with equilibration buffer (20 mM HEPES,150 mM NaCl, pH 7.4), and loaded with 5 ㎕ of HserG/Lser ($SL335_{\Delta ds}$-hGH fusion). Elution was performed with equilibration buffer at 0.35 Mpa alarm pressure and 0.5 ㎕/min running flow rate. Fraction number 13, 16, 19 and 23 were analyzed by SDS-PAGE as described below.

[80]

[81] *1-(8) Affinity measurement by biolayer interferometry*

[82] Real-time binding assays between the purified SL335 and the antigens (human SA, rat SA or mouse SA) were performed using biolayer interferometry with an Octet RED system (ForteBio, Menlo park, CA, USA) as previously described except that AR2G (Amine Reactive Second-Generation) sensors were used (Costin *et al.*, (2013) *J Virol.* 87, 52-66). Briefly, the predetermined concentration of SL335 was coupled to kinetics grade AR2G biosensors, and unbound Fab fragments were removed from the surfaces of the sensors by incubating in the kinetics buffer (1 M ethanolamine, pH 8.5). The probes were then allowed to bind to human SA, rat SA or mouse SA at the predetermined concentrations under pH 6.0 or pH 7.4 conditions (human SA concentration at pH 6 and pH 7.4: 200 nM, 100 nM, 50 nM, 25 nM and 12.5 nM; rat SA concentration at pH 6: 4 mM, 1 mM, 500 nM, 250 nM and 125 nM; rat SA concentration at pH 7.4: 4 mM, 2 mM, 1 mM, 500 nM and 125 nM; mouse SA concentration at pH 6 and pH 7.4: 20 mM, 10 mM, 5 mM, 2.5 mM and 12.5 mM), followed by dissociation in PBS containing 0.1% BSA, pH 6 or pH 7.4. The binding and dissociation kinetics were calculated using the Octet QK software package, which fit the observed binding curves to a 1:1 binding model to calculate the association rate constants. The association and dissociation rate constants were calculated using at least three different concentrations of human SA, rat SA or mouse SA. The equilibrium dissociation

constants were calculated as the kinetic dissociation rate constant divided by the kinetic association rate constant.

[83]

[84] *1-(9) Generation of the SL335-hGH fusion constructs*

[85] To create SL335ds, the mutant Fd (Cys^{233} Ser^{233} substitution), termed Hser, was obtained by PCR amplification from the codon-optimized Fd chain gene of SL335 using a set of PCR primer #9 (5'-ggggaatt catgaaatatctgctgcctacggcggcggcgggcctgct-gctgctggctgcacaa-3' (SEQ ID NO:29)) and #10 (5'-gggaagcttttagctgctcttcggttccacgcgtt-3' SEQ ID NO:30)). The ~750 bp PCR product was treated with *Eco*R I/ *Hind* III and ligated with pHEKA. The mutant L chain (Cys^{214} → Ser^{214} substitution), termed Lser, was also obtained by PCR amplification from the codon-optimized L chain gene of SL335 using a set of PCR primer #11 (5'- gggggatc-catgaaaaaaactgcgattgcgattgcggtgctggccggctttg - 3' (SEQ ID NO:31)) and #12 (5'-gggctcgagttagctttcgc cgcggttaaagctctttg - 3' (SEQ ID NO:32)), cut with *Bam*H I/*Xho* I and cloned into pHEKA containing Hser. The cloning procedures for generating the HcysG/Lcys construct were as follow: the wild type Fd with Cys^{233}, termed Hcys, was PCR amplified from the codon-optimized Fd of SL335 using a set of PCR primer #9 and #13 (5'-agatccaggagctggtgcagaaccgcagctcttcggttccacgcgtt-3' (SEQ ID NO: 33)), and the hGH containing a linker sequence was also PCR amplified from the codon-optimized hGH gene using a set of PCR primer #14 (5'-ggttctgcaccagctcctggatcttttccgaccattccgctgagccg-3' (SEQ ID NO: 34)) and #15 (5'-gggaagcttttagaagccgcaggagccctcca-3' (SEQ ID NO: 35)). The Hcys and the hGH genes were linked together to generate HcysG by assembly PCR using a set of PCR #9 and #15 primers, cut with *Eco*R I/*Hind* III, and cloned into pHEKA containing the wild type L chain with Cys^{214} of SL335, termed Lcys. To generate the LcysG/Hcys construct, Lcys, was PCR amplified from the codon-optimized L chain of SL335 using a set of PCR primer #11 and #16 (5'-agatccaggagctggtgcagaaccgcattcgccgcggttaaagctcttt-3' (SEQ ID NO: 36)), and the hGH containing a linker sequence was also PCR amplified from the codon-optimized hGH gene using a set of PCR primer #14 and #17 (5'-gggctcgagttagaagccgcaggagccctcca-3' (SEQ ID NO: 37)). Lcys and the hGH gene were linked to generate LcysG by assembly PCR using a set of PCR #11 and #17 primers, cut with *Bam*H I/*Xho* I and cloned into pHEKA containing the wild type Fd. To create the HserG/Lcys construct, Hser was PCR amplified from the codon-optimized wild type Fd chain using a set of PCR primer #9 and #18 (5'-gggctcgagttagaagccgcaggagccctcca-3' (SEQ ID NO: 38)). The PCR amplification of the hGH containing a linker sequence, assembly PCR and cloning of HserG were performed as creating the HcysG/Lcys construct. To generate the LserG/Hcys

construct, Lser was PCR amplified from the codon-optimized L chain of SL335 using a set of PCR primer #11 and #19 (5'-agatccaggagctggtgcagaaccgctgctcttcggttccacgcgtt-3' (SEQ ID NO: 39)). PCR amplification of the hGH containing a linker sequence, assembly PCR and cloning of LserG were performed as in creating the LcysG/Hcys construct. To generate the HerG/Lser construct, the PCR amplification of HserG and the hGH, and assembly PCR were performed as creating the HserG/Lcys construct except that pHEKA containing Lser was used for cloning. LserG/Hser was also constructed as the creation of the LserG/Hcys construct except that pHEKA containing Hser was used for cloning. The PCR primers for preparing SL335-hGH fusion constructs and $SL335_{\Delta ds}$-hGH fusion constructs are shown in Table 2 below.

[86]

[87] Table 2

[Table 2]

PCR primers for SL335-hGH or $SL335_{\Delta ds}$-hGH fusion constructs

Constructs	Primers	Oligonucleotide sequence
$SL335_{\Delta ds}$	Primer 9	5'-ggggaattcatgaaatatctgctgcctacggcggcggcgggcctgctgctgctggctgcacaa-3' (SEQ ID No:29)
	Primer 10	5'-gggaagcttttagctgctcttcggttccacgcgtt-3' (SEQ ID No:30)
	Primer 11	5'-gggggatccatgaaaaaaactgcgattgcgattgcggtgctggccggctttg-3' (SEQ ID No:31)
	Primer 12	5'-gggctcgagttagctttcgc cgcggttaaagctctttg-3' (SEQ ID No:32)
$SL335_{wt}$-hGH fusion	Primer 13	5'-agatccaggagctggtgcagaaccgcagctcttcggttccacgcgtt-3' (SEQ ID No:33)
	Primer 14	5'-ggttctgcaccagctcctggatcttttccgaccattccgctgagccg-3' (SEQ ID No:34)
	Primer 15	5'-gggaagcttttagaagccgcaggagccctcca-3' (SEQ ID No:35)
	Primer 16	5'-agatccaggagctggtgcagaaccgcattcgccgcggttaaagctcttt-3' (SEQ ID No:36)
	Primer 17	5'-gggctcgagttagaagccgcaggagccctcca-3' (SEQ ID No:37)
$SL335_{\Delta ds}$-hGH fusion	Primer 18	5'-agatccaggagctggtgcagaaccgctgctcttcggttccacgcgtt-3' (SEQ ID No:38)
	Primer 19	5'-agatccaggagctggtgcagaaccgctttcgccgcggttaaagctctttg-3' (SEQ ID No:39)

[88]

[89] *1-(10)-Generation of the SL335-GCSF fusion constructs*

[90] The cloning procedures for generating the HcysGF/Lcys construct were as follow; Hcys was PCR amplified from the codon-optimized H chain of SL335 using a set of PCR primer #9 and #20 (5'-agatccaggagctggtgcagaaccgctttcgccgcggttaaagctctttg-3' (SEQ ID NO: 40)), and the G-CSF containing a linker sequence was also PCR amplified from the codon-optimized G-CSF gene using a set of PCR primer #21 (5'-ggttctgcaccagctcctggatctgcgcctacctatcgcgcgagca-3' (SEQ ID NO:41)) and #22 (5'-gggaagcttattaaggctgtgccagatggcgcag-3' (SEQ ID NO:42)). The Hcys and the G-CSF genes were linked together by assembly PCR using a set of PCR #9 and #22 primers, cut with *Eco*R I/*Hind* III, and cloned into pHEKA containing the L chain of SL335. To generate the LcysGF/Hcys construct, Lcys was PCR amplified from the codon-optimized L chain of SL335 using a set of PCR primer #11 and #23 (5'-agatccaggagctggtgcagaaccgcattcgccgcggttaaagctcttt-3' (SEQ ID NO: 43)), and the G-CSF containing a linker sequence was also PCR amplified from the codon-optimized G-CSF gene using a set of PCR primer #21 and #24 (5'-taacagatctgcggccgcactcgagattaaggctgtgccagatggcgcag-3' (SEQ ID NO: 44)). The Lcys and G-CSF genes were linked by assembly PCR using a set of PCR primer #11 and #25 (5'-agatccaggagctggtgcagaaccgctgctcttcggttccacgcgtt-3' (SEQ ID NO: 45)), cut with *Bam*H I/*Xho* I and cloned into pHEKA containing the Fd of SL335. To create the HserGF/Lser construct, Hser was PCR amplified from the codon-optimized Fd of SL335 using a set of PCR #9 and #25 primers. The Hser and the G-CSF genes were linked together by assembly PCR using a set of PCR #9 and #22 primers, cut with EcoR I/Hind III, and cloned into pHEKA containing Lser. To generate the LserGF/ Hser construct, Lser was PCR amplified from the codon-optimized L chain of SL335 using a set of PCR primer #11 and #26 (5-agatccaggagctggtgcagaaccgctttcgccgcggttaaagctctttg-3(SEQ ID NO: 46)), and the G-CSF containing a linker sequence was also PCR amplified from the codon-optimized G-CSF gene using a set of PCR #21 and #24 primers. The Lcys and G-CSF genes were linked by assembly PCR using a set of PCR #11 and #25 primers, cut with *Bam*H I/*Xho* I and cloned into pHEKA containing Hser. The PCR primers for preparing SL335-GCSH fusion constructs and $SL335_{\Delta ds}$-GCSF fusiong constructs are shown in Table 3 below.

[91]

[92] Table 3

[Table 3]

PCR primers for SL335-GCSH or $SL335_{\Delta ds}$-GCSF fusion constructs

Constructs	Primers	Oligonucleotide sequence
$SL335_{wt}$-GCSF fusion	Primer 20	5'-agatccaggagctggtgcagaaccgcagctcttcggttccacgcgtt-3' (SEQ ID No:40)
	Primer 21	5'-ggttctgcaccagctcctggatctgcgcctacctatcgcgcgagca-3' (SEQ ID No:41)
	Primer 22	5'-gggaagcttattaaggctgtgccagatggcgcag-3' (SEQ ID No:42)
	Primer 23	5'-agatccaggagctggtgcagaaccgcattcgccgcggttaaagctcttt-3' (SEQ ID No:43)
	Primer 24	5'-taacagatctgcggccgcactcgagattaaggctgtgccagatggcgcag-3' (SEQ ID No:44)
$SL335_{\Delta ds}$-GCSF fusion	Primer 25	5'-agatccaggagctggtgcagaaccgctgctcttcggttccacgcgtt-3' (SEQ ID No:45)
	Primer 26	5'-agatccaggagctggtgcagaaccgctttcgccgcggttaaagctctttg-3' (SEQ ID No:46)

[93]

[94] *1-(11) Generation of the SL335-IFN-b fusion constructs*

[95] The cloning procedures for generating the HcysIFNb/Lcys construct were as follow. Hcys was PCR amplified from the codon-optimized H chain of SL335 using a set of primer #9 and #27 (5'-agatccaggagctggtgcagaaccgcagctcttcggttccacgcgtt-3' (SEQ ID NO: 47)), and the IFN-*b* containing a linker sequence was also PCR amplified from the codon-optimized IFN-*b*1a gene using a set of PCR primer #28 (5'- ggttctgcaccagctcctg-gatcttcatacaacctgctgggcttcctg -3' (SEQ ID NO:48)) and #29 (5'- gggaagcttttagttgcgca-gatagccggtcag -3' (SEQ ID NO:49)). Hcys and the IFN-*b*1a genes were linked together by assembly PCR using a set of PCR #9 and #29 primers, cut with *Eco*R I/*Hind* III, and cloned into the pHEKA containing Lcys. To create the HserIFN-*b*/Lser construct, Hser was PCR amplified from the codon-optimized H chain of SL335 using a set of PCR primer #9 and #30 (5'- agatccaggagctggtgcagaaccgctgctcttcggttccacgcgtt -3' (SEQ ID NO:50)). Hser and the IFN-b 1a genes were linked together by assembly PCR using a set of PCR #9 and #29 primers, cut with EcoR I/Hind III, and cloned into the pHEKA containing Lser. The PCR primers for preparing SL334-IFNb fusion constructs and $SL335_{\Delta ds}$-IFNb fusion constructs are shown in Table 4 below.

[96]

[97] Table 4

[Table 4]

PCR primers for SL335-IFNb or $SL335_{\Delta ds}$-IFNb fusion constructs

Constructs	Primers	Oligonucleotide sequence
$SL335_{\Delta ds}$-IFNb and SL335-IFNb fusion	Primer 27	5'-agatccaggagctggtgcagaaccgcagctcttcggttccacgcgtt-3' (SEQ ID NO: 47)
	Primer 28	5'-ggttctgcaccagctcctggatcttcatacaacctgctgggcttcctg-3' (SEQ ID NO: 48)
	Primer 29	5'-gggaagcttttagttgcgcagatagccggtcag-3' (SEQ ID NO: 49)
	Primer 30	5'-agatccaggagctggtgcagaaccgctgctcttcggttccacgcgtt-3' (SEQ ID NO: 50)

[98]

[99] *1-(12) Generation of the EGL4-hGH and the 1b28-hGH fusion constructs*

[100] EGL4, a human anti-EGFR Fab, and 1*b*28, a human anti-IL-1b Fab, had been isolated from HuDVFab-8L antibody library (unpublished, AprilBio Co.). To create $EGL4_{wt}$ and $EGL4_{\Delta ds}$, Hcys and Hser were PCR amplified from the H chain gene of EGL4 cDNA using a set of PCR primer #5 and #6, and #5 and #31 (5'-gggaagcttattaactagatttgggctcaactctcttg-3' (SEQ ID NO: 51)), respectively. The ~ 750 bp PCR products were treated with *Eco*R I/*Hind* III and ligated with pHEKA, followed by transforming MC1061 competent cells. Lcys and Lser were also PCR amplified the L chain gene of EGL4 cDNA using a set of PCR primer #11 and #32 (5'-gggctcgagttagcattcgccgcggttaaagctcttt-3' (SEQ ID NO: 52)), and #11 and #33 (5'-gggctcgagttagctttcgccgcggttaaagctcttt-3' (SEQ ID NO: 53)), respectively. They were cut with *Bam*H I/*Xho* I and cloned into the pHEKA containing Hcys or Hser of EGL4, respectively. To create the $EGL4_{wt}$-hGH fusion construct, the cloning procedures for generating the HcysG/Lcys construct were as follow. Hcys was PCR amplified from the H chain of EGL4 cDNA using a set of PCR primer #5 and #34 (5'-agatccaggagctggtgcagaaccacaagatttgggctcaactctcttgtc-3' (SEQ ID NO: 54)), and the hGH containing a linker sequence was also PCR amplified from the codon-optimized hGH gene using a set of PCR #14 and #15 primers. The Hcys and the hGH genes were linked together by assembly PCR using a set of PCR #5 and #15 primers, cut with *Eco* R I/*Hind* III, and cloned into the pHEKA containing Lcys of EGL4. For creating the $EGL4_{\Delta ds}$-hGH fusion construct construct, Hser was PCR amplified from the H chain of EGL4 cDNA using a set of PCR primer #5 and #35 (5'-agatccaggagctggtgcagaaccactagatttgggctcaactctcttgtc-3' (SEQ ID NO: 55)), and the hGH containing a linker sequence was also PCR amplified from the codon-optimized

HGH gene using a set of PCR #14 and #15 primers. The Hser and the hGH genes were linked together by assembly PCR using a set of PCR #5 and #15 primers, cut with *Eco* R I/*Hind* III, and cloned into the pHEKA containing Lser of EGL4$_{\Delta ds}$. 1*b*28 $_{wt}$, 1*b*28$_{\Delta ds}$, 1*b*28$_{wt}$-hGH and 1*b*28$_{\Delta ds}$-hGH were created as EGL4-hGH fusions using the same PCR primer sets except that 1*b*28 cDNA was served for PCR templates. The PCR primers for preparing EGL4-hGH and the 1*b*28-hGH fusion constructs are shown in Table 5 below,

[101]

[102] Table 5

[Table 5]

PCR primers for repaing EGL4-hGH and the 1b28-hGH fusion constructs

Constructs	Primers	Oligonucleotide sequence
EGL4-hGH and1*b*28-hGH fusion	Primer 31	5'-gggaagcttattaactagatttgggctcaactctcttg - 3' (SEQ ID NO. 51)
	Primer 32	5' -gggctcgagttagcattcgccgcggttaaagctcttt - 3' (SEQ ID NO. 52)
	Primer 33	5' -gggctcgagttagctttcgccgcggttaaagctcttt - 3' (SEQ ID NO. 53)
	Primer 34	5' - agatccaggagctggtgcagaaccacaagatttgggctcaactctcttgtc - 3' (SEQ IN NO. 54)
	Primer 35	5' - agatccaggagctggtgcagaaccactagatttgggctcaactctcttgtc - 3' (SEQ ID NO. 55)

[103]

[104] *1-(13) SDS-PAGE and western blot analyses*

[105] For SDS-PAGE analysis, purified SL335$_{wt}$-hGH and SL335$_{\Delta ds}$-hGH proteins were re-suspended in NuPAGE® LDS Sample Buffer (Invitrogen) with or without NuPAGE® Sample Reducing Agent (Invitrogen), and loaded onto the gel at 7 μg/well concentration. The protein bands were visualized by using Coomassie Blue staining (Bio-Rad). For the western blot analysis, 500 ng of affinity-purified SL335$_{wt}$-hGH and SL335$_{\Delta ds}$-hGH were loaded onto each well as above, and transferred to nitrocellulose membrane. After blocking the membrane with 3% skimmed milk (Bio-Rad) in PBS containing 0.01% Tween (Sigma-Aldrich), proteins were detected by incubation with a goat anti-human kappa L chain pAb conjugated with AP (Bethyl). The nitro blue tetrazolium chloride (NBT)/5-bromo-4-chloro-3-indolyl phosphate (BCIP) substrate (Duchefa) was added onto the membrane to visualize the binding signals.

[106]

[107] *1-(14) Chip-based capillary electrophoresis*

[108] Chip-based capillary electrophoresis was carried out with the Agilent 2100 Bio-analyzer system (Agilent Technologies, Santa Clara, CA, USA). The protein samples were prepared according to the manufacturers protocol and analyzed on the Protein 80 kit, which is recommended for the analysis of proteins between 5 to 80 kDa. Briefly, the samples were mixed with sample buffer in the presence or absence of DTT for reducing or non-reducing electrophoresis, respectively. The samples were denatured at 95℃ and loaded on the chip which had been filled with proper reagents including the fluorescent dye and gel solution. The chip was then inserted into the system and run on the system using the Expert 2100 software. The results were plotted to reflect fluorescence intensity units against protein size.

[109]

[110] *1-(15) MALDI-TOF mass spectrometry*

[111] MALDI-TOF mass spectrometry was performed on an Autoflex III Smartbeam device (Bruker Daltonics, Billerica, MA, USA). Sample was mixed with the same volume of MALDI matrix (10 mg/mL of a-cyano-4-hydroxycinnamic acid) and spotted on a MALDI target plate. External calibration was performed with a Peptide and Protein MALDI-MS Calibration Kit (Sigma-Aldrich). Mass spectra in the m/z range of 15000160000 and 1000070000 were acquired for $SL335_{wt}$-hGH fusion and $SL335_{\Delta ds}$-hGH fusion, respectively, in the positive ion mode.

[112]

[113] *1-(16) In vitro hGH bioactivity assay*

[114] Nb2-11 rat lymphoma cells (Sigma-Aldrich) were grown in complete DMEM supplemented with 5% horse serum (Sigma-Aldrich) and 1% PenicillinStreptomycin (Invitrogen) in a humidified 5% CO_2 incubator at 37℃ (Tanaka*et al.*, 1980). The cells were washed two times with DMEM, centrifuged at 1,000 *g* for 5 min and resuspended in DMEM containing 5% (v/v) horse serum at 8×10^4 cells/ml. A 50 ㎍ aliquot of the cell suspension was added to each well of 96-well plates, and incubated overnight. The cells were then treated with increasing concentrations (0 - 20 nM) of Growtropin® (a unmodified rhGH; Dong-A Pharmaceuticals, Seoul, South Korea) or $SL335_{\Delta ds}$-hGH in 50 ml DMEM containing 5% horse serum for 48 h at 37℃. Following the incubation, 10 ㎕ of CCK-8 (Dojindo, Mashiki-machi, Japan) was added to each well, and incubated for 4 h. The absorbance was recorded on a microplate reader (Bio-Rad) at a wavelength of 450 nm.

[115]

[116] *1-(17) Serum stability of SL335Δds-hGH*

[117] $SL335_{wt}$ and $SL335_{\Delta ds}$-hGH (10 ㎍/ml final concentration) were resuspended in fetal bovine serum (FBS) (Thermo Scientific, Waltham, MA, USA) containing 0.03%

sodium azide, and incubated for 16 days at 37°C. Small aliquots (50 ml) were taken every day and stored at -20°C before use. The binding reactivity to HSA was determined by ELISA, and the *in vitro* hGH bioactivity was measured using Nb2-11 cells(Sigma-Aldrich) as described above.

[118]

[119] *1-(18) In vivo pharmacokinetics assay*

[120] The PK studies were performed at a certified CRO company (ChemOn, Suwon, South Korea). The animals were fed a standard diet of rodent pellets and water *ad libitum* and kept in a room of constant humidity and temperature with controlled lighting (12 h light followed by 12 h dark). Briefly, SL335 and Neg Fab (an irrelevant human Fab) were intravenously (I.V.) or subcutaneously (S.C.) injected separately into groups of three Sprague Dawley rats at 1 mg/kg, and serum samples were obtained at several time points (5 min, 15 min, 30 min, 1 h, 2 h, 4 h, 8 h, 24 h, 48 h, 96 h and 144 h for I.V., and 5 min, 15 min, 30 min, 1 h, 2 h, 4 h, 8 h, 24 h, 48 h, and 96 h for S.C.). The concentration of SL335 and Neg Fab in the serum samples was measured by sandwich ELISA using the mouse anti-human IgG Fd mAb and the goat anti-human kappa L chain pAb conjugated with HRPO as a capture and detecting antibodies, respectively. Human Fab fragments of known concentration were also included in the assay to obtain a standard curve. Curves of serum concentration versus time were fitted for a noncompartment model using WinNonlin software (SL335 and Neg Fab) and plotted using Sigma Plot software. Similarly, Growtropin® and $SL335_{\Delta ds}$-hGH were intravenously or subcutaneously injected separately into group of three to four rats. The dosages of Growtropin® and $SL335_{\Delta ds}$-hGH for I.V. administration were 0.3 mg/kg, and for S.C. administration were 0.6 mg/kg, respectively. Serum samples were obtained at several time points (5 min, 15 min, 30 min, 1 h, 2 h, 3 h, 4 h, 6 h and 8 h for Growtropin® and 5 min, 30 min, 1 h, 2 h, 4 h, 8 h, 24 h, 48 h, 96 h and 144 h for $SL335_{\Delta ds}$-hGH. The amount of Growtropin® in the serum samples was measured using the hGH ELISA detection kit (Genway, San Diego, CA, USA), and that of $SL335_{\Delta ds}$-hGH was measured by sandwich ELISA as described above. A serum concentration versus time curve was fitted for a one compartment model using Phoenix™ WinNonlin software (Version 6.2).

[121]

[122] *1-(19) In vivo pharmacodynamics assay*

[123] The ability of daily dosing of Growtropin® and once-weekly dosing of $SL335_{\Delta ds}$-hGH to promote weight gain was analyzed in hypophysectomized rats by using S.C. administration at ChemOn as previously described (see Clark et al., (1996) *J. Biol. Chem.* 271, 21969-21977). Briefly, young hypophysectomized Sprague Dawley rats (Harlan, Tokyo, Japan) were purchased, and any animal gaining more than 7 g over the

first 15 days following surgery was excluded from the study. The animals were randomized for five treatment groups (Excipient only, daily injection of 0.3 mg/kg Growtropin® and once-weekly injection of 0.6 mg/kg, 1.2 mg/kg or 2.4 mg/kg SL335 $_{\Delta ds}$-hGH). The body weights were recorded daily after starting dosage regimen. The tibia bone growth was carefully measured with a bone caliper. Statistical comparisons were made using an analysis of variance followed by Dunnetts Multiple Comparison Test, and *p* values less than 0.05 were considered significant.

[124]

[125] **2. Experimental results**

[126] *2-(1) Isolation of anti-SA Fab clones*

[127] The HuDVFab-8L antibody library was selected against the magnetic beads conjugated with human SA, rat SA or mouse SA at pH 6 or pH 7.4. After three rounds of biopanning, a monoclonal phage ELISA was performed to identify the phage antibody clones that were specific for the antigens. More than 60 positive clones were identified by the ELISA (data not shown), and a DNA sequencing analysis of the V_H and the V_L genes identified eight discrete phage antibodies, termed SA138, SA139, SA140, SA141, SL18, SL301, SL310 and SL335, respectively. The binding reactivity of these clones to human SA, rat SA, mouse SA or bovine SA was confirmed by a monoclonal phage ELISA under pH 6 or pH 7.4 conditions (Fig. 1A & 1B). Three phage antibody clones, SA138, SA139 and SA141, were reactive only to human SA regardless of pH conditions. SA140 also recognized human SA only at pH 7.4, but its binding reactivity disappeared at pH 6. On the other hand, SL18, SL310 and SL335 bound to human SA, rat SA and mouse SA under both pH conditions with slightly different intensities. SL301 was significantly reactive to human SA and rat SA at both pH, and weakly to mouse SA at pH 7.4 only. None of eight Fab clones were reactive to bovine SA. SL18, SL301, SL310 and SL335 were further characterized because of their cross-reactivity to SAs from at least two different species. The Fd and the L chain genes of four phage antibody clones were subcloned into the pHEKA vector for periplasmic expression in *E. coli*, and the soluble Fab fragments were prepared from the culture supernatant or periplasmic extracts. After affinity purification, an ELISA was performed to compare the binding reactivity of these fragments to human SA, rat SA or mouse SA under pH 6 (Fig. 2A) and pH 7.4 conditions (Fig. 2B). HSA, RSA, MSA or BSA at 5 ㎍/ml concentrations was immobilized in each well of the microtiter plates, and four purified Fab molecules (SL18, SL301, SL310 and SL335) were allowed to bind to the antigens at pH 6.0 (Fig. 2A) or at pH 7.4 (Fig. 2B). The goat antihuman kappa L chain pAb HRPO conjugate was used as a secondary antibody. The binding signals were visualized using TMB substrate, and the absorbance at 450 nm was measured using an ELISA reader (Bio-Rad). The data represent the average

standard deviation of three experiments. In the human SA binding, the order of binding signals was SL335 > SL310 > SL301 > SL18 at both pH 6 and pH 7.4. In the rat SA binding, the order was SL335 > SL310 > SL301 > SL18 at pH 6, and SL335 = SL310 > SL301 = SL18 at pH 7.4. In the mouse SA binding, the order was SL18 > SL335 > SL310 at pH 6, and SL335 > SL310 > SL18 at pH 7.4. In accordance with Fig. 2, SL301 failed to bind to mouse SA at pH 6, yet very weakly at pH 7.4. SL335 was found to be the best binder among four the Fab clones to both human SA and rat SA regardless of the pH condition. SL335 bound to human SA at pH 6 twice as strongly than it did at pH 7.4 (50% binding signal at 20 ng/ml *vs.* 40 ng/ml), 20-fold stronger than to rat SA under the same pH condition (50% binding signal at 20 ng/ml *vs.* 400 ng/ml), and four-fold stronger than to rat SA at pH 7.4 (50% binding signal at 40 ng/ml *vs.* 160 ng/ml).

[128]

[129] *2-(2) Cross-reactivity and binding affinity of SL335*

[130] Since SL335 was the best binder among four anti-human SA Fab clones, its cross-reactivity was further analyzed by ELISA. Binding reactivity to human SA, rat SA and mouse SA was reproduced as shown in Fig. 2. It was also found that SL335 intensely recognized cynomolgus monkey SA and weakly bound to canine SA. However, SL335 did not recognize rabbit SA as well as other irrelevant antigens including EGFR, EpCAM, IL-15Ra, IL-1b, CD16a or c-MET. The binding affinities of SL335 to human SA, rat SA and mouse SA at pH 6 or pH 7.4 were further measured *via* biolayer interferometry by passing through different concentration of the antigens on biosensors that were coated with SL335 (see Table 6 below). The results correlated well with the ELISA data in Fig.2 in that the dissociation constants of SL335 to HSA were 9 nM at pH 6 and 13 nM at pH 7.4, respectively, and those to RSA were 122 nM and 65 nM at pH 6 and pH 7.4, respectively. The binding affinities of SL335 for MSA were approximately 10 mM at pH 6 and 1.6 mM at pH 7.4, but these data were not included in Table 6 due to lack of reliability.

[131]

[132] Table 6

[Table 6]

Determination of binding affinity of SL335 and HserG/Lser by Biolayer interferometry binding assay

Binder	Antigen	pH condition	KD(M)	K on(1/Ms)	K off(1/s)	Full R^2	Chi2 values
SL335	HSA	pH 6.0	8.68E-09	1.79E+05	1.55E-03	0.920807	0.479289
		pH 7.4	1.30E-08	1.17E+05	1.52E-03	0.966233	0.378597
	RSA	pH 6.0	1.22E-07	4.71E+04	5.76E-03	0.882417	1.299042
		pH 7.4	6.53E-08	4.32E+04	2.82E-03	0.839612	2.718799
HserG /Lser	HSA	pH 6.0	1.68E-09	5.00E+05	8.41E-04	0.951998	1.015294
		pH 7.4	1.51E-09	6.73E+05	1.02E-03	0.915507	0.652098
	RSA	pH 6.0	4.99E-07	6.96E+04	3.47E-02	0.980042	0.214899
		pH 7.4	8.36E-08	9.33E+04	7.80E-03	0.836744	1.101016

[133] The binding kinetics and the dissociation kinetics were calculated using the Octet QK software package.

[134]

[135] *2-(3) In vivo pharmacokinetics of SL335*

[136] Of all of the plasma proteins, HSA has an exceptionally long half-life through the FcRn-mediated recycling mechanism, and is commonly used as a fusion partner for extending the half-lives of therapeutic proteins. In addition, antibody fragments that are associated with serum albumin have been known to have an extended serum half-life. Thence, a pharmacokinetic analysis was performed to verify whether SL335 also has a long serum half-life. Human Fab with an unknown binding specificity was included as a negative control (Neg Fab). SL335 and Neg Fab were intravenously or subcutaneously injected separately into group of three rats at 1 mg/kg, and serum samples were collected at several time points (5 min, 15 min, 30 min, 1 h, 2 h, 4 h, 8 h, 24 h, 48 h, 96 h and 144 h for I.V., and 5 min, 15 min, 30 min, 1 h, 2 h, 4 h, 8 h, 24 h,

48 h, and 96 h for S.C.). The concentration of SL335 and Neg Fab in the serum samples was measured by sandwich ELISA using the mouse anti-human IgG Fd mAb and the goat anti-human kappa L chain pAb conjugated with HRPO as a capture and detecting antibodies, respectively. Human Fab fragments of known concentration were also included in the assay to obtain a standard curve. Curves of serum concentration versus time were fitted for a one compartment model using WinNonlin software (SL335 and Neg Fab) and a two-compartment model using Sigma Plot software. In intravenous administration, the terminal half-life ($t_{1/2}$) of SL335 was 37 h and its area under the curve ($AUC_{0\rightarrow\infty}$) was 187 h mg/ml, representing a ten-fold increase in the $t_{1/2}$ and a 26-fold increase in $AUC_{0\rightarrow\infty}$ compared to Neg Fab (3.8 h and 7 h mg/ml, respectively) (Fig. 3A). The subcutaneous injection of SL335 produced similar measurements, including a nine-fold increase in $t_{1/2}$ (120 h *vs.* 13 h) and a 44-fold increase $AUC_{0\rightarrow\infty}$ compared to Neg Fab (87 *vs.* 2 h mg/ml) (Fig. 3B). These results clearly showed an extended serum half-life of SL335, and implied that SL335 would not interfere with the interaction between RSA and FcRn in rats.

[137]

[138] *2-(4) Production of the SL335-hGH fusions*

[139] SL335 was used to create two SL335-hGH fusions and four additional SL335-hGH fusions by genetically fusing the recombinant hGH (27 - 191 aa) to the N- or C-terminus of the Fd or the L chain *via* a short peptide linker. Recombinant hGH cDNA (27 - 191 aa) was fused to the C-terminus of the H or L chain of $SL335_{wt}$ in a classic Fab form *via* a short peptide linker, resulting in construction of two fusion formats (HcysG/Lcys and LcysG/Hcys). Four additional fusion formats (HserG/Lcys, LserG/Hcys, HserG/Lser and LserG/Hser) were also constructed as above except for using SL335 in a null form ($SL335_{null}$) or a ds Fab form ($SL335_{\Delta ds}$) of which Cys^{233} at the C-terminal C_{H1} and/or Cys^{214} at the C-terminal C_{Lk} were replaced with Ser. For periplasmic expression of the fusion proteins, the ompA (MKKTAIAIAVLAGFATVAQA (SEQ ID No:56)) leader sequence was located at the upstream of the L chain or the L-hGH fusions, and the pelB leader sequence (MKYLLPTAAAGLLLLAAQPAMA (SEQ IN No:57)) was located at the upstream of the H chain or the H-hGH fusions. In these preliminary experiments, the genetic linking of hGH to the N-terminus of the Fd or the L chain resulted in low or no expression of soluble fusion proteins. The fusion of hGH to the C-terminus of the Fd also showed low expression yields, and seemed to interrupt the folding of the hGH domain probably due to aberrant disulfide bonding in the SL335-hGH fusion (data not shown). Previously, it had been reported that the removal of the interchain disulfide bond of a Fab by mutating the C-terminal Cys residues in the C_{H1} and the C_{Lk} (Cys^{233} and Cys^{214}, respectively) does not affect the levels of periplasmic production, stability upon ex-

traction and purification, serum stability or serum half-life (see Kabat *et al.*, (1991) *Sequences of Proteins of Immunological Interest*; Humphreys *et al.*, (1997) *J. Immunol. Methods.* 209, 193202; Humphreys *et al.*, (2007) *Protein Eng Des Sel.* 20, 227234.). By replacing both Cys^{233} of the C_{H1} and Cys^{214} of the C_{Lk} with serine (Cys^{233} Ser^{233} and Cys^{214} Ser^{214} substitutions), we tested whether these Cys residues in SL335 modulate the soluble expression and appropriate folding of SL335-hGH fusions. Fig. 4 illustrates six SL335-hGH fusion constructs. Other than $SL335_{wt}$ and $SL335_{\Delta ds}$, one more SL335 variant, termed $SL335_{null}$, was also created by substituting either Cys^{233} of the C_{H1} or Cys^{214} of the C_{Lk}, with Ser to elucidate the effect of each cysteine residues (Cys^{233} or Cys^{214}) separately. Two $SL335_{wt}$ fusion derivatives were HcysG/Lcys (HCys ^{233}-hGH fusion paired with $LCys^{214}$) and LcysG/Hcys ($LCys^{214}$-hGH fusion paired with $HCys^{233}$), two $SL335_{null}$ fusion derivatives were HserG/Lcys ($HSer^{233}$-hGH fusion paired with $LCys^{214}$) and LserG/Hcys ($LSer^{214}$-hGH fusion paired with $HCys^{233}$). Finally, two $SL335_{\Delta ds}$ fusion derivatives were HserG/Lser ($HSer^{233}$-hGH fusion paired with $LSer^{214}$) and LserG/Hser ($LSer^{214}$-hGH fusion paired with $HSer^{233}$). These six SL335-hGH fusion constructs were expressed in the *E. coli* SUPEX5 host cells, the yields and HSA-binding reactivity of these six SL335-hGH fusion proteins in the culture supernatant were analyzed by ELISA. *E. coli* clones expressing SL335-hGH fusion proteins were grown under the identical conditions in the presence of IPTG, and culture supernatant was harvested by brief centrifugation. The concentration of soluble SL335-hGH fusions was measured by sandwich ELISA using the mouse anti-human Fd mAb as a capturing Ab and the goat anti-human kappa L chain pAb conjugated with HRPO was used as a detecting antibody (Fig. 5A). No soluble Fab forms were detected from LcysG/Hcys or LserG/Hcys. Although the data were not presented, the western blot using the *E. coli* cell lysates revealed that Cys^{233} of the Fd were responsible for heavy degradation and no secretion of the Fd fragments probably due to protein aggregation. The yield of HcysG/Lcys was 0.5 μg/ml, and those of HserG/Lcys and LserG/Hser were approximately 1.8 μg/ml and 1.4 μg/ml, respcectively(Fig. 5A). Interestingly, the yield of HserG/Lser was about 4 μg/ml which was eight-fold higher than that of HcysG/Lcys. The periplasmic extracts showed the identical expression pattern, although the total yields were only ~30% to those present in the culture supernatant (data not shown). In the repeated experiments, it was confirmed that the difference in the yields between HcysG/Lcys and HserG/Lser was independent of the clonal variation or growth rate of the *E. coli* clones. The binding reactivity of SL335-hGH fusions to HSA were compared using the microtiter plates coated with 5 μg/ml HSA, and incubated with the serial dilutions of the culture supernatant containing SL335-hGH fusions. SL335-hGH fusions bound to HSA were then detected using the goat anti-human kappa L chain pAb conjugated with HRPO. As expected,

the detection of HserG/Lser that bound to HSA with the anti-human κL pAb produced an eight-fold stronger binding signal than that of HcysG/Lcys and approximately four-fold stronger binding signal than those of HserG/Lcys and LserG/Hser (Fig. 5B). Similar binding signal patterns were also observed when T-20, a goat pAb specific for the C-terminus of the hGH was used to detect the SL335-hGH fusions (Fig. 5C). In the detection with NYThGH, a mouse mAb specific for full-length hGH, however, HserG/Lser produced a 30-fold higher binding signal than those of both HserG/Lcys and LserG/Hser and 60-fold higher binding signal than that of HcysG/Lcys (Fig. 5D), suggesting that the binding of NYThGH to the hGH domain of HcysG/Lcys was interfered by the presence of the interchain disulfide bond in SL335. Since HcysG/Lcys and HserG/Lser represent the utilization of $SL335_{wt}$ and $SL335_{\Delta ds}$ for creating the SL335-hGH fusions, they were named as $SL335_{wt}$-hGH fusion and $SL335_{\Delta ds}$-hGH fusion, respectively, hereafter (Fig 5).

[140] To determine the high yield of soluble $SL335_{\Delta ds}$-hGH fusion was dependent upon removal of the interchain disulfide bond in SL335, host *E. coli* strains or induction temperature, $SL335_{wt}$, $SL335_{\Delta ds}$, $SL335_{wt}$-hGH fusion and $SL335_{\Delta ds}$-hGH fusion were expressed in the parental MC1061 as well as the mutant SUPEX5 cells at 20℃ (Fig. 6A), 25℃ (Fig. 6B) or 30℃ (Fig. 6C) and the amount of Fab molecules in the culture supernatant was measured by ELISA. The yield of $SL335_{wt}$ expressed in the MC1061 strain was 1 ㎍/ml at 20℃, which was about three-fold higher than that at 25℃ and 30℃. This implied induction of $SL335_{wt}$ below 25℃ is advantageous especially when MC1061 was used as a host strain. Similar results were also obtained with the SUPEX5 strain. In the case of $SL335_{\Delta ds}$, the yield was about 1.3 ㎍/ml at 20℃ regardless of the host *E. coli* strains and induction temperature. These results indicated that the presence or absence of the interchain disulfide bond in a Fab did not significantly influence the yield of soluble Fab production at 20℃ regardless of the *E. coli* host strains. The yield of $SL335_{wt}$-hGH fusion was about 0.3 - 0.5 ㎍/ml regardless of the host *E. coli* strains and induction temperature. On the other hand, the yield of $SL335_{\Delta ds}$-hGH fusion expressed in the MC1061 strain was 1.8 ㎍/ml at both 20℃ and 25℃, and 1.5 ㎍/ml at 30℃, showing minor temperature-dependency, whereas, the yield of $SL335_{\Delta ds}$-hGH fusion expressed in the SUPEX5 strain was 4.0 ㎍/ml at both 20℃ and 25℃, and 3.5 ㎍/ml at 30℃. These results meant that utilization of the $SL335_{\Delta ds}$ form and the *E. coli* SUPEX5 strain enabled about 12-fold higher yield of the SL335-hGH fusion protein compared to the combination of the $SL335_{wt}$ form and the *E. coli* MC1061 strain.

[141]

[142] *2-(5) Generation of the SL335-GCSF, SL335-IFNb, EGL4-hGH and 1b28-hGH fusion constructs*

[143] To demonstrate the beneficial effect of a $Fab_{\Delta ds}$ form and the SUPEX5 strain on improving soluble expression of a Fab-effector fusion protein, diverse Fab-effector fusioncon structs were generated. First, two SL335-GCSF fusion variants (HcysGCSF/Lcys that termed as $SL335_{wt}$-GCSF, HserGF/Lser that termed as $SL335_{\Delta ds}$ -GCSF) and two SL335-IFNb fusion variants (HcysIFNb/Lcys that termed as $SL335_{wt}$ - IFNb, HserIFNb/Lser that termed as $SL335_{\Delta ds}$-IFNb) were created as the same way as generating $SL335_{wt}$-hGH and $SL335_{\Delta ds}$-hGH fusions to determine the influence of an effector domain. Induction temperature was set to optimal 20°C and the expression yields of these fusion proteins in the *E. coli* culture supernatant were compared by ELISA. The yields of $SL335_{wt}$-GCSF were 0.3 and 0.6 mg/ml in MC1061 and SUPEX5, respectively, and those of $SL335_{\Delta ds}$-GCSF were 0.6 and 1.5 mg/ml in MC1061 and SUPEX5, respectively (Fig. 7A). Whereas, the yield of $SL335_{wt}$-IFNb was approximately 0.16 mg/ml in both MC1061 and SUPEX5, and those of $SL335_{\Delta ds}$ - IFNb were 0.2 and 0.5 mg/ml in MC1061 and SUPEX5, respectively (Fig. 7B). Therefore, the combination of $SL335_{\Delta ds}$-GCSF fusion and SUPEX5 strain produced about 5-fold higher yield of a SL335-GCSF fusion form compared to the combination of $SL335_{wt}$-GCSF fusion and the MC1061 strain, and the combination of $SL335_{\Delta ds}$ - IFNb fusion and SUPEX5 strain produced about 3-fold higher amount of a SL335-IFNb fusion form compared to the combination of $SL335_{wt}$-IFNb fusion and the MC1061 strain. Second, we also created two Fab-hGH fusion constructs using EGL4, a human anti-EFGR Fab, and 1b28, a human anti-IL-1b Fab to determine the influence of a Fab. As the same way as generating $SL335_{wt}$-hGH and $SL335_{\Delta ds}$-hGH fusions, the two EGL4-hGH fusion constructs were $EGL4_{wt}$-hGH fusion in the HcysG/Lcys format and $EGL4_{\Delta ds}$-hGH fusion in the HserG/Lser format. Likewise, the 1b284-hGH fusion constructs were $1b28_{wt}$-hGH fusion in the HcysG/Lcys format and $1b28_{\Delta ds}$-hGH fusion in the HserG/Lser format. The yield of $EGL4_{wt}$-hGH fusion was 8090 ng/ml in the MC1061 and SUPEX5 strains, and the yields of $EGL4_{\Delta ds}$-hGH fusion were 140 ng/ml in the MC1061 strain and 220 ng/ml in the SUPEX5 strain (Fig. 8A), indicating that the combination of $EGL4_{\Delta ds}$-hGH fusion and the SUPEX5 host cell produced 2.4-fold higher amount of a EGL4-hGH fusion protein in the culture supernatant compared to the combination of $EGL4_{wt}$-hGH fusion and the MC1061 host cell. In the case of the 1b28-hGH fusion constructs, the yield of $1b284_{wt}$-hGH fusion was 50 ng/ml in the MC1061 and 100 ng/ml SUPEX5 strains, respectively, and the yields of $1b28_{\Delta ds}$-hGH fusion were 900 ng/ml in the MC1061 strain and 4 mg/ml in the SUPEX5 strain (Fig. 8B), indicating that the combination of $1b28_{\Delta ds}$-hGH fusion and the SUPEX5 host cell produced 800-fold higher amount of a 1b28-hGH fusion form in the culture supernatant compared to the combination of $1b28_{wt}$-hGH fusion and the MC1061 host cell.

[144]

[145] *2-(6) Molecular Characterization of SL335wt-hGH and SL335$_{\Delta ds}$-hGH*

[146] SL335$_{wt}$-hGH and SL335$_{\Delta ds}$-hGH fusions were further characterized at the molecular level. The fusion proteins in the culture supernatant were affinity-purified by passing through the resins coated with HSA, and analyzed by SDS-PAGE and western blot under the reducing and non-reducing conditions. HcysG/Lcys (lane 1) and HserG/Lser (lane 2) were affinity-purified from the culture supernatnat with HSA-immobilized sepharose beads, and SDS-PAGE was carried out using 4-12% Bis-Tris gel under the reducing or non-reducing condition. Protein bands were visualized with Coomassie Blue staining (Fig. 9A). The proteins of the separate SDS-PAGE were transferred to nitrocellulose membrane, and the goat anti-human kappa L Ab-conjugated with AP was used to detect Lcys and Lser (Fig. 9B). The binding signals were visualized with a NBT/BCIP substrate. In SDS-PAGE analysis, both SL335$_{wt}$-hGH and SL335$_{\Delta ds}$-hGH produced two major protein bands at 46kDa and 23kDa in size which correspond to the Fd-hGH fusions and the L chains, respectively, under the reducing conditions. Under the non-reducing conditions, SL335$_{\Delta ds}$-hGH expectedly produced two identical protein bands due to the absence of an interchain disulfidebond. In the case of SL335$_{wt}$-hGH, a major 70 kD a protein band which corresponds to a correct heterodimeric form of SL335$_{wt}$-hGH was visible. Yet, many different size of SL335$_{wt}$-hGH derivatives were also found, including four obvious protein bands ranging from 24 kDa to 45 kDa of unknown identity and a couple of weak protein bands corresponding to 100 kDa and 135 kDa in size. The proteins at 15 kDa and 12.5 kDa in size were also visible from all of the samples. Western blot analysis was then performed using an anti-human Fd mAb, the anti-kappa L chain pAb and the anti-hGH pAb, T-20. The blot with the anti-human Fd mAb detected only HcysG and HserG of 46 kDa in size under both non-reducing and reducing conditions (data not shown). On the other hand, four proteins bands ranging from 24 kDa to 45 kDa as well as those larger than 70 kDa in the SL335 $_{wt}$-hGH sample were all detected by the anti-kappa L chain pAb under the non-reducing condition (Fig. 9B). This result indicated that Cys^{214} of the L chain is responsible for the formation of the diverse multimeric L chains, at least, *via* aberrant disulfide bond formations. The blot with T-20 anti-hGH pAb correctly recognized the 70 kDa heterodimeric form of SL335$_{wt}$-hGH and the ~ 45 kDa monomeric HerG of SL335$_{\Delta ds}$-hGH under the non-reducing condition (Fig. 9C). The proteins at 15 kDa and 12.5 kDa in size were not detected by any of those antibodies, suggesting that they were either the degraded products from the fusions or the contaminants from *E. coli* host proteins.

[147] A chip-based capillary electrophoresis confirmed the SDS-PAGE analysis. HcysG/Lcys (Fig. 10A) and HserG/Lser (Fig. 10B) were prepared with sample buffer in the presence or absence of DTT for reducing or non-reducing electrophoresis, and chip-

based capillary electrophoresis was carried out with the Agilent 2100 Bioanalyzer system according to the manufacturers protocol using the Protein 80 kit. The results were plotted to reflect fluorescence intensity units against protein size. $SL335_{wt}$-hGH produced several $SL335_{wt}$-hGH derivatives ranging from 27.1 kDa to 52.4 kDa in size under the non-reducing condition, and many of them disappeared under the reducing condition in the presence of DTT (Fig. 10A). $SL335_{\Delta ds}$-hGH produced almost identical protein peaks between the non-reducing and reducing conditions except for minor changes in molecular weights (Fig. 10B).

[148] $SL335_{wt}$-hGH and $SL335_{\Delta ds}$-hGH were further analyzed using MALDI-TOF mass spectrometry. MALDI-TOF mass spectrometry was performed on an Autoflex III Smartbeam device (Bruker Daltonics, Billerica, MA, USA). Affinity-purified HcysG/ Lcys (Fig. 11A) and HserG/Lser (Fig. 11B) were mixed with the MALDI matrix, and spectra were acquired over the m/z range 10000 - 150000 Da in the positive ion mode. Mass spectra in the m/z range of 10000 - 70000 were acquired for $SL335_{\Delta ds}$-hGH. For $SL335_{wt}$-hGH, those of 15000 - 160000 were obtained because the $SL335_{wt}$-hGH sample showed the protein bands larger than 70 kDa as shown in Fig. 8A. Molecular masses of Lcys, HcysG and $SL335_{wt}$-hGH were identified as 23,226 Da, 46226 Da and 69,837 Da, respectively (Fig. 11A). The size of three discrete proteins those are bigger than the correct $SL335_{wt}$-hGH were found to be 92,824 Da, 117,455 Da and 139,347 Da. In the case of $SL335_{\Delta ds}$-hGH, molecular masses of Lser and HserG were identified as 23,334 Da and 46,667 Da, respectively (Fig. 11B). The low peak of HserG compared to Lser might represent lower ionizing efficiency of larger molecules, or the presence of lower molar ratio of HserG than Lser in the sample.

[149] Affinity-purified $SL335_{\Delta ds}$-hGH was further purified by passing through SephacrylS-200HR column using FPLC. Gel filtration of HserG/Lser was performed after affinity purification using Sephacryl™ S-200HR Prepacked Column and AKTA FPLC (GE Healthcare, Wauwatosa, WI, USA). The column was equilibrated with equilibration buffer (20 mM HEPES pH 7.4 containing 150 mM NaCl), and loaded with affinity-purified HserG/Lser. Elution was performed with equilibration buffer at 0.5 ml/min running flow rate. Arrows indicate the fractions chosen for SDS-PAGE analysis (Fig. 12A). Fraction #13, #16, #19 and #23 that retrieved from two distinctive peaks were analyzed by 4-12% Bis-Tris gel under the reducing condition (Fig. 12B). Protein bands were visualized with Coomassie Blue staining. Two peaks that correspond to approximately 66 kDa and 25 kDa were visible from the fraction #12 to #27 (Fig. 9A). Thence, four fractions (fraction #13, #16, #19 and #23) were analyzed by SDS-PAGE under the reducing condition to determine protein contents in the fractions (Fig. 9B). The results showed that the fractions from the 66 kDa peak (fraction #13, #16 and #19) contained the heterodimeric $SL335_{\Delta ds}$-hGH, and the fraction from the 25 kDa peak

(fraction #23) mainly contained the monomeric Lser.

[150]

[151] *2-(7) In vitro functional characterization of SL335Δds-hGH*

[152] To determine whether removal of an interchain disulfide bond in $SL335_{wt}$ and the fusion of the hGH affect binding affinities to HSA or RSA, a biolayer interferometry assay was performed using $SL335_{\Delta ds}$-hGH under pH 6 and pH 7.4 conditions (see the Table 6 below). The dissociation constants of $SL335_{\Delta ds}$-hGH to HSA were 1.7 nM at pH 6 and 1.5 nM at pH 7.4, showing a five-fold and an 8.7-fold increase of affinity compared to those of SL335, respectively. The dissociation constants to RSA were 499 nM and 83.6 nM under pH 6 and pH 7.4, showing a 4.2-fold and a 1.3-fold decrease of affinity compared with those of SL335, respectively.

[153] The *in vitro* hGH activity of $SL335_{ds}$-hGH was also measured using the Nb2-11 rat lymphoma cells that proliferate upon hGH treatment in a concentration-dependent manner. Nb2-11 rat lymphoma cells were resuspended in DMEM containing 5% (v/v) horse serum at 8×10^4 cells/ml, and a 50 $\mu\ell$ aliquot of the cell suspension was added into each well of the 96-well plates, followed by overnight incubation. The cells were then treated with increasing concentrations of Growtropin® or HserG/Lser (0 - 20 nM) in 50 ml DMEM containing 5% horse serum for 48 h at 37°C. Following incubation, 10 $\mu\ell$ of CCK-8 solution was added to each well, and cells were incubated for 4 h. The absorbance was recorded on a microplate reader at a wavelength of 450 nm. The data represent the average SD of three experiments. In the absence of HSA, $SL335_{\Delta ds}$-hGH was able to stimulate the growth of Nb2-11 with an apparent EC_{50} of 50 pM (3.5 ng/ml) (Fig. 13A). This value is 6.7-fold less potent than that of Growtropin®, the rhGH standard (7.5 pM). In the presence of 10 mM HSA, the respective potencies of Growtropin® and $SL335_{\Delta ds}$-hGHwere largely unaffected, although $SL335_{\Delta ds}$-hGH represented an approximately five-fold reduction in potency compared to that of Growtropin® (Fig. 13B). SL335 that was used as a negative control did not show any proliferative effect. These results clearly demonstrated a functional hGH bioactivity of $SL335_{\Delta ds}$-hGH.

[154] The serum stability was then determined by incubating $SL335_{\Delta ds}$-hGH at 37°C for 16 days. FBS was used instead of human serum for resuspending the samples because the binding capabilities of $SL335_{\Delta ds}$-hGH and SL335 to HSA in human serum would complicate the subsequent experiments. Samples were collected once a day, and the HSA-binding reactivity and *in vitro* bioactivity were measured by ELISA (Fig. 14A) and the Nb2-11 cell proliferation assay (Fig. 14B), respectively. SL335 was also included as a control. Similar to SL335, the binding reactivity to HSA and the Nb2-11 proliferative activity of $SL335_{\Delta ds}$-hGH did not change even after 16 days of incubation at 37°C, demonstrating that $SL335_{\Delta ds}$-hGH is as stable as SL335 despite the absence of

the interchain disulfide bond.

[155]

[156] *2-(8) Pharmacokinetics and pharmacodynamics studies in rats*

[157] Because SL335$_{\Delta ds}$-hGH was shown to be a promising candidate for a long-acting hGH, *in vivo* efficacy studies were performed. Firstly, the pharmacokinetics of Growtropin® and SL335$_{\Delta ds}$-hGH were compared in rats by measuring serum levels of each analog as a function of time after a single intravenous or subcutaneous injection. Each group of rats (four in a group) was given subcutaneous injection (Fig. 15A) of a single bolus dose of 0.6㎎/㎏ of Growtropin or SAFAtropin, or intravenous injection (Fig. 15B) of a single bolus dose of 0.3㎎/㎏ of Growtropin or SAFAtropin. Serum samples were taken over intervals extending to 144h depending upon the protein. Serum samples were analyzed at indicated times for Growtropin® or SAFAtropin® by an ELISA as described above. The pharmacokinetic parameters are shown in Table 7.

[158]

[159] Table 7

[Table 7]

Pharmacokinetic parameters in rats given a single intravenous or subcutaneous injection of Growtropin or SAFAtropin

		$t_{1/2}$ (h)	Cmax (ng/ml)	$AUC_{0\rightarrow\infty}$ (h ng/ml)	Cl/f (ml/hr/kg)
I.V.	Growtropin	0.23±0.05	5168.69±61.32	1759.97±145.03	171.04±13.66
	SAFAtropin	16.6±1.5	882.2±81.8	19580.3±999.3	15.34±0.76
S.C.	Growtropin	1.35±0.13	283.42±28.84	821.8±52.56	714.79±45.63
	SAFAtropin	97.16±30.86	83.2±23.12	7689.4±2640.71	56.11±25.39

[160] Values shown are averages standard deviation. Abbreviations are as follow: Cmax: maximum concentration; $t_{1/2}$: terminal half-life; $AUC_{0\rightarrow\infty}$: area under the concentration-time curve extrapolated to infinity; Cl/f: apparent total plasma clearance.

[161] SL335$_{\Delta ds}$-hGH showed dramatically extension of the $t_{1/2}$ irrespective of the route of administration. In intravenous administration, SL335$_{\Delta ds}$-hGH represented an 83-fold increase in the $t_{1/2}$ compared to Growtropin (16.6 h vs. 0.2 h) and a 69-fold increase in the subcutaneous administration (97.2 h *vs.* 1.4 h).

[162] SL335$_{\Delta ds}$-hGH also exhibited a ~ 10-fold increase in $AUC_{0\rightarrow\infty}$ and a more than

10-fold slower clearance rate (Cl/f) compared to those of Growtropin® regardless of the route of administration. Each group of rats (four in a group) was given subcutaneous injection of a single bolus dose of 0.6 mg/kg of Growtropin or SAFAtropin, or intravenous injection of a single bolus dose of 0.3 mg /kg of Growtropin or SAFAtropin. Serum samples were taken over intervals extending to 144 h depending upon the protein. Serum samples were analyzed at indicated times for Growtropin® or SAFAtropin® by an ELISA as described above. Interestingly, the C_{max} values of SL335$_{\Delta ds}$-hGH were 6-fold and 3-fold lower than those of Growtropin® depending on the route of administration.

[163] Next, the growth rates of hypophysectomized rats were compared over ten days after daily S.C. administration of Growtropin® or an excipient buffer control (Excipient only), or once-weekly S.C. administration of SL335$_{ds}$-hGH. Hypophysectomized rats were treated with Excipient only or 0.3 mg/kg Growtropin® daily, or with increasing dose of SAFAtropin® on days 0 and 7 (Fig. 16). Solid lines indicate the mean percentage change in body weight. Error bars represent standard deviation. The excipient-treated rats showed an approximately 5% weightloss. Whereas, those receiving daily injection of Growtropin® (0.3 mg/kg) showed a 5% weight gain, resulting in a total 10% weight gain over the Excipient Only group. Once-weekly injections of SL335$_{\Delta ds}$-hGH produced dose-dependent weight gains in that the 2.4 mg/kg dosage produced a 15% weight gain, and the 0.6 mg/kg dosage produced a 3.5 % weight gain. An equimolar SL335$_{\Delta ds}$-hGH (1.2 mg/kg) dosage regimen resulted in a 5% weight gain which was comparable to that obtained by daily injections of Growtropin®.

[164] Fig. 17 shows that the once-weekly administration of 0.6 mg/kg SL335$_{\Delta ds}$-hGH achieved equivalent increases in tibia length as those achieved by the daily administration of Growtropin®. Solid bars indicate the mean of measured tibia bone length. Error bars represent standard deviation.

[165]

Industrial Applicability

[166] The present invention would be used to develop bioactive protein or polypeptide therapeutic agents, since the fusion constructs of the invention can be prepared to comprise various types of effector moieties including human growth hormone, interferon, erythropoietin, colony stimulating factors or derivaties therof, and antibody derivatives, etc.

[167]

[168] The teachings of all patents, published applications and references cited herein are incorporated by reference in their entirety.

[169]

[170]

BUDAPEST TREATY ON THE INTERNATIONAL RECOGNITION OF THE DEPOSIT OF MICROORGANISMS FOR THE PURPOSE OF PATENT PROCEDURE

INTERNATIONAL FORM

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT

issued pursuant to Rule 7.1

TO : **CHA, Sang Hoon**
AprilBio Co., Ltd.
1, Kangwondaehak-gil, Chuncheon-si, Gangwon-do 200-701
Republic of Korea

I. IDENTIFICATION OF THE MICROORGANISM	
Identification reference given by the DEPOSITOR: ***Escherichia coli* SUPEX5**	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: **KCTC 12657BP**
II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION	
The microorganism identified under I above was accompanied by: [x] a scientific description [ ] a proposed taxonomic designation (Mark with a cross where applicable)	
III. RECEIPT AND ACCEPTANCE	
This International Depositary Authority accepts the microorganism identified under I above, which was received by it on **August 20, 2014.**	
IV. RECEIPT OF REQUEST FOR CONVERSION	
The microorganism identified under I above was received by this International Depositary Authority on and a request to convert the original deposit to a deposit under the Budapest Treaty was received by it on	
V. INTERNATIONAL DEPOSITARY AUTHORITY	
Name: **Korean Collection for Type Cultures** Address: Korea Research Institute of Bioscience and Biotechnology (KRIBB) 125 Gwahak-ro, Yuseong-gu, Daejeon 305-806 Republic of Korea	Signature(s) of person(s) having the power to represent the International Depositary Authority of authorized official(s): D. S. Park PARK, Doo Sang, Director Date: **August 21 2014**

Form BP/4 (KCTC Form 17) side page

Claims

[Claim 1] An antigen binding fragment (Fab) to a serum albumin (SA), wherein the Fab comprises,
(a) a heavy chain variable domain (V_H domain) having the amino acid sequence selected from the group consisting of SEQ ID NO. 1, SEQ ID NO. 2, SEQ ID NO. 3, SEQ ID NO. 4, SEQ ID NO. 5 and SEQ ID NO.6; and
(b) a light chain variable domain (V_L domain) having the amino acid sequence selected from the group consisting of SEQ ID NO. 7, SEQ ID NO. 8, SEQ ID NO. 9, SEQ ID NO. 10, SEQ ID NO.11 and SEQ ID NO. 12, wherein the Fab binds specifically to serum albumin.

[Claim 2] An antigen binding fragment (Fab) binding to a serum albumin (SA), wherein the Fab comprises,
(a) the amino acid sequences of SEQ ID NOS. 13 (CDR1), 14 (CDR2) and 15 (CDR15) determining the CDRs of V_H domain; and
(b) the amino acid sequences of SEQ ID NOS. 16 (CDR1), 17 (CDR2) and 18 (CDR3) determining the CDRs of V_L domain.

[Claim 3] The Fab according to claim 2, wherein the V_H domain is bound to the heavy chain constant 1 domain (C_{H1} domain), and V_L domain is bound to light chain constant domain ($C_{\kappa L}$ domain).

[Claim 4] The Fab according to claim 3, wherein the V_H domain has the amino acid sequence of SEQ ID NO. 6 and the V_L domain has the amino acid sequence of SEQ ID NO. 12.

[Claim 5] The Fab according to any one of claims 1 to 4, wherein the amino acid of Cystein of C_{H1} domain and $C_{\kappa L}$ domain are deleted or substituted with any other amino acid residues, including serine, except for cysteine.

[Claim 6] The Fab according to claim 5, wherein the amino acid of Cystein of C_{H1} domain is the 233th amino acid starting from the N-terminus of the C_{H1} domain, and the Cystein of $C_{\kappa L}$ domain is the 214th amino acid starting from the N-terminus of the $C_{\kappa L}$ domain.

[Claim 7] A fusion construct of an antigen binding fragment (Fab) and a bioactive effector moiety, wherein the amino acid of Cystein of C_{H1} domain and the amino acid of Cystein of $C_{\kappa L}$ domain of the Fab are deleted or substituted with any other amino acid residue, including serine, except for cysteine; and wherein the bioactive effector moiety is a protein or a (poly)peptide; and wherein the Fab and the bioactive effector moiety

are covalently linked by genetic fusion.

[Claim 8] A fusion construct of the antigen binding fragment (Fab) of any one of claims 1 to 6 and a bioactive effector moiety; wherein the bioactive effector moiety is a protein or a (poly)peptide; and wherein the Fab and the bioactive effector moiety are covalently linked by genetic fusion.

[Claim 9] The fusion construct according to claim 7 or claim 8, wherein the Fab and the bioactive effector moiety are covalently linked by genetic fusion using a peptide linker of 0 to 20 amino acids.

[Claim 10] The fusion construct according to any one of claims 7 to 9, wherein the bioactive effector moiety is one selected from the group consisting of hormone, cytokine, enzyme, antibody, growth factor, transcription factor, blood factor, vaccine, structure protein, ligand protein, and receptor.

[Claim 11] The fusion construct according to claim 7 to 9, wherein the bioactive effector moiety is one selected from the group consisting of human growth hormone(hGH), growth hormone releasing hormone (GHRH), growth hormone releasing peptide, interferons, interferon receptors, colony stimulating factors (CSFs), glucagon-like peptides, G-protein-coupled receptor, interleukins, interleukin receptors, enzymes, interleukin binding proteins, cytokine binding proteins, macrophage activating factor, macrophage peptide, B cell factor, T cell factor, protein A, allergy inhibitor, cell necrosis glycoproteins, immunotoxin, lymphotoxin, tumor necrosis factor, tumor suppressors, metastasis growth factor, alpha-1 antitrypsin, albumin, alpha-lactalbumin, apolipoprotein-E, erythropoietin, highly glycosylated erythropoietin, angiopoietins, hemoglobin, thrombin, thrombin receptor activating peptide, thrombomodulin, factor VII, factor VIIa, factor VIII, factor IX, factor XIII, plasminogen activating factor, fibrin-binding peptide, urokinase, streptokinase, hirudin, protein C, C-reactive protein, renin inhibitor, collagenase inhibitor, superoxide dismutase, leptin, platelet-derived growth factor, epithelial growth factor, epidermal growth factor, angiostatin, angiotensin, bone growth factor, bone stimulating protein, calcitonin, insulin, atriopeptin, cartilage inducing factor, elcatonin, connective tissue activating factor, tissue factor pathway inhibitor, follicle stimulating hormone, luteinizing hormone, luteinizing hormone releasing hormone, nerve growth factors, parathyroid hormone, relaxin, secretin, somatomedin, insulin-like growth factor, adrenocortical hormone, glucagon, cholecystokinin, pancreatic polypeptide, gastrin

releasing peptide, corticotropin releasing factor, thyroid stimulating hormone, autotaxin, lactoferrin, myostatin, receptors, receptor antagonists, cell surface antigens, virus derived vaccine antigens, monoclonal antibodies, polyclonal antibodies, and antibody fragments.

[Claim 12] The fusion construct according to claim 11, wherein the bioactive effector moiety is hGH, GCSFs, or IFNs.

[Claim 13] The fusion construct according to any one of claims 7 to 12, wherein the molar ratio of the bioactive (poly)peptide (or protein) to the Fab is between 1:1 and 10:1, preferably between 1:1 and 4:1.

[Claim 14] An expression vector comprising: (a) promoter; (b) a first nucleic acid sequence encoding the Fab of any one of claims 1 to 5; and (c) a second nucleic acid sequence encoding a bioactive (poly)peptide or protein and optionally a linker, wherein the promoter, the first nucleic acid sequence and the second nucleic acid sequence are operably linked.

[Claim 15] A host cell comprising the expression vector of claim 14.

[Claim 16] The host cell according to claim 15, in which the host cell is *E. coli.*

[Claim 17] The host cell according to claim 17, in which the host cell is SUPEX5 (KCTC 12657BP).

[Claim 18] A method of increasing soluble expression of a bioactive protein or a (poly)peptide in periplasm of *E. coli*, the method comprising introducing an expression vector into *E. coli*; wherein the expression vector comprises (a) promotor, (b) a first nucleic acid sequence encoding an antigen binding fragment (Fab), and (c) a second nucleic acid sequence encoding a linker and the bioacive (poly)peptide or protein; wherein the promotor, the first nucleic acid sequence and the second nucleic acid sequence are operably linked; and wherein the amino acids of Cystein of C_{H1} domain and $C_{\kappa L}$ domain of the Fab are deleted or substituted with Serine residues.

[Claim 19] The method of increasing soluble expression of a bioactive protein or a (poly)peptide in periplasm of *E. coli* of claim 18, wherein the *E. coli* is SUPEX5 (KCTC 12657BP).

[Claim 20] A method of increasing soluble expression of a bioactive protein or a (poly)peptide in periplasm of *E. coli*, which comprises introducing the expression vector of claim 13 into *E. coli.*

[Claim 21] The method of increasing soluble expression of a bioactive protein or a (poly)peptide in periplasm of *E. coli* of claim 20, wherein the *E. coli* is SUPEX5 (KCTC 12657BP).

[Claim 22] The method according to any one of claims claim 18 to 21, wherein the

bioactive protein or (poly)peptide is one selected from the group consisting of hormone, cytokine, enzyme, antibody, growth factor, transcription factor, blood factor, vaccine, structure protein, ligand protein, and receptor.

[Claim 23] The method according to any one of claim 18 to 21, in which the bioactive protein or (poly)peptide is one selected from the group consisting of human growth hormone(hGH), growth hormone releasing hormone (GHRH), growth hormone releasing peptide, interferons, interferon receptors, colony stimulating factors (CSFs), glucagon-like peptides, G-protein-coupled receptor, interleukins, interleukin receptors, enzymes, interleukin binding proteins, cytokine binding proteins, macrophage activating factor, macrophage peptide, B cell factor, T cell factor, protein A, allergy inhibitor, cell necrosis glycoproteins, immunotoxin, lymphotoxin, tumor necrosis factor, tumor suppressors, metastasis growth factor, alpha-1 antitrypsin, albumin, alpha-lactalbumin, apolipoprotein-E, erythropoietin, highly glycosylated erythropoietin, angiopoietins, hemoglobin, thrombin, thrombin receptor activating peptide, thrombomodulin, factor VII, factor VIIa, factor VIII, factor IX, factor XIII, plasminogen activating factor, fibrin-binding peptide, urokinase, streptokinase, hirudin, protein C, C-reactive protein, renin inhibitor, collagenase inhibitor, superoxide dismutase, leptin, platelet-derived growth factor, epithelial growth factor, epidermal growth factor, angiostatin, angiotensin, bone growth factor, bone stimulating protein, calcitonin, insulin, atriopeptin, cartilage inducing factor, elcatonin, connective tissue activating factor, tissue factor pathway inhibitor, follicle stimulating hormone, luteinizing hormone, luteinizing hormone releasing hormone, nerve growth factors, parathyroid hormone, relaxin, secretin, somatomedin, insulin-like growth factor, adrenocortical hormone, glucagon, cholecystokinin, pancreatic polypeptide, gastrin releasing peptide, corticotropin releasing factor, thyroid stimulating hormone, autotaxin, lactoferrin, myostatin, receptors, receptor antagonists, cell surface antigens, virus derived vaccine antigens, monoclonal antibodies, polyclonal antibodies, and antibody fragments.

[Claim 24] A method of increasing *in vivo* half-life of a bioactive protein or a (poly)peptide, which comprises linking a bioactive protein or a (poly)peptide to the Fab of any one of claims 1 to 6 by genetic fusion.

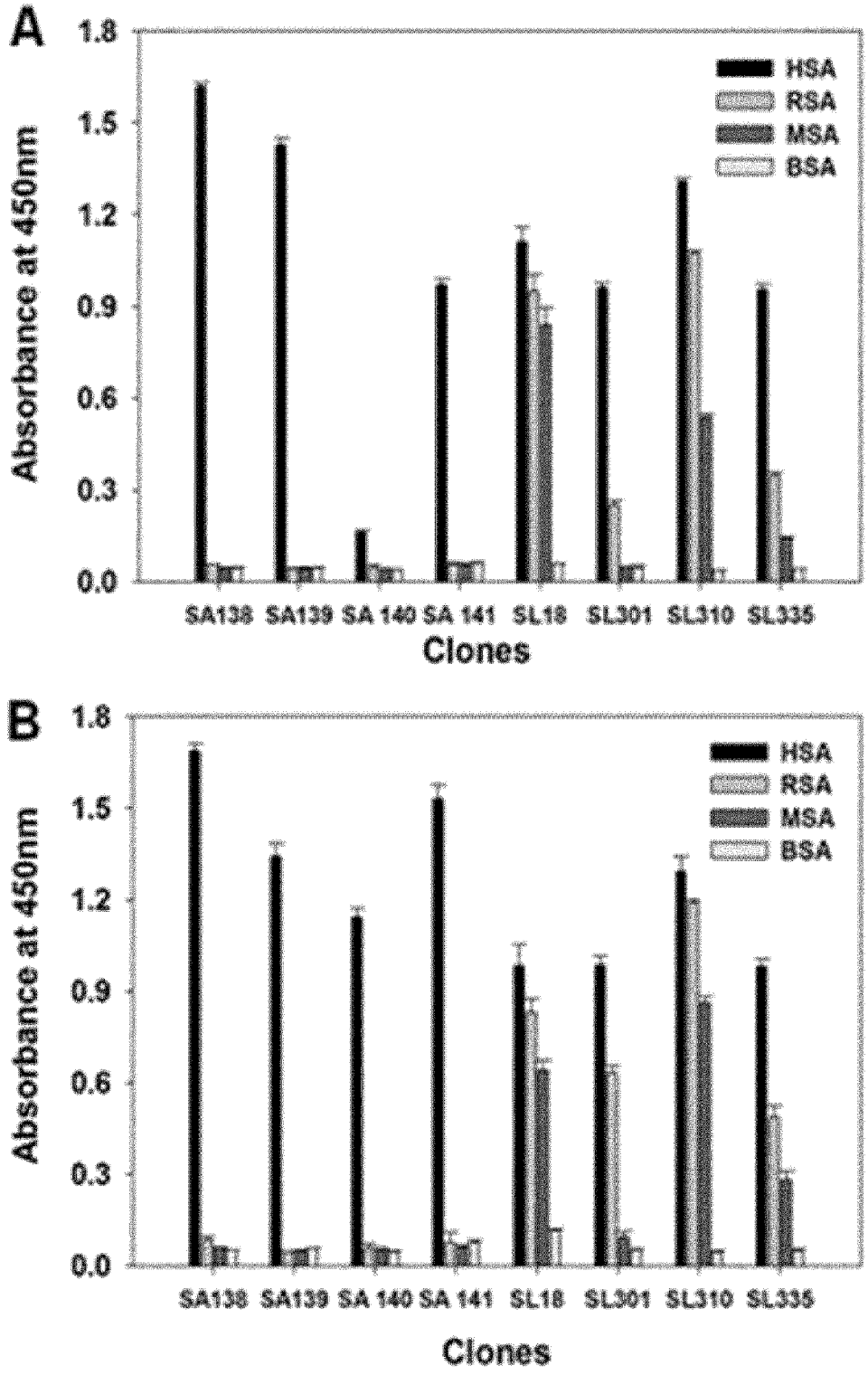
[Claim 25] The method of increasing *in vivo* half-life of a bioactive protein or a

(poly)peptide according to claim 24, wherein the bioactive protein or the (poly)peptide is linked to the Fab by a peptide linker of 0 to 20 amino acids.

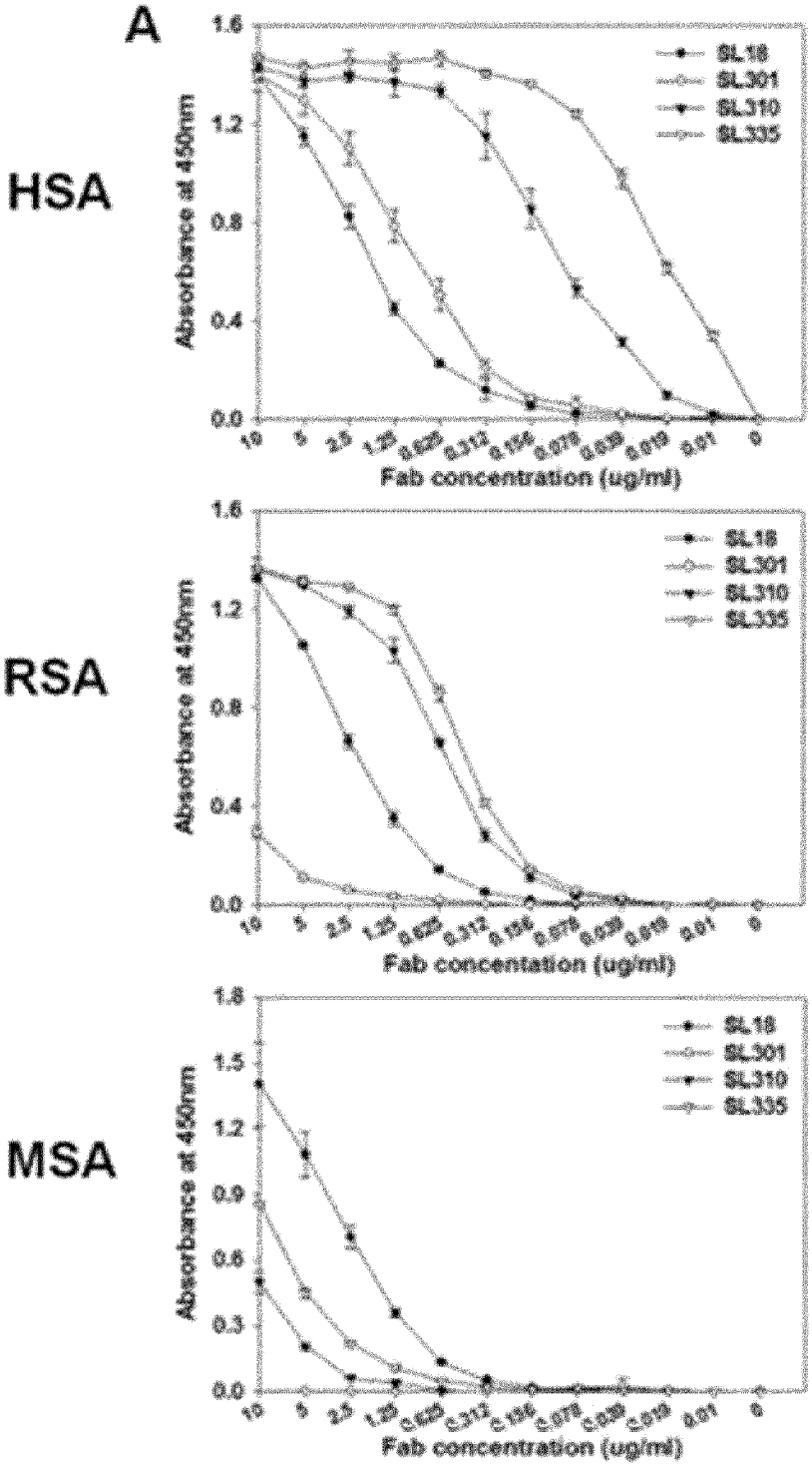
[Claim 26] The method according to claim 24 or claim 25, wherein the bioactive protein or (poly)peptide is one selected from the group consisting of human growth hormone (hGH), growth hormone releasing hormone (GHRH), growth hormone releasing peptide, interferons (IFNs), interferon receptors, colony stimulating factors (CSFs), granulocyte-colony stimulating factors (GCSFs), glucagon-like peptides, G-protein-coupled receptor, interleukins, interleukin receptors, enzymes, interleukin binding proteins, cytokine binding proteins, macrophage activating factor, macrophage peptide, B cell factor, T cell factor, protein A, allergy inhibitor, cell necrosis glycoproteins, immunotoxin, lymphotoxin, tumor necrosis factor, tumor suppressors, metastasis growth factor, alpha-1 antitrypsin, albumin, alpha-lactalbumin, apolipoprotein-E, erythropoietin, highly glycosylated erythropoietin, angiopoietins, hemoglobin, thrombin, thrombin receptor activating peptide, thrombomodulin, factor VII, factor VIIa, factor VIII, factor IX, factor XIII, plasminogen activating factor, fibrin-binding peptide, urokinase, streptokinase, hirudin, protein C, C-reactive protein, renin inhibitor, collagenase inhibitor, superoxide dismutase, leptin, platelet-derived growth factor, epithelial growth factor, epidermal growth factor, angiostatin, angiotensin, bone growth factor, bone stimulating protein, calcitonin, insulin, atriopeptin, cartilage inducing factor, elcatonin, connective tissue activating factor, tissue factor pathway inhibitor, follicle stimulating hormone, luteinizing hormone, luteinizing hormone releasing hormone, nerve growth factors, parathyroid hormone, relaxin, secretin, somatomedin, insulin-like growth factor, adrenocortical hormone, glucagon, cholecystokinin, pancreatic (poly)peptide, gastrin releasing peptide, corticotropin releasing factor, thyroid stimulating hormone, autotaxin, lactoferrin, myostatin, receptors, receptor antagonists, cell surface antigens, virus derived vaccine antigens, monoclonal antibodies, polyclonal antibodies, and antibody fragments.

[Claim 27] A pharmaceutical composition comprising the fusion construct of any one of claims 7 to 13, and pharmaceutically accepted excipient.

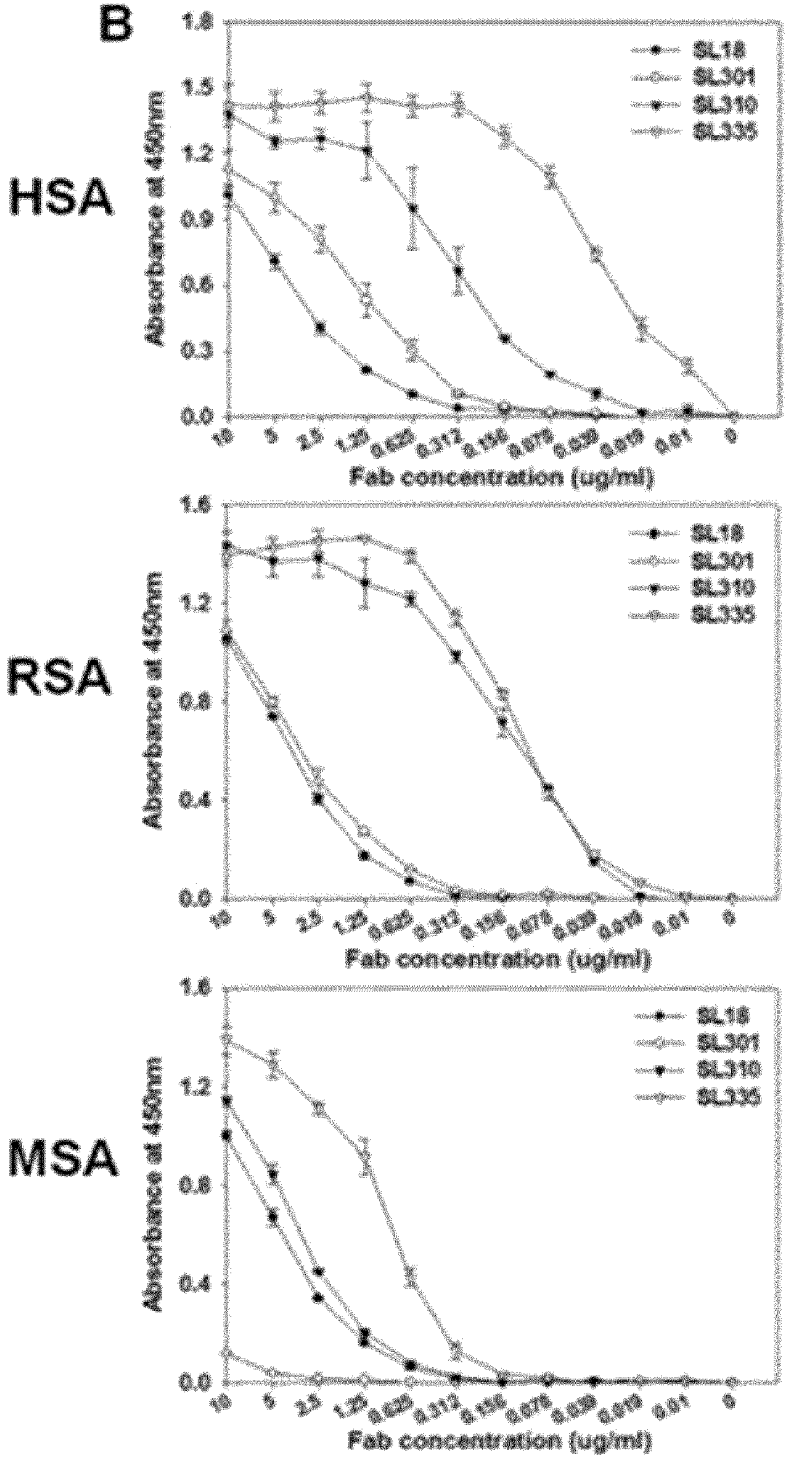
[Fig. 1]


A
Absorbance at 450nm
1.8
1.5
1.2
0.9
0.6
0.3
0.0
HSA
RSA
MSA
BSA
SA138 SA139 SA 140 SA 141 SL18 SL301 SL310 SL335
Clones
B
Absorbance at 450nm
1.8
1.5
1.2
0.9
0.6
0.3
0.0
HSA
RSA
MSA
BSA
SA138 SA139 SA 140 SA 141 SL18 SL301 SL310 SL335
Clones

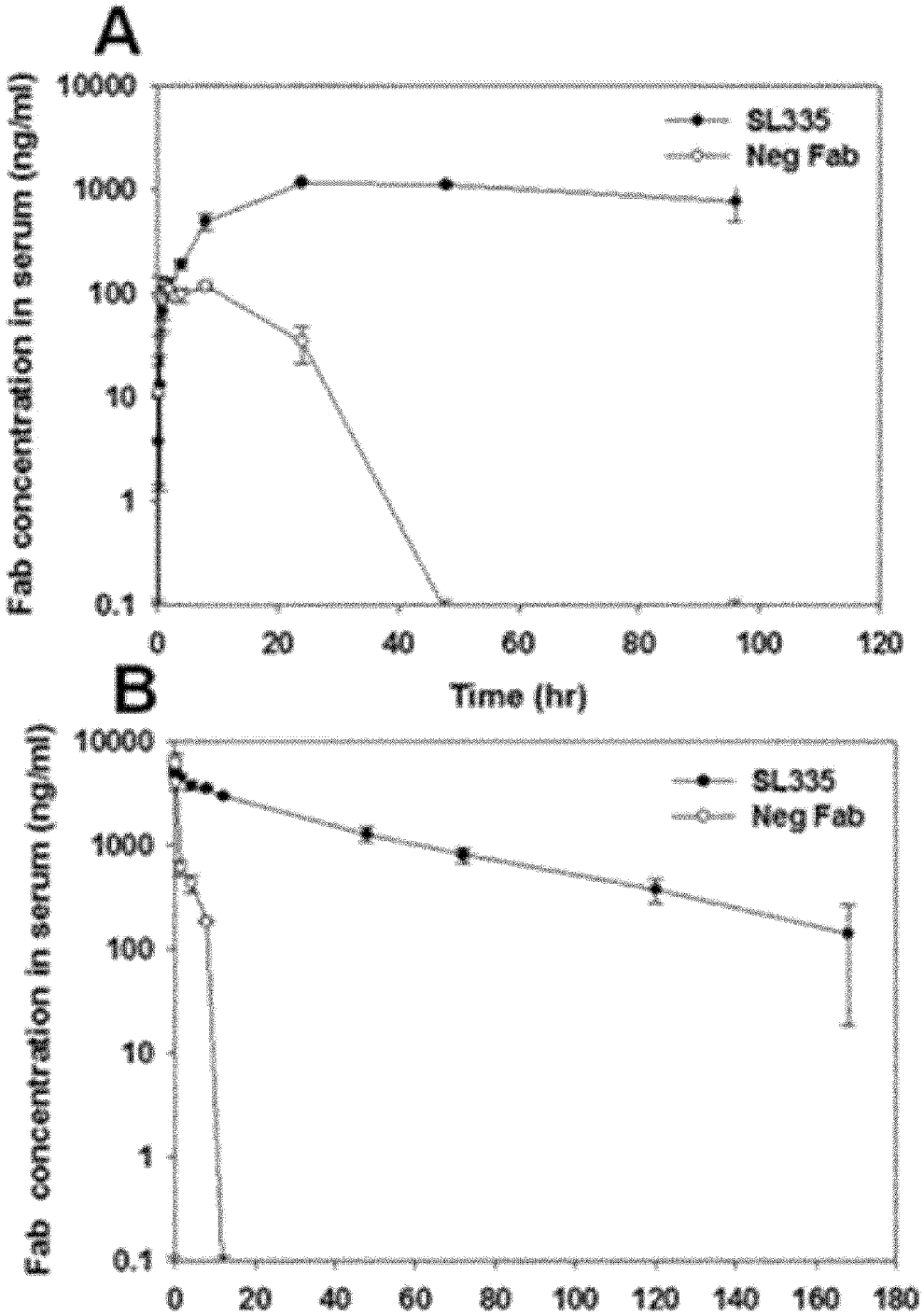
[Fig. 2a]


A
HSA
RSA
MSA
SL18
SL301
SL310
SL335
Absorbance at 450nm
Fab concentration (ug/ml)

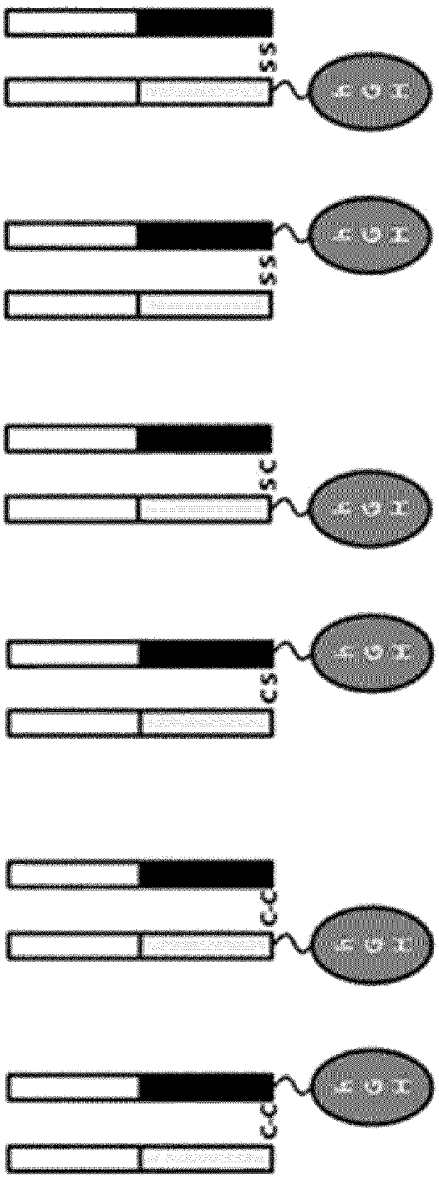
[Fig. 2b]


B
HSA
RSA
MSA
Absorbance at 450nm
Fab concentration (ug/ml)
SL18
SL301
SL310
SL335

[Fig. 3]


A
Fab concentration in serum (ng/ml)
10000
1000
100
10
1
0.1
0
20
40
60
80
100
120
Time (hr)
SL335
Neg Fab
B
Fab concentration in serum (ng/ml)
10000
1000
100
10
1
0.1
0
20
40
60
80
100
120
140
160
180
SL335
Neg Fab

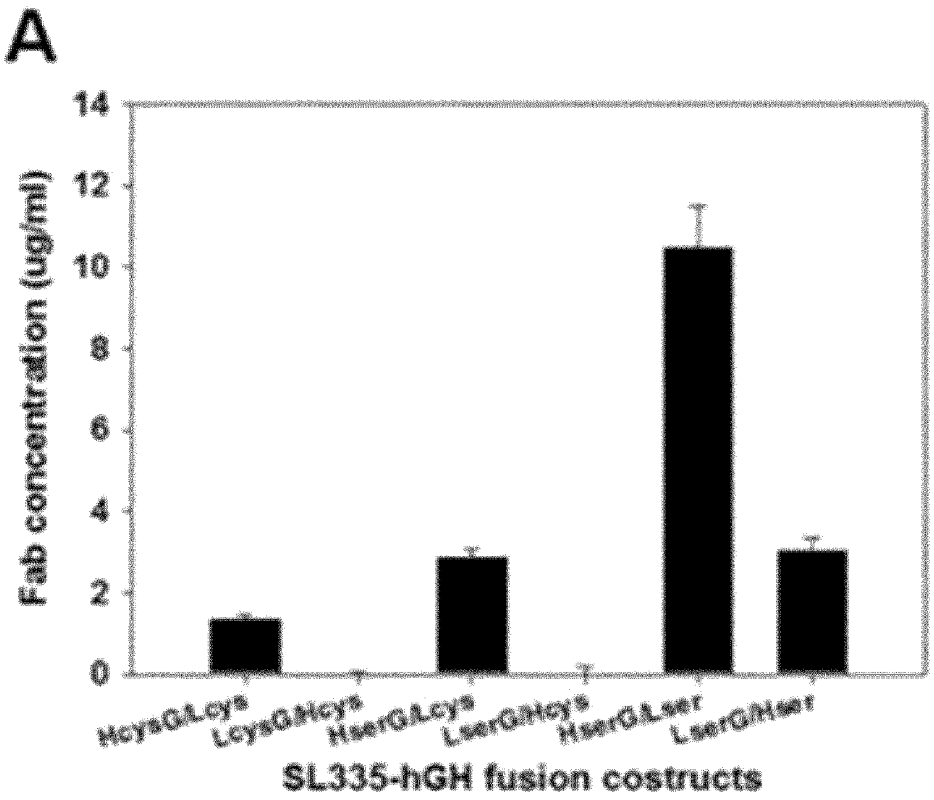
[Fig. 4]



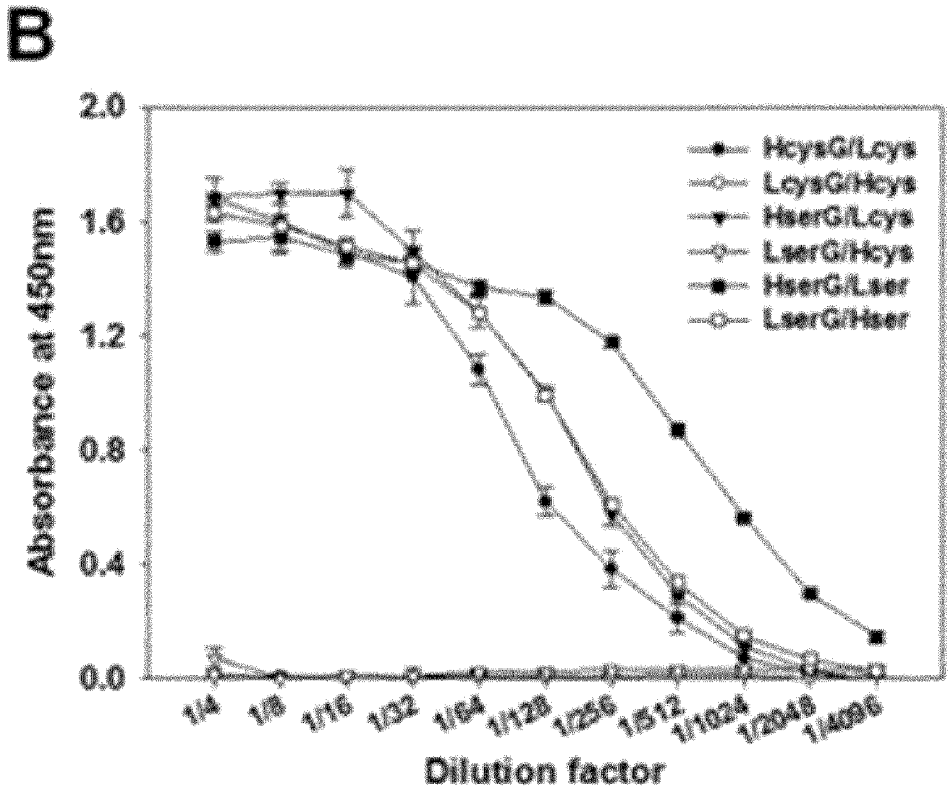
SL335	Classic Fab (SL335)		Null Fab (SL335$_{null}$)		Ads Fab (SL335$_{Ads}$)	
Fab-hGH format	Hcys/Lcys	Lcys/Hcys	Hser/Lcys	Lser/Hcys	Hser/Lser	Lser/Hser
L chain	335Lcys	335Lcys-hGH	335Lcys	335Lser-hGH	335Lser	335Lser-hGH
H chain	335Hcys-hGH	335Hcys	335Hser-hGH	335Hcys	335Hser-hGH	335Hser

Hcys = H chain with Cys^{233}, Lcys = L chain with Cys^{214}, Hser = H chain with Ser^{233}, Lser = L chain with Ser^{214}

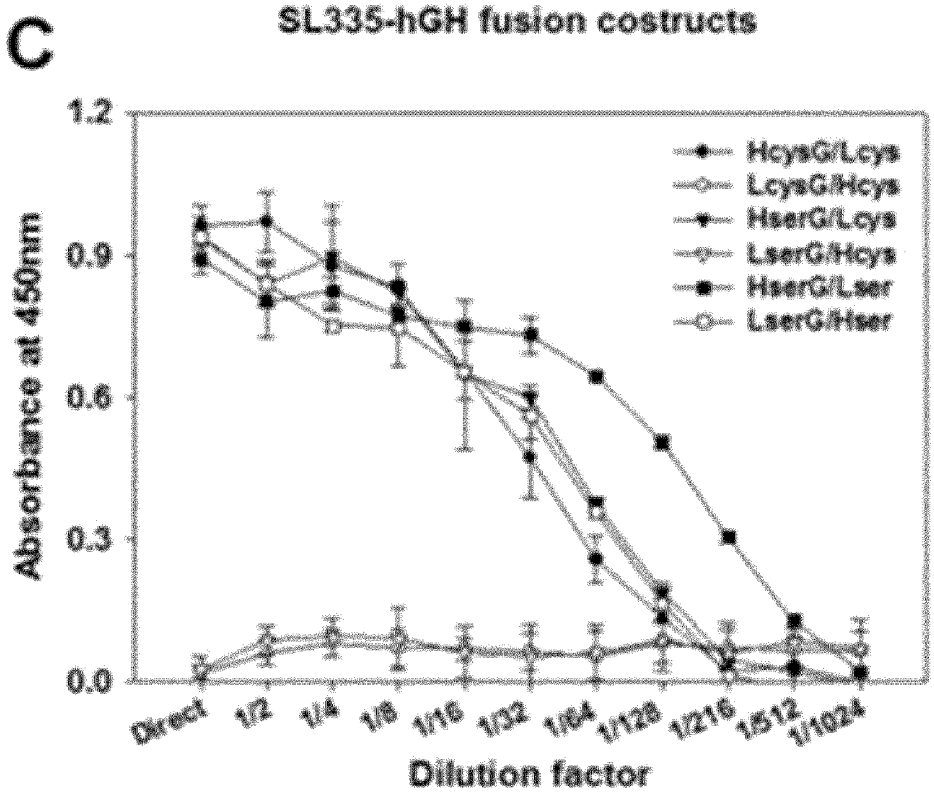
[Fig. 5a]


A
Fab concentration (ug/ml)
0
2
4
6
8
10
12
14
HcysG/Lcys
LcysG/Hcys
HserG/Lcys
LserG/Hcys
HserG/Lser
LserG/Hser
SL335-hGH fusion costructs

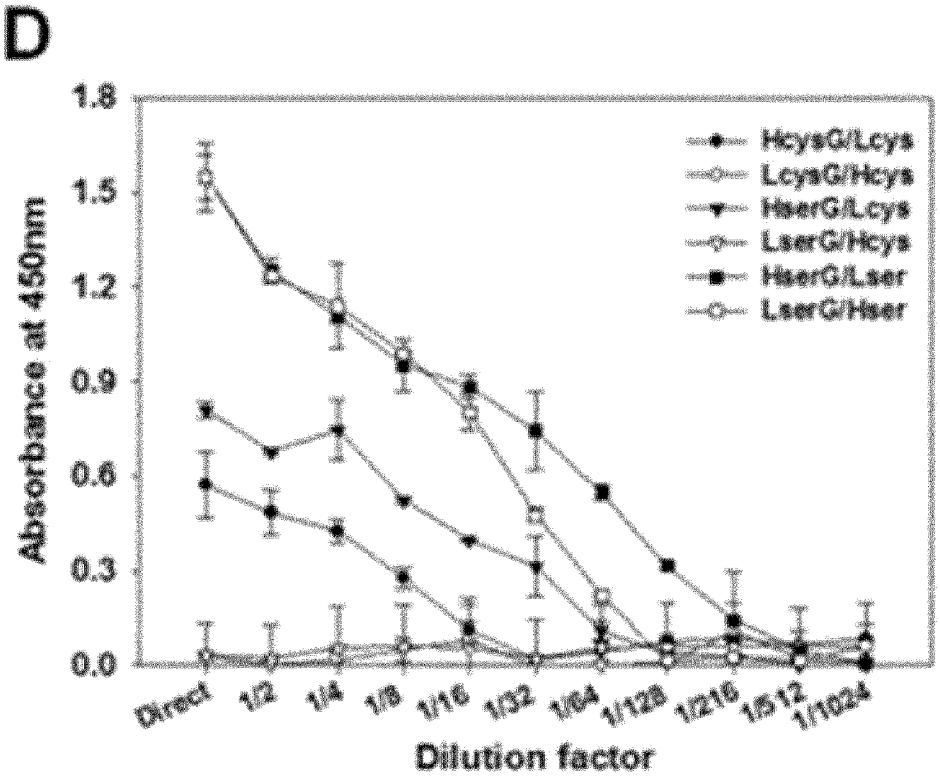
[Fig. 5b]


B
Absorbance at 450nm
0.0
0.4
0.8
1.2
1.6
2.0
HcysG/Lcys
LcysG/Hcys
HserG/Lcys
LserG/Hcys
HserG/Lser
LserG/Hser
1/4
1/8
1/16
1/32
1/64
1/128
1/256
1/512
1/1024
1/2048
1/4096
Dilution factor

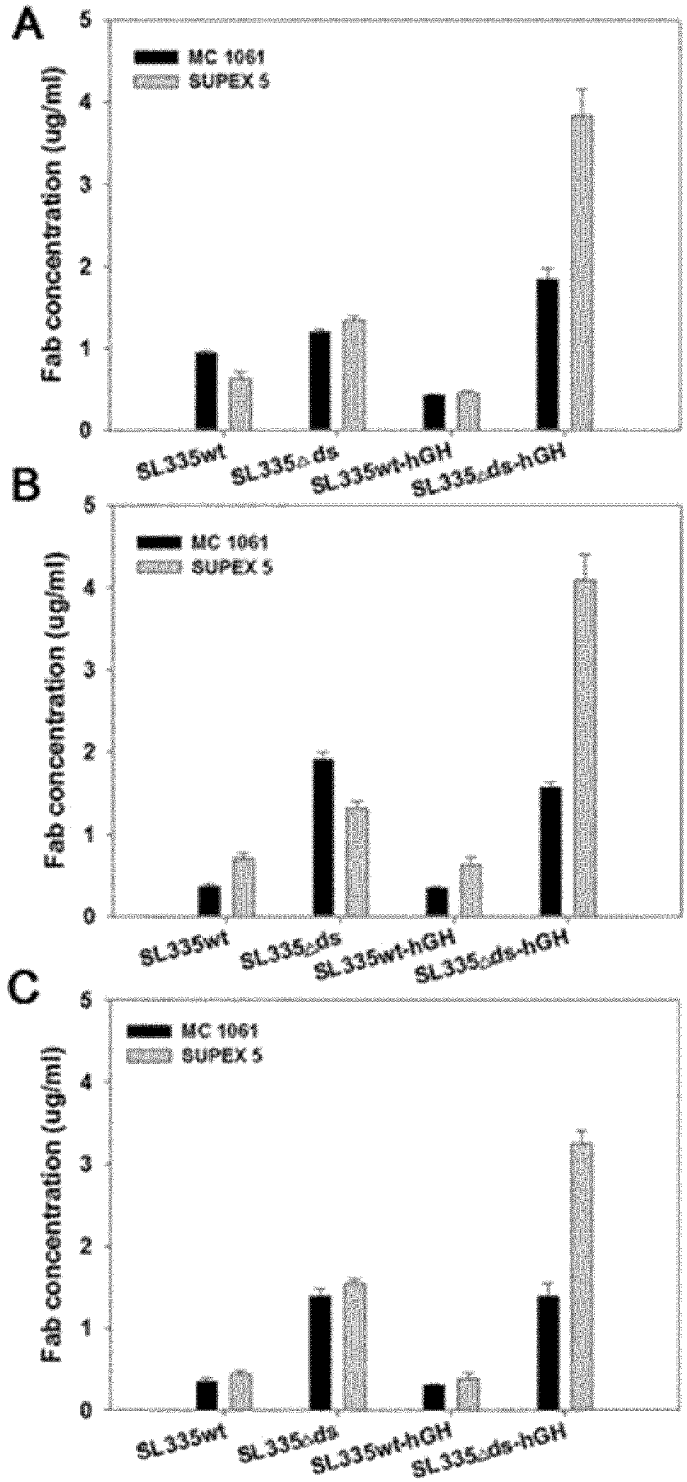
[Fig. 5c]


C
SL335-hGH fusion constructs
Absorbance at 450nm
1.2
0.9
0.6
0.3
0.0
HcysG/Lcys
LcysG/Hcys
HserG/Lcys
LserG/Hcys
HserG/Lser
LserG/Hser
Direct
1/2
1/4
1/8
1/16
1/32
1/64
1/128
1/256
1/512
1/1024
Dilution factor

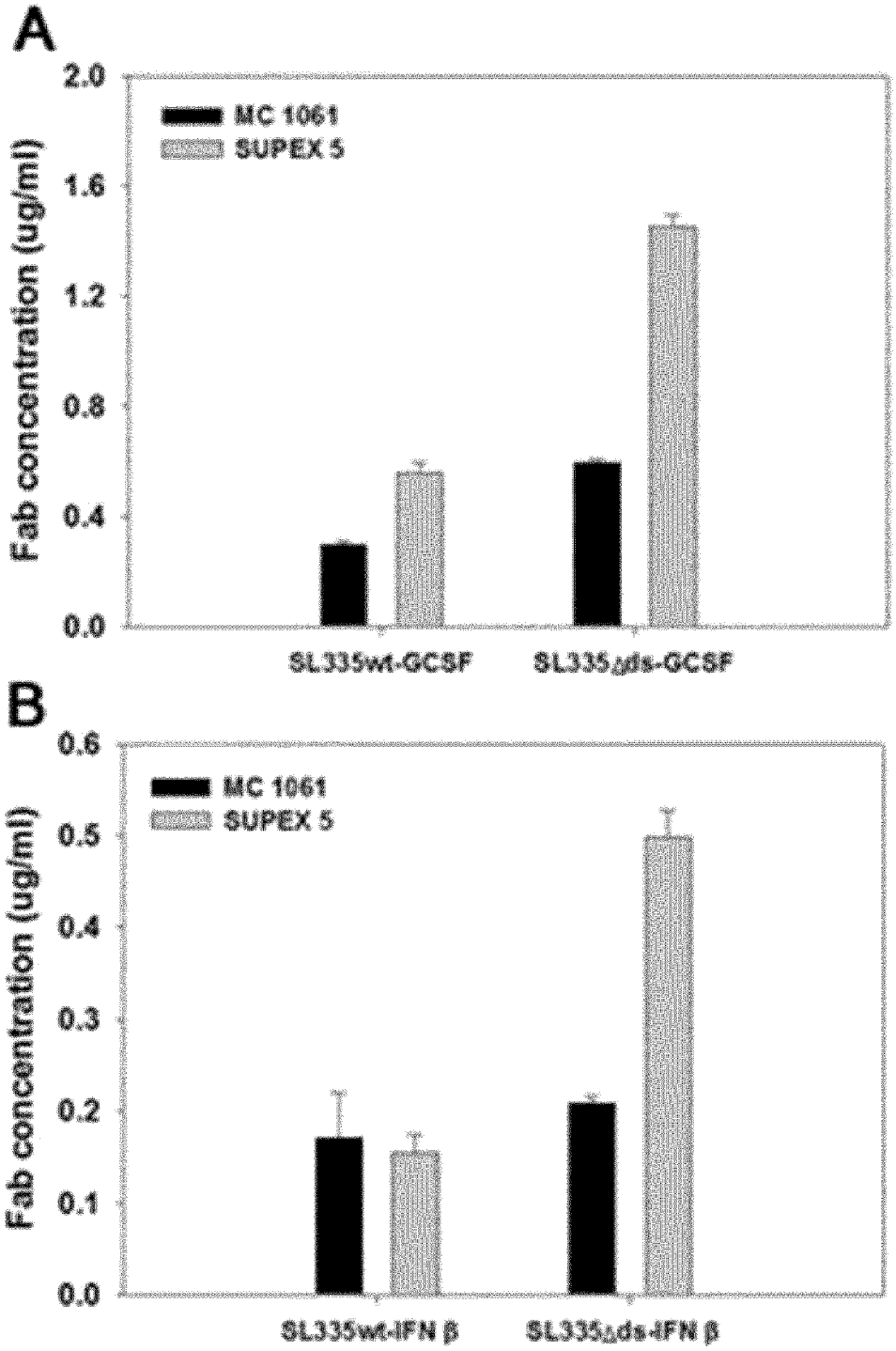
[Fig. 5d]


D
Absorbance at 450nm
1.8
1.5
1.2
0.9
0.6
0.3
0.0
HcysG/Lcys
LcysG/Hcys
HserG/Lcys
LserG/Hcys
HserG/Lser
LserG/Hser
Direct
1/2
1/4
1/8
1/16
1/32
1/64
1/128
1/256
1/512
1/1024
Dilution factor

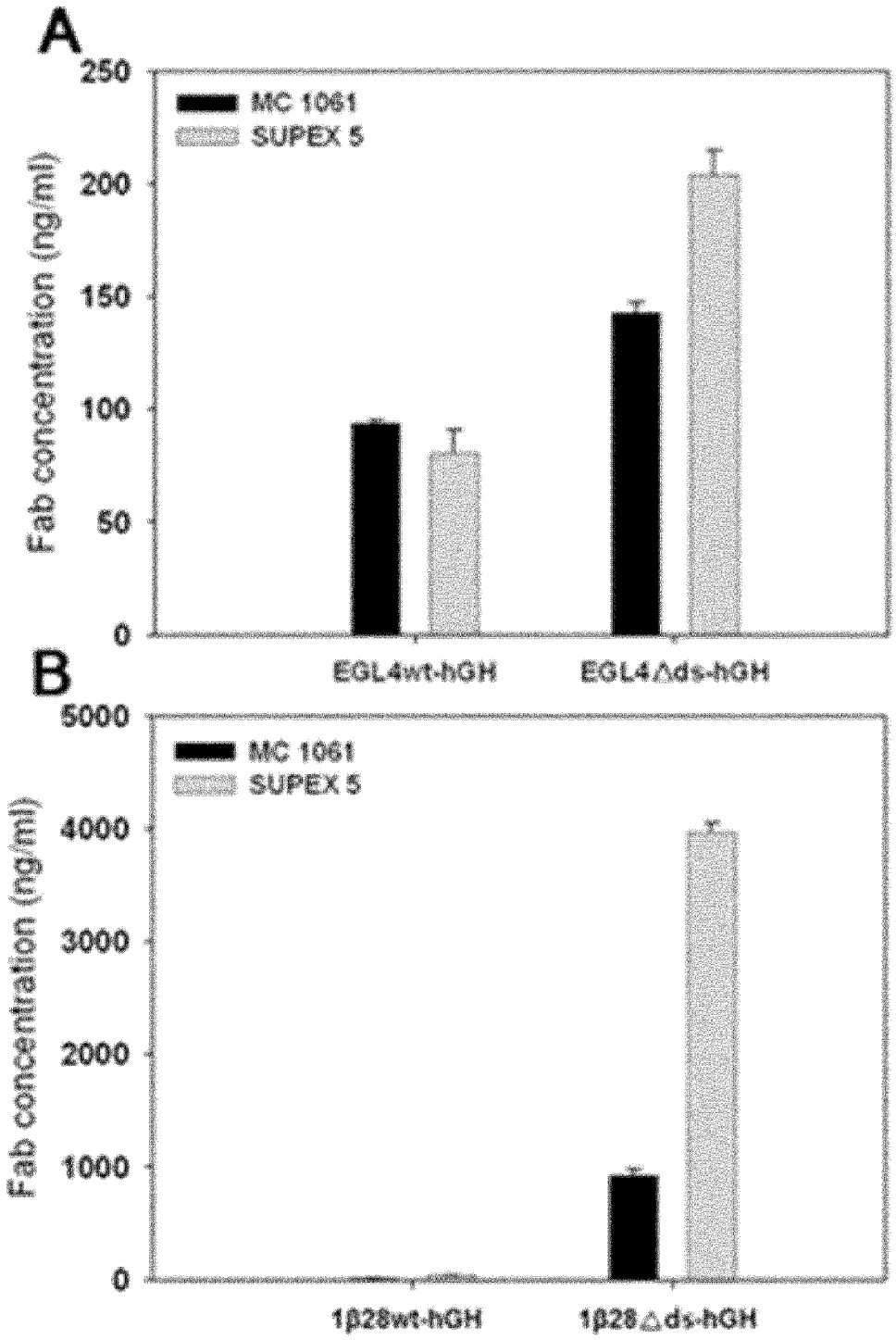
[Fig. 6]


A
B
C
MC 1061
SUPEX 5
Fab concentration (ug/ml)
0
1
2
3
4
5
SL335wt
SL335△ds
SL335wt-hGH
SL335△ds-hGH

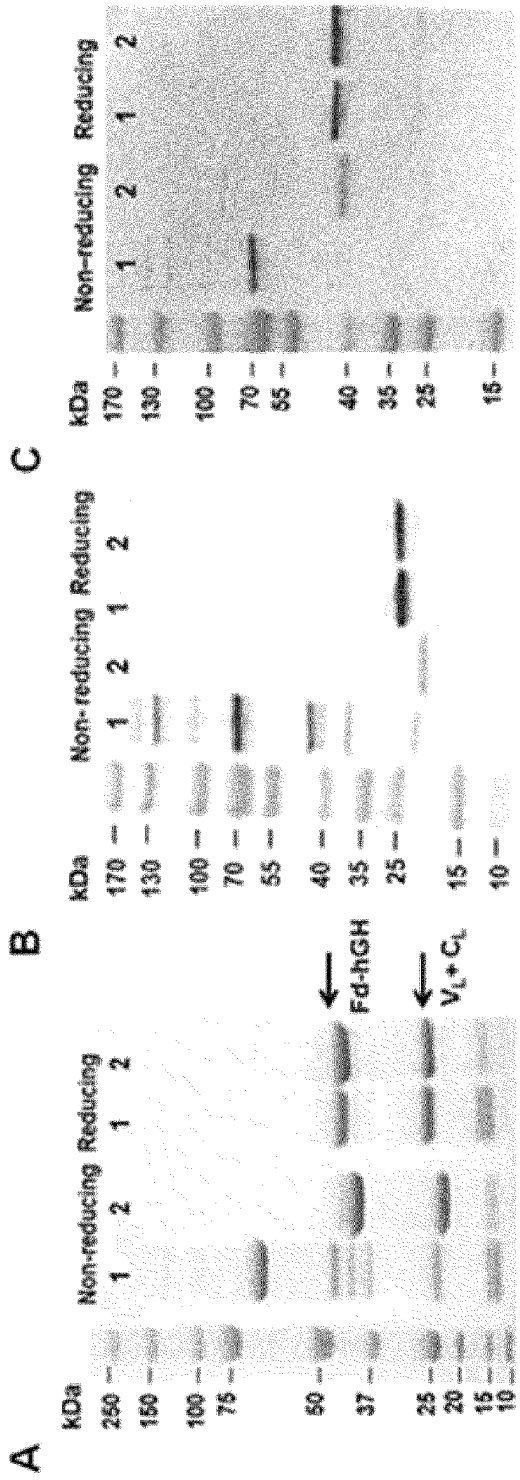
[Fig. 7]


A
Fab concentration (ug/ml)
2.0
1.6
1.2
0.8
0.4
0.0
MC 1061
SUPEX 5
SL335wt-GCSF
SL335Δds-GCSF
B
Fab concentration (ug/ml)
0.6
0.5
0.4
0.3
0.2
0.1
0.0
MC 1061
SUPEX 5
SL335wt-IFN β
SL335Δds-IFN β

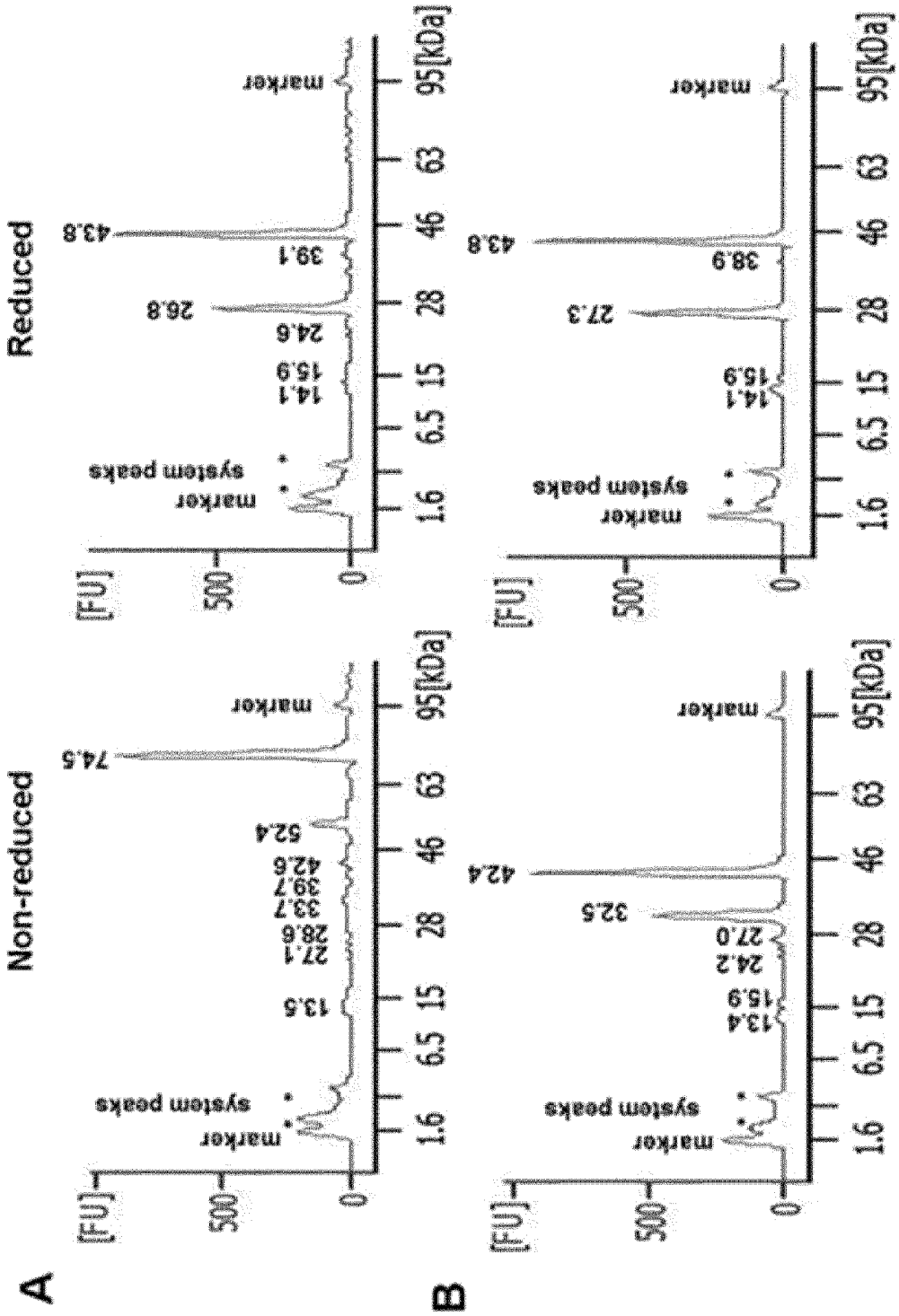
[Fig. 8]


A
250
200
150
100
50
0
Fab concentration (ng/ml)
MC 1061
SUPEX 5
EGL4wt-hGH
EGL4△ds-hGH
B
5000
4000
3000
2000
1000
0
Fab concentration (ng/ml)
MC 1061
SUPEX 5
1β28wt-hGH
1β28△ds-hGH

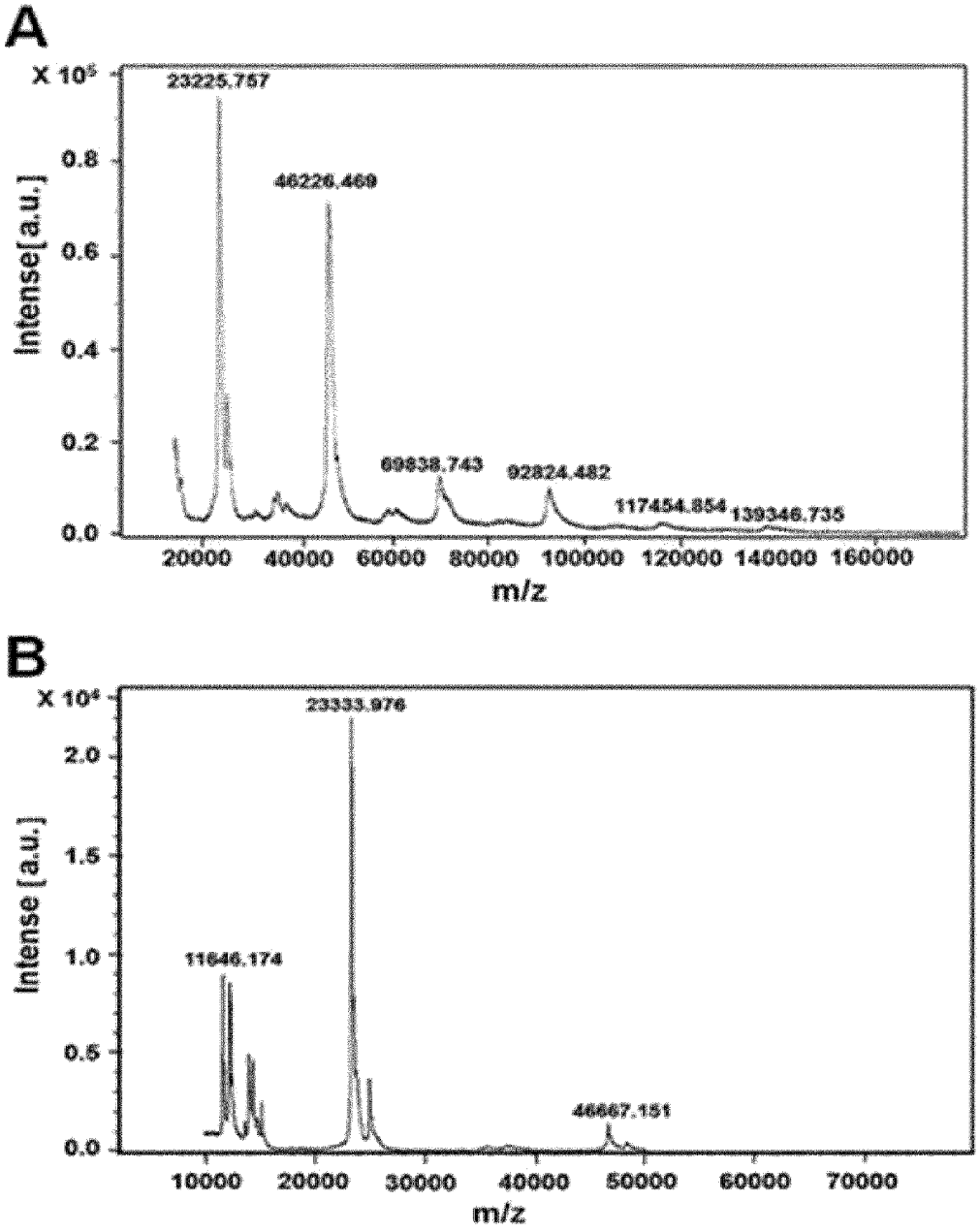
[Fig. 9]


A
kDa
250
150
100
75
50
37
25
20
15
10
Non-reducing
Reducing
1
2
Fd-hGH
$V_L + C_L$
B
kDa
170
130
100
70
55
40
35
25
15
10
Non-reducing
Reducing
1
2
C
kDa
170
130
100
70
55
40
35
25
15
Non-reducing
Reducing
1
2

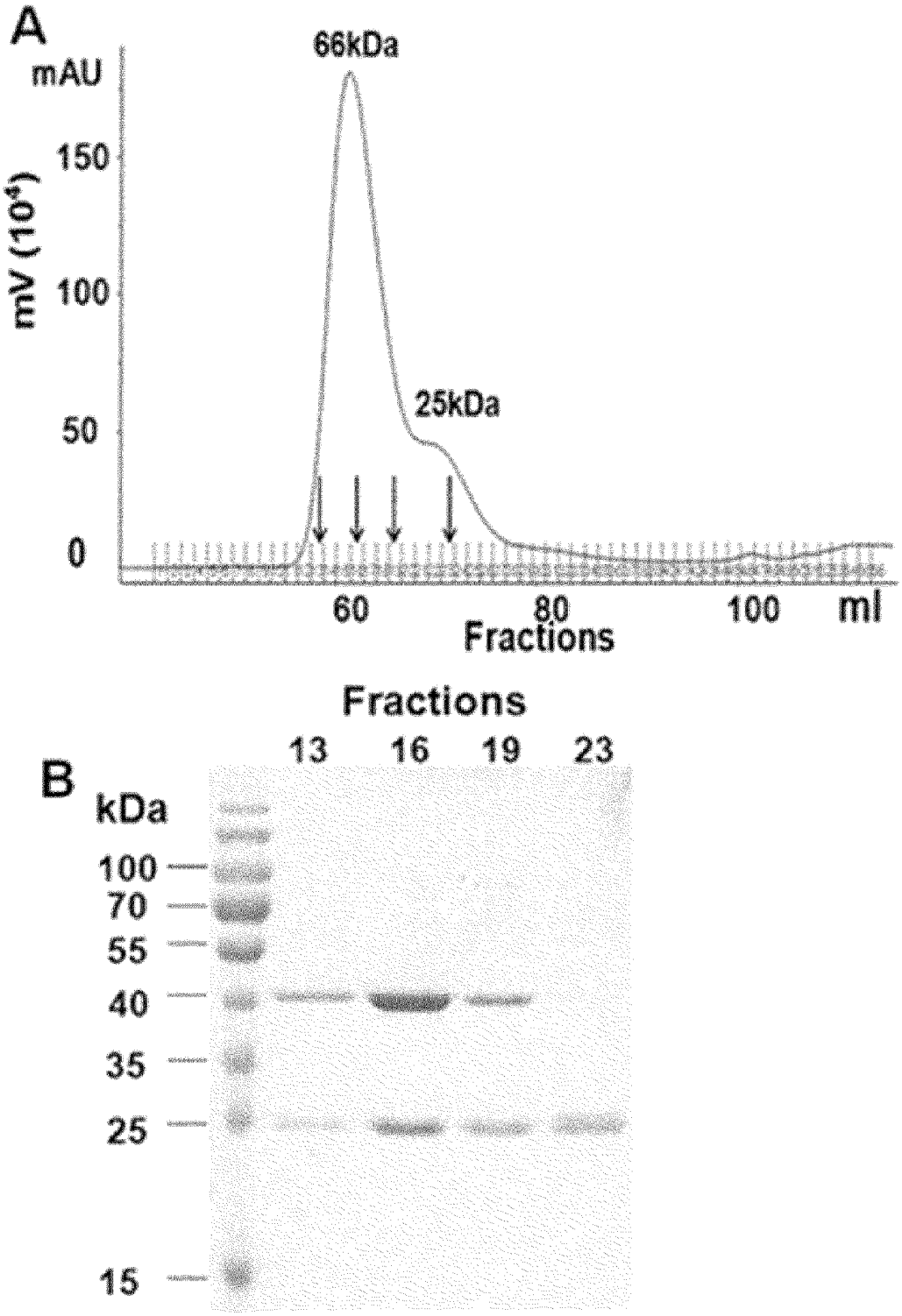
[Fig. 10]


A
B
Reduced
Non-reduced
[FU]
500
0
1.6
6.5
15
28
46
63
95[kDa]
marker
system peaks
43.8
39.1
26.8
24.6
15.9
14.1
74.5
52.4
42.6
39.7
33.7
28.6
27.1
13.5
42.4
32.5
27.0
24.2
15.9
13.4
38.9
27.3

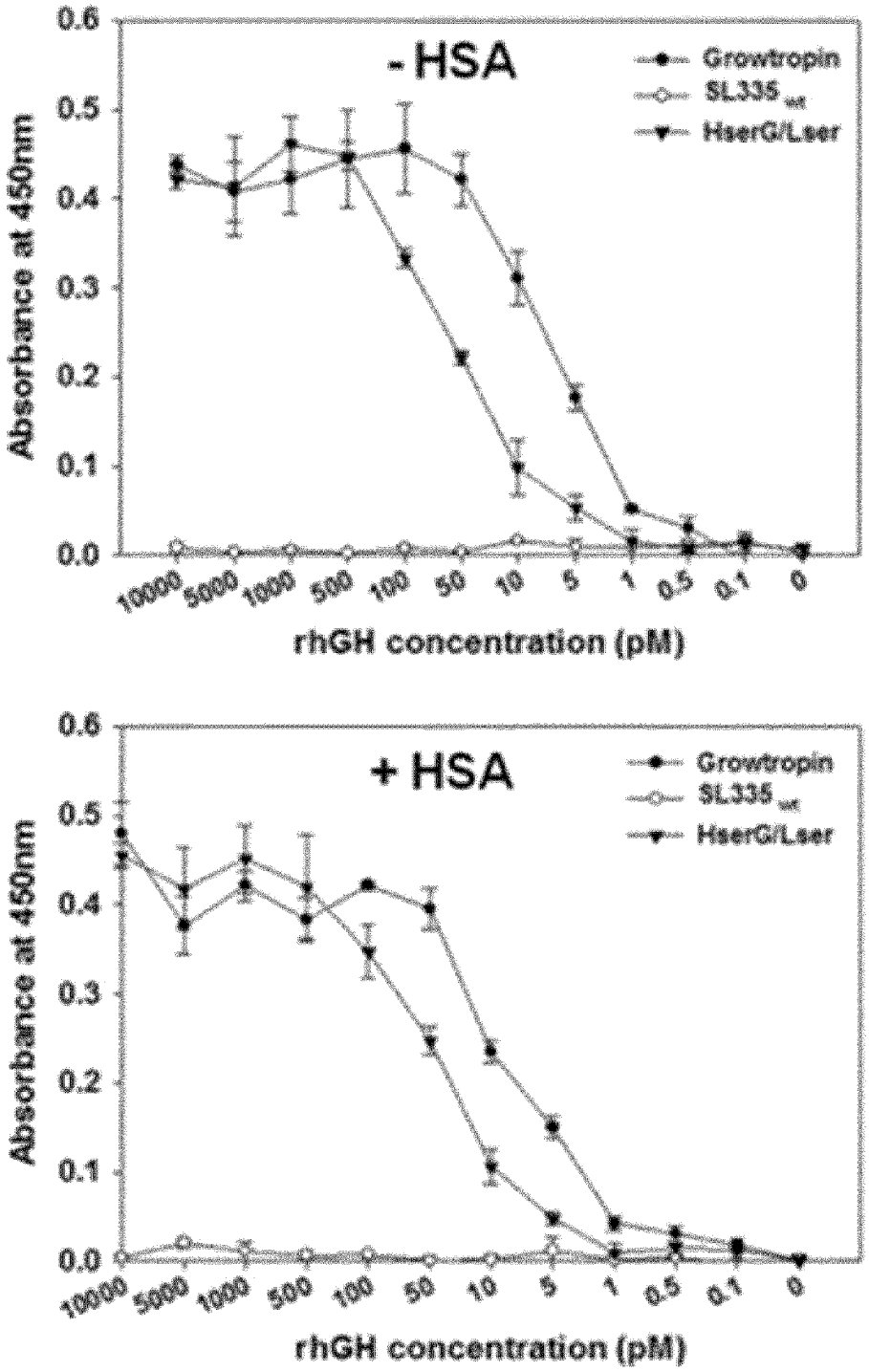
[Fig. 11]


A
X 10^5
Intense[a.u.]
23225.757
46226.469
69838.743
92824.482
117454.854
139346.735
m/z
B
X 10^4
Intense [a.u.]
23333.976
11646.174
46667.151
m/z

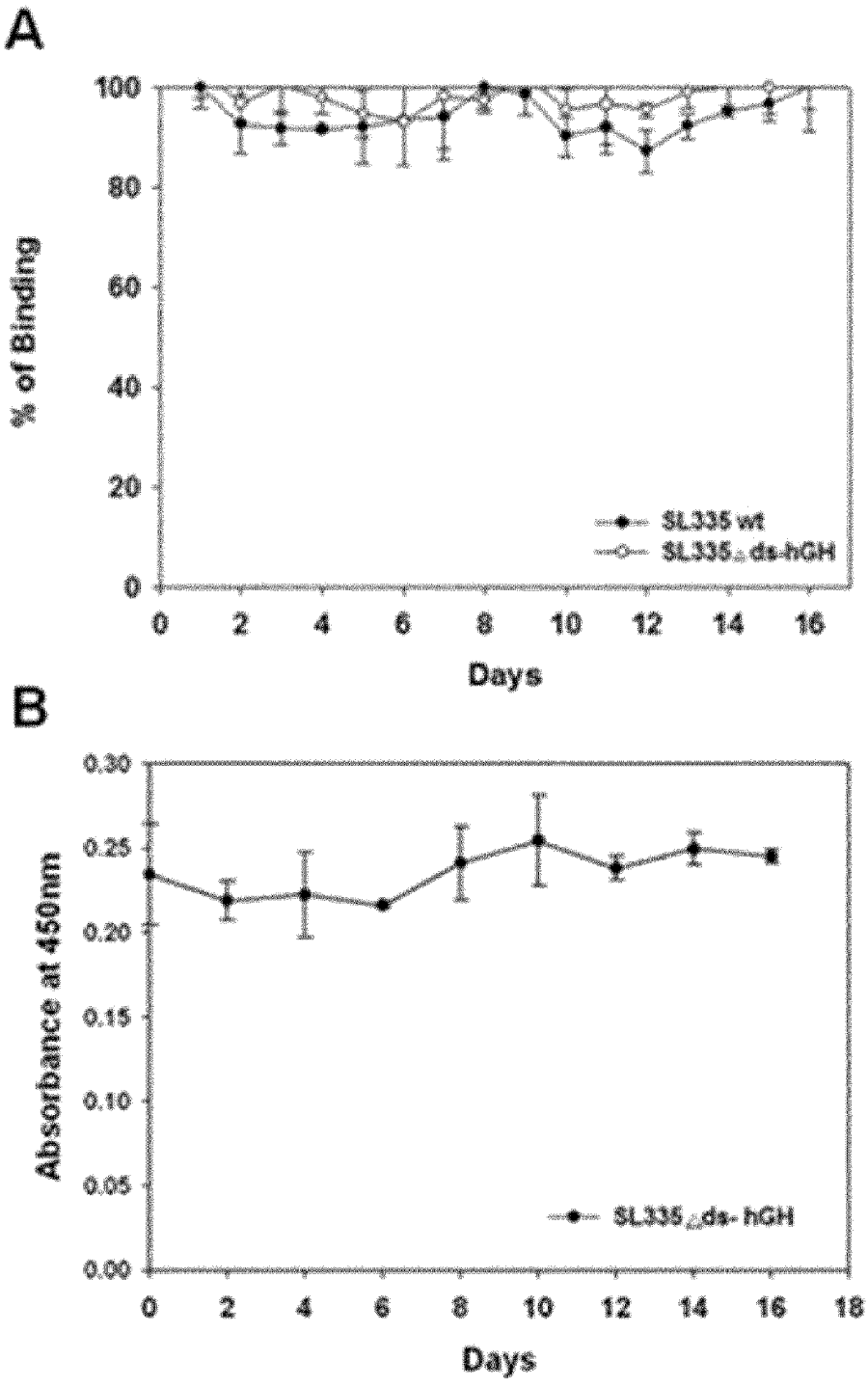
[Fig. 12]


A
mAU
150
100
50
0
mV (10⁴)
66kDa
25kDa
60
80
100
ml
Fractions
Fractions
13
16
19
23
B
kDa
100
70
55
40
35
25
15

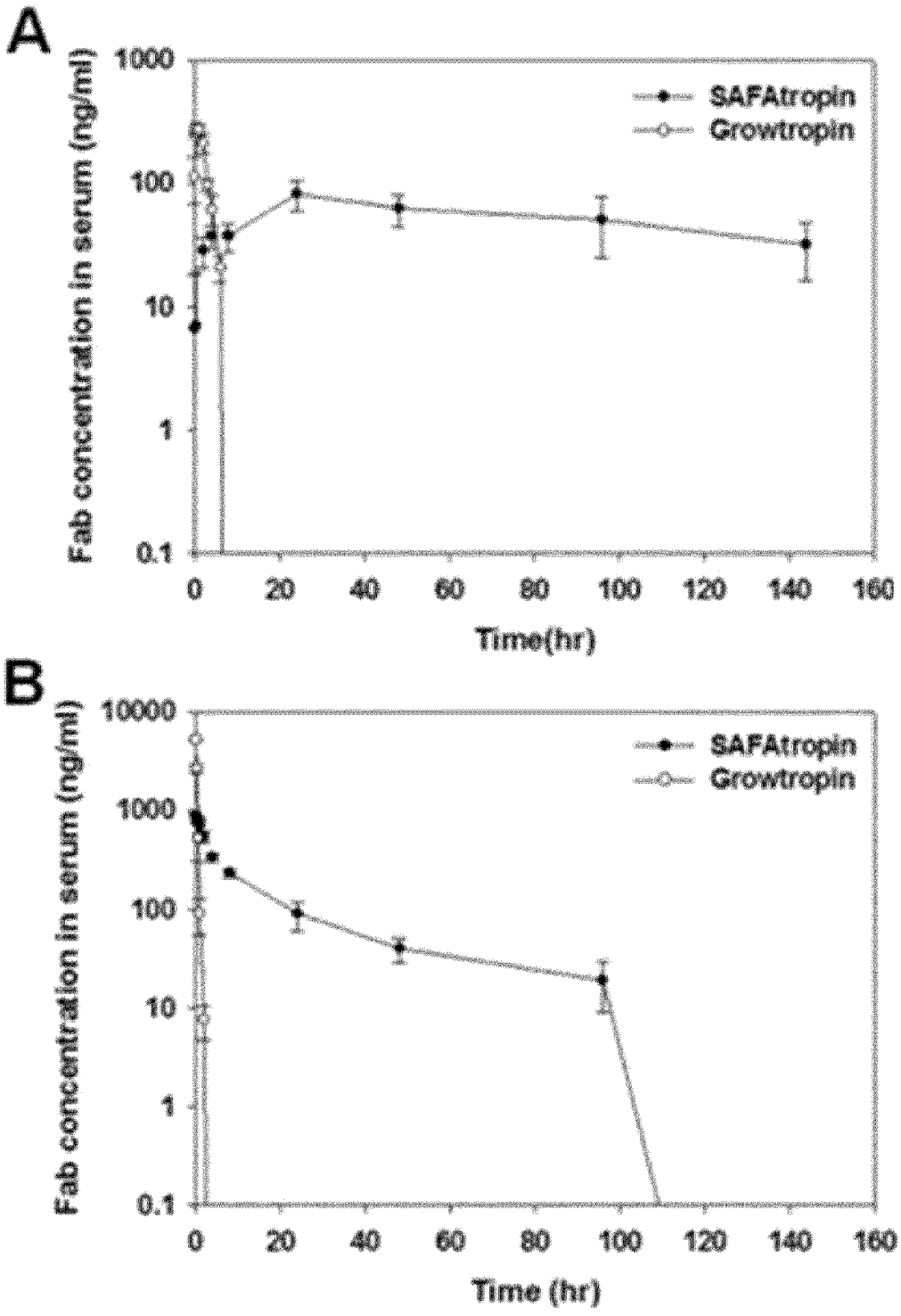
[Fig. 13]


- HSA
Growtropin
SL335 wt
HserG/Lser
Absorbance at 450nm
0.6
0.5
0.4
0.3
0.2
0.1
0.0
10000
5000
1000
500
100
50
10
5
1
0.5
0.1
0
rhGH concentration (pM)
+ HSA
Growtropin
SL335 wt
HserG/Lser
Absorbance at 450nm
0.6
0.5
0.4
0.3
0.2
0.1
0.0
10000
5000
1000
500
100
50
10
5
1
0.5
0.1
0
rhGH concentration (pM)

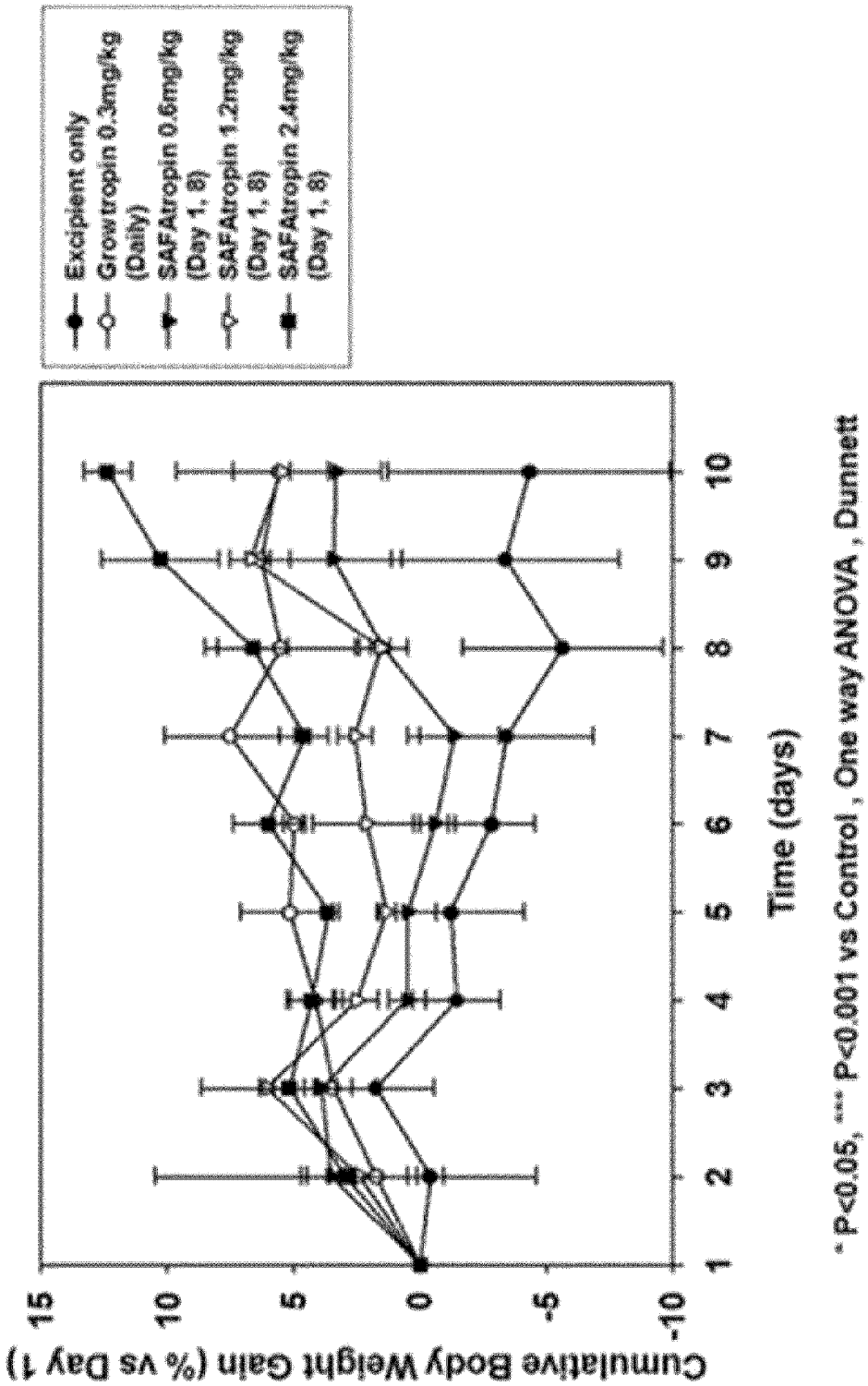
[Fig. 14]


A
% of Binding
100
80
60
40
20
0
0 2 4 6 8 10 12 14 16
Days
SL335 wt
SL335△ds-hGH
B
Absorbance at 450nm
0.30
0.25
0.20
0.15
0.10
0.05
0.00
0 2 4 6 8 10 12 14 16 18
Days
SL335△ds- hGH

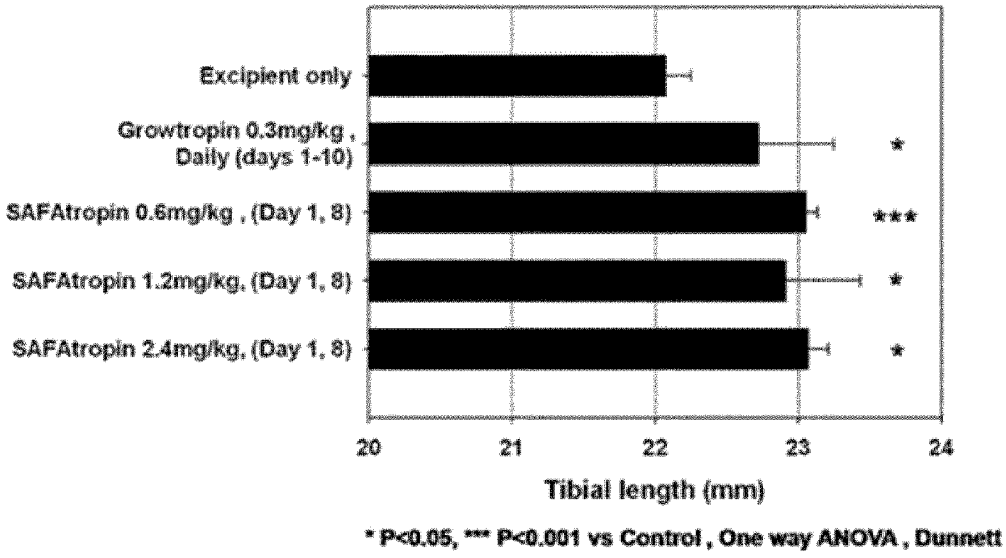
[Fig. 15]


A
Fab concentration in serum (ng/ml)
1000
100
10
1
0.1
0
20
40
60
80
100
120
140
160
Time(hr)
SAFAtropin
Growtropin
B
Fab concentration in serum (ng/ml)
10000
1000
100
10
1
0.1
0
20
40
60
80
100
120
140
160
Time (hr)
SAFAtropin
Growtropin

[Fig. 16]

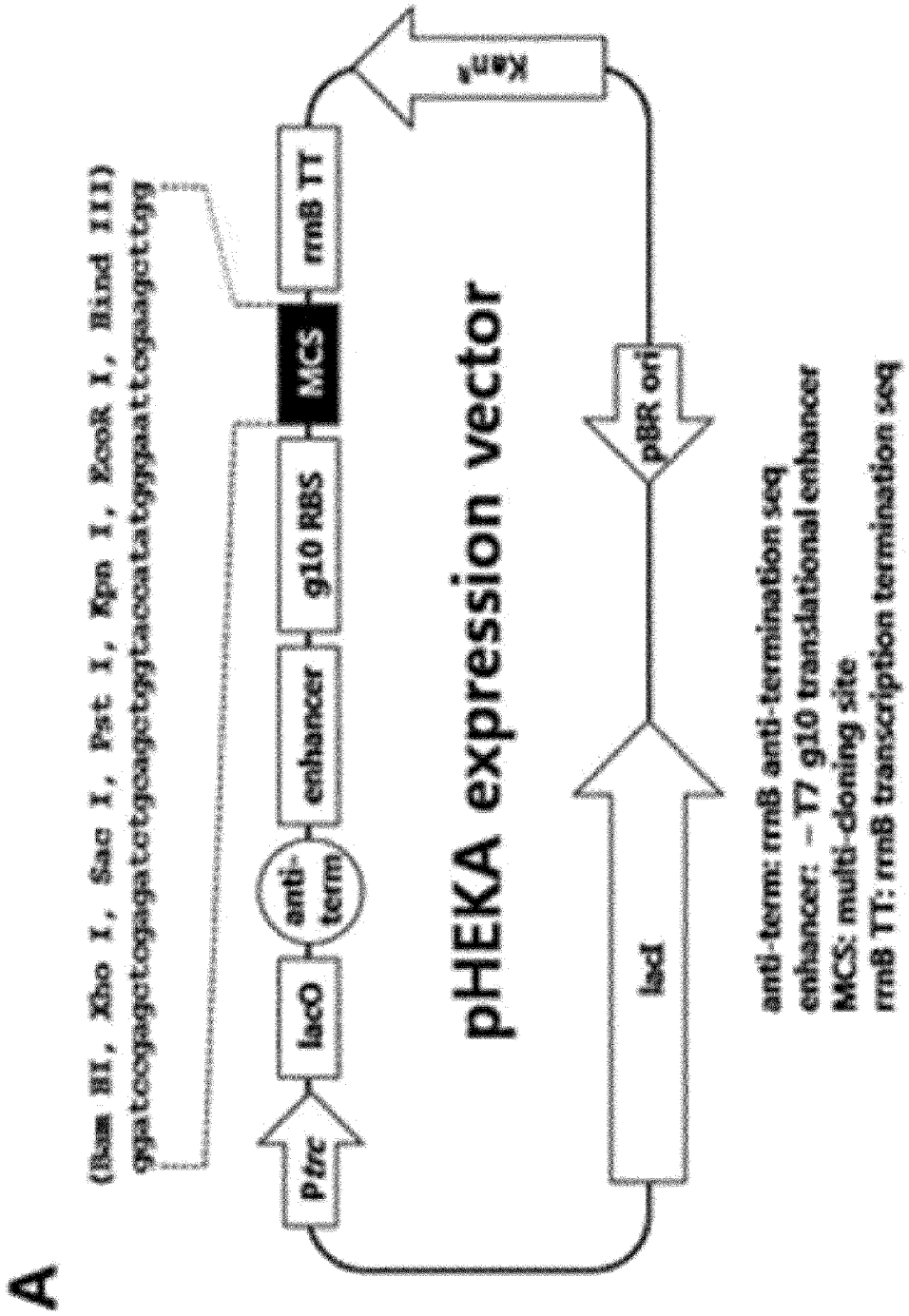

Excipient only
Growtropin 0.3mg/kg (Daily)
SAFAtropin 0.6mg/kg (Day 1, 8)
SAFAtropin 1.2mg/kg (Day 1, 8)
SAFAtropin 2.4mg/kg (Day 1, 8)
Cumulative Body Weight Gain (% vs Day 1)
15
10
5
0
-5
-10
1
2
3
4
5
6
7
8
9
10
Time (days)
* P<0.05, *** P<0.001 vs Control , One way ANOVA , Dunnett

[Fig. 17]



* P<0.05, *** P<0.001 vs Control , One way ANOVA , Dunnett

[Fig. 18]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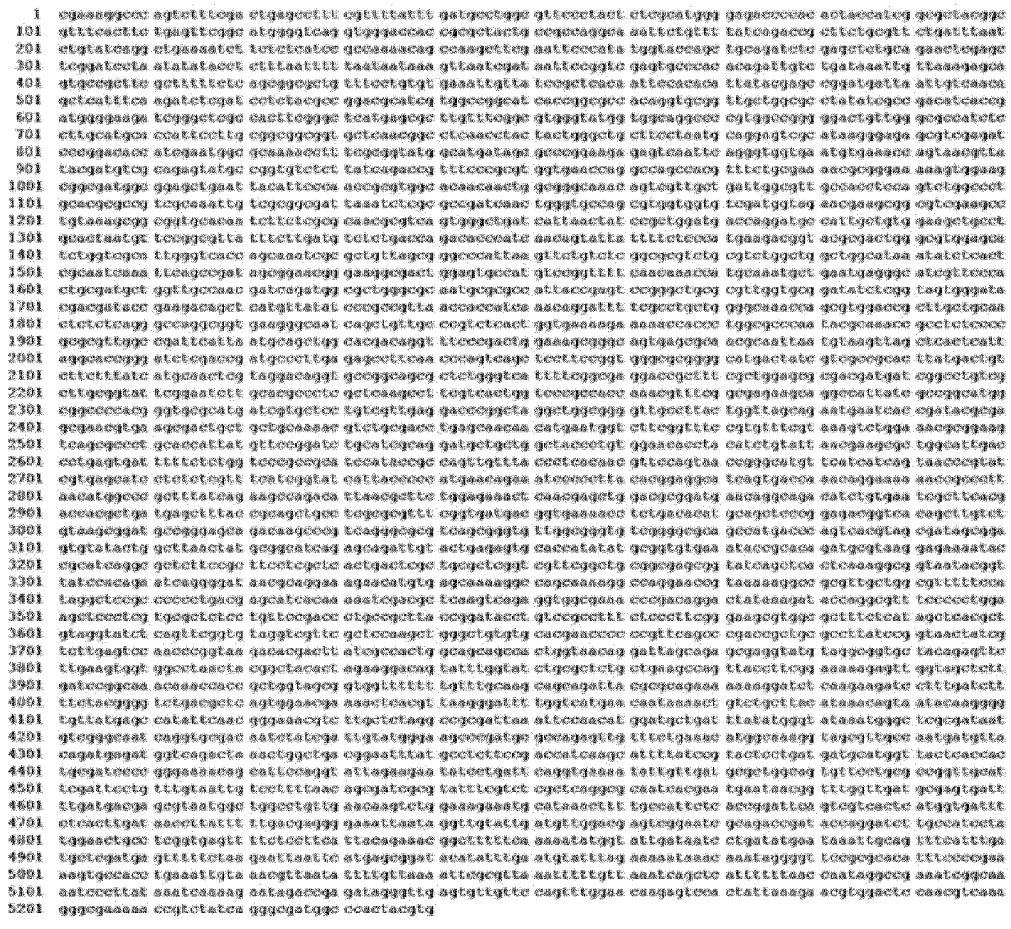
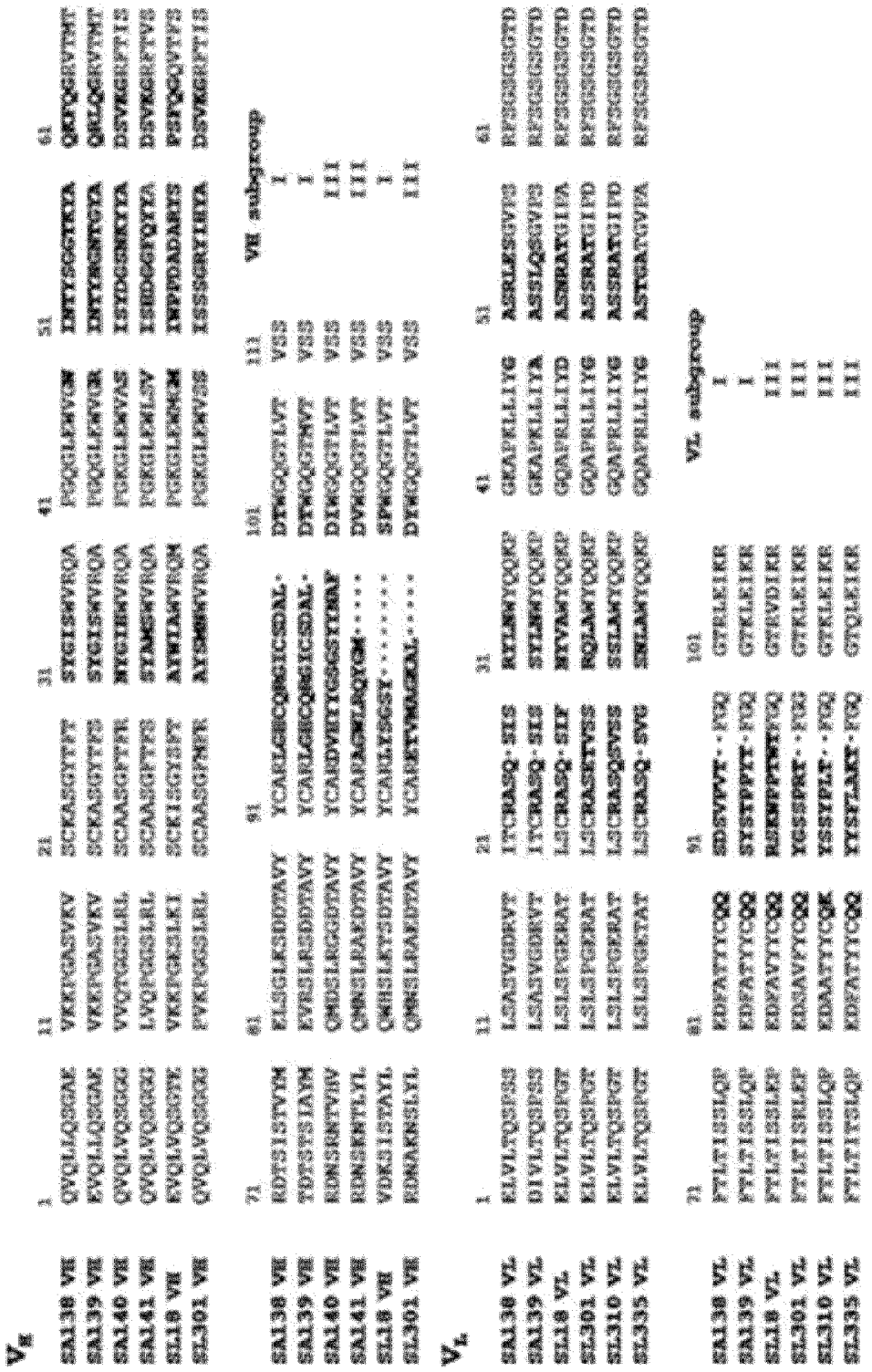
pHEKA expression vector
Kan^R
pBR ori
lacI
P trc
lacO
anti-term
enhancer
g10 RBS
MCS
rrnB TT
(Bam HI, Xho I, Sac I, Pst I, Kpn I, EcoR I, Hind III)
ggatccgagctcgagatctgcagctggtaccatatgggaattcgaagcttgg
anti-term: rrnB anti-termination seq
enhancer: ~T7 g10 translational enhancer
MCS: multi-cloning site
rrnB TT: rrnB transcription termination seq

[Fig. 19]

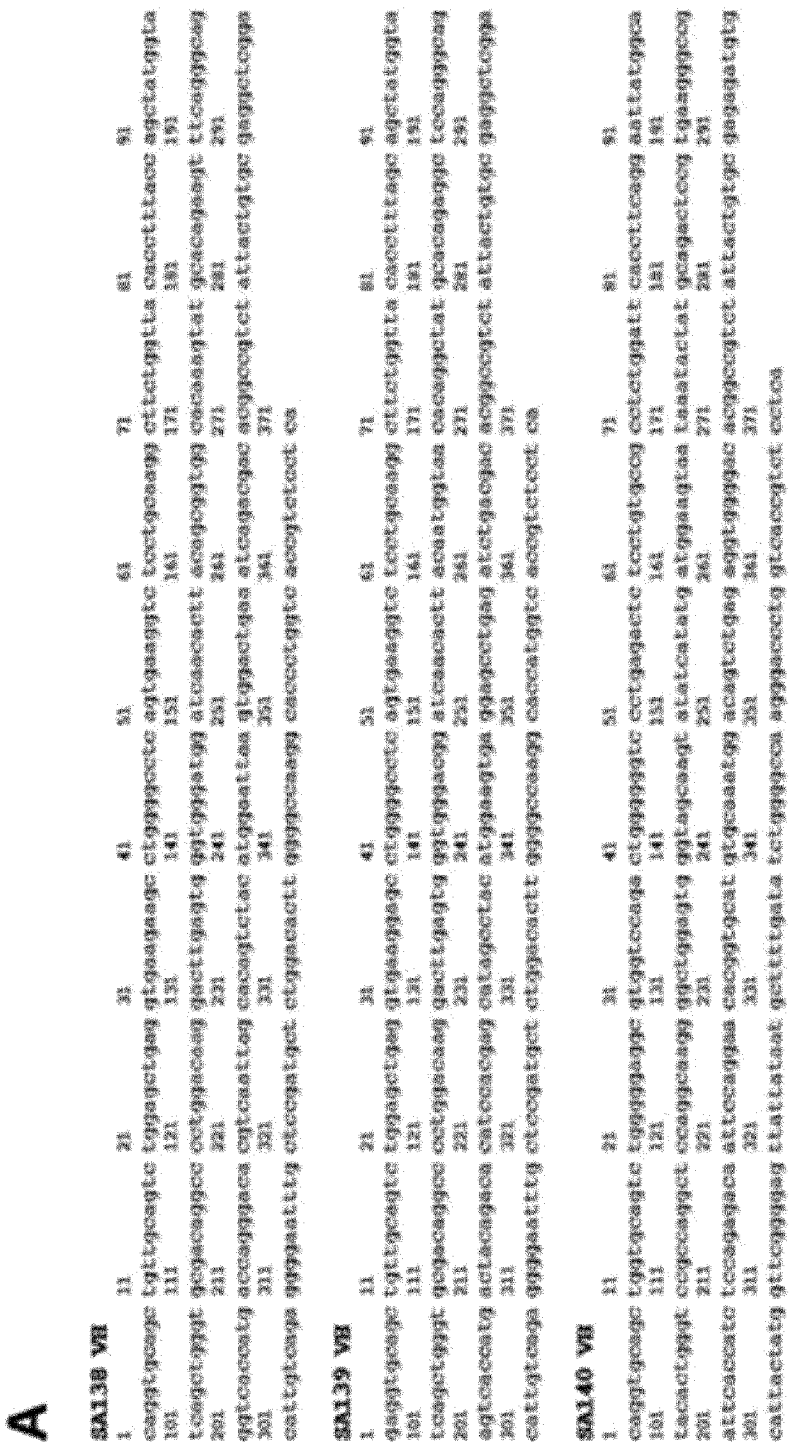
B pHEKA sequence (5240 bp)



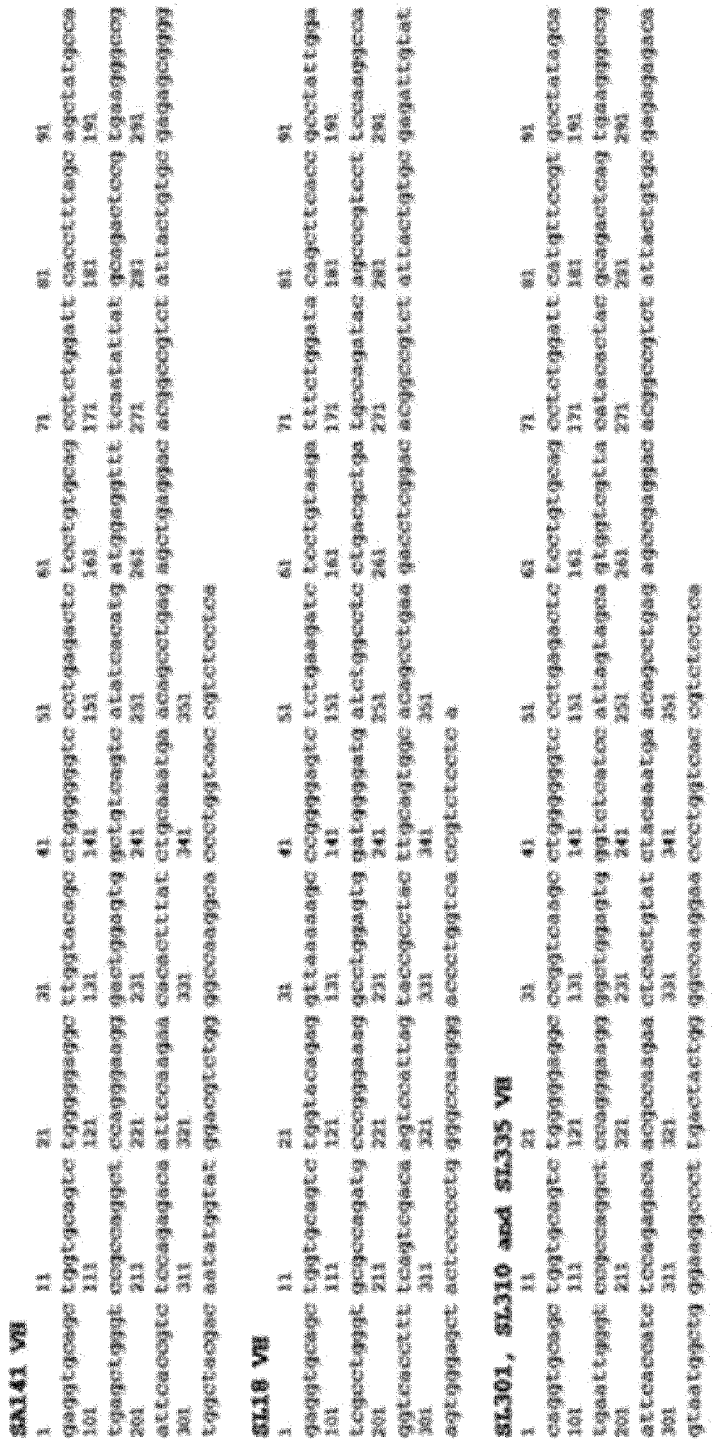
[Fig. 20]



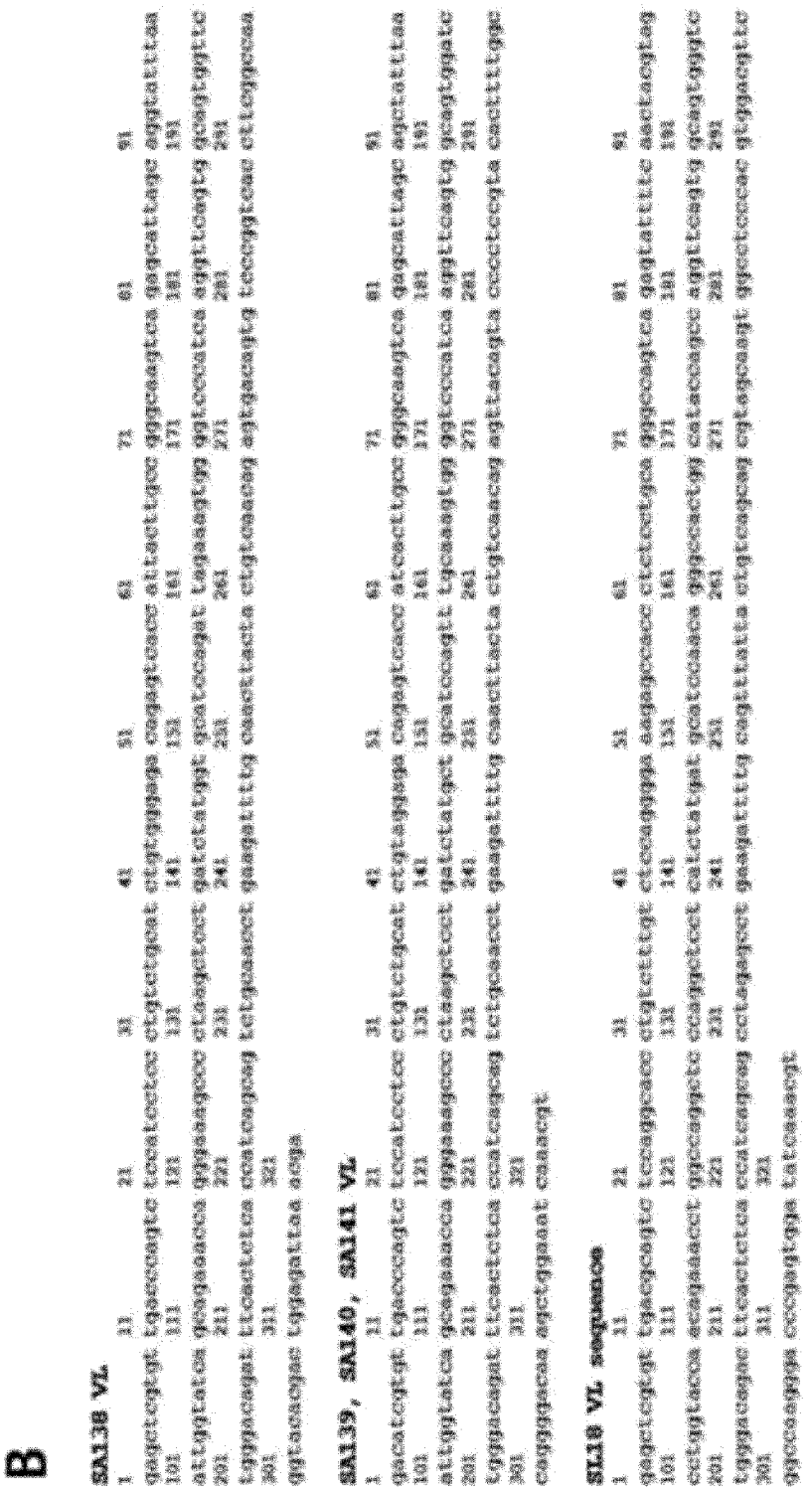
[Fig. 21a]



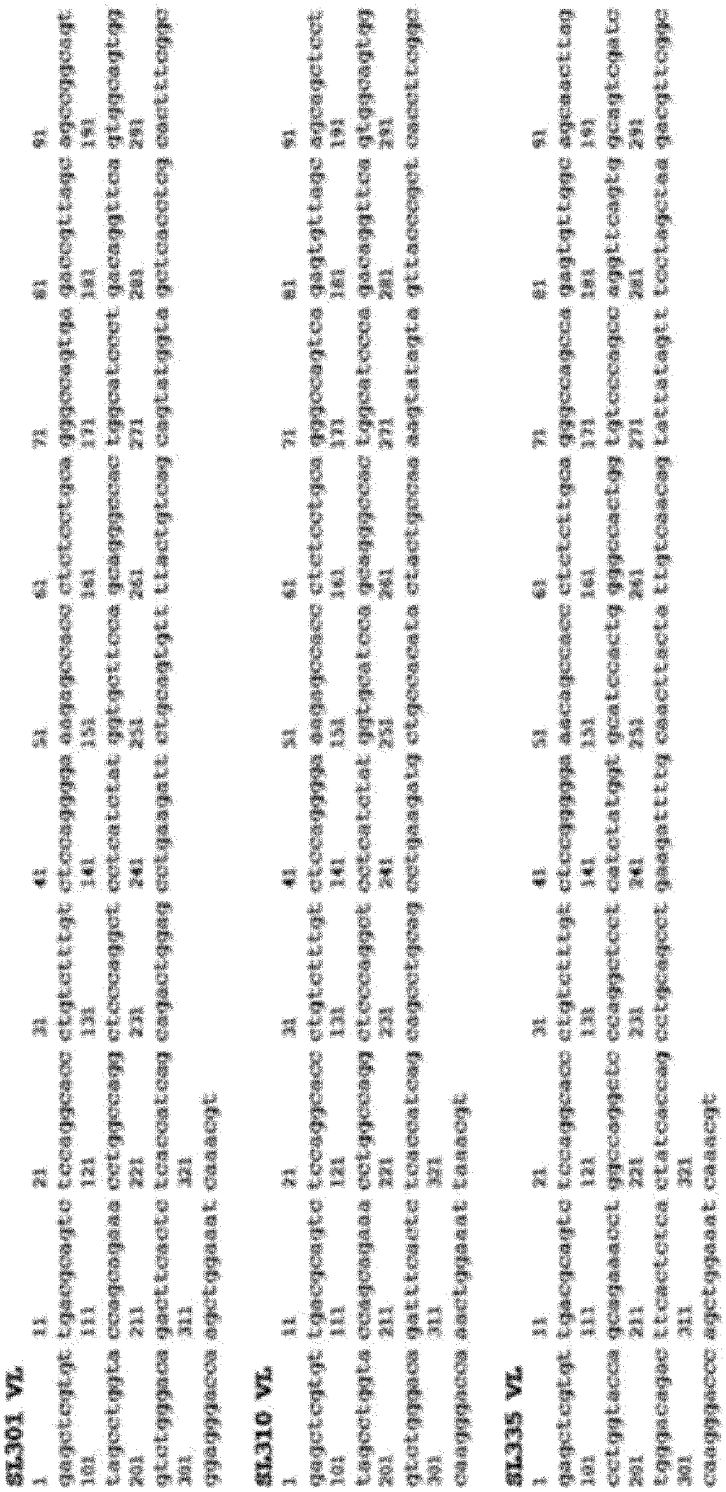
[Fig. 21b]



[Fig. 21c]



[Fig. 21d]



[Fig. 22a]

1) SL335$_{wt}$-hGH

Heavy chain (Hcys + hGH format)	**DNA**	CAAGTTCAGCTGGTTCAGAGCGGTGGCGGCCCGGTGAAACCAGGTGGCAGCCTGCG TCTGTCCTGCGCGGCGAGCGGTTTTATGTTTCGTGCGTATAGCATGAACTGGGTGCG CCAGGCGCCGGGCAAAGGCCTGGAATGGGTGAGCAGCATTAGCAGCAGTGGCCGC TATATTCATTATGCCGACAGTGTTAAAGGTCGTTTTACCATTTCTCGTGACAATGCG AAAAACAGCCTGTATCTGCAAATGAATAGCCTGCGCGCGGAAGACACCGCGGTGTA CTACTGTGCGCGCGAAACCGTGATGGCGGGCAAAGCACTGGATTATTGGGGTCAGG GCACCCTGGTGACCGTGAGCAGCGCGAGCACCAAAGGCCCGAGCGCGAGCACCAA AGGCCCGAGCGTGTTTCCGCTGGCACCTAGTTCGAAATCAACGAGCGGTGGCACCG CGGCTCTGGGCTGCCTGGTGAAAGATTATTTCCCGGAACCTGTTACCGTGAGCTGGA ACAGCGGTGCGTTGACGAGTGGTGTGCATACCTTTCCCGCAGTTCTGCAATCGAGC GGCCTGTACTCACTGAGCAGCGTGGTTACGGTCCCGAGCAGTAGCCTGGGTACACA GACCTATATTTGTAACGTGAACCACAAGCCTTCGAACACGAAAGTTGACAAACGCG TGGAACCGAAGAGC**TGC**GGTTCTGCACCAGCTCCTGGATCT**TTTCCGACC**ATTCCGC TGAGCCGCCTGTTCGATAACGCGATGCTGCGCGCCCACCGCCTGCATCAACTGGCCT TTGATACCTATCAGGAGTTTGAGGAAGCGTACATCCCGAAGGAACAGAAATATTCT TTTCTGCAGAACCCACAGACGAGCCTGTGCTTTAGCGAATCTATCCCGACCCCGTCC AACCGCGAAGAAACCCAACAGAAGTCTAACCTGGAACTGCTGCGTATCTCTCTGCT GCTGATTCAATCCTGGCTGGAACCGGTTCAATTTCTGCGTAGCGTGTTTGCGAACTC TCTGGTGTATGGCGCGTCTGACTCTAACGTGTATGACCTGCTGAAAGATCTGGAAG AAGGCATCCAAACTCTGATGGGCCGTCTGGAGGACGGCTCTCCACGTACCGGCCAG ATCTTTAAACAGACCTATAGCAAATTTGACACCAATTCTCACAACGATGATGCGCTG CTGAAAAACTATGGCCTGCTGTATTGCTTCCGTAAAGACATGGATAAAGTTGAAAC GTTCCTGCGCATTGTTCAGTGCCGTTCCGTGGAGGGCTCCTGCGGCTTC
	Amino acid	QVQLVQSGGGPVKPGGSLRLSCAASGFMFR**AYSMN**WVRQAPGKGLEWV**SSISSS** **GRYIHYADSVKG**RFTISRDNAKNSLYLQMNSLRAEDTAVYYCAR**FTVMAGKALDY**W GQGTLVTVSSASTKGPSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWN SGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKRVEPKS CGSAPAPGSFPTIPLSRLFDNAMLRAHRLHQLAFDTYQEFEEAYIPKEQKYSFLQNPQTS LCFSESIPTPSNREETQQKSNLELLRISLLLIQSWLEPVQFLRSVFANSLVYGASDSNVYD LLKDLEEGIQTLMGRLLDGSPRTGQIFKQTYSKFDTNSHNDDALLKNYGLLYCFRKDM DKVETFLRIVQCRSVEGSCGF
Light chain (Lcys format)	**DNA**	GATATCGTTCTGACCCAATCTCCGGGTACGCTGAGCCTGAGCCCGGGCGAAACCGC GACCCTGAGCTGCCGCGCGAGCCAAAGCGTGGGTTCTAATCTGGCTTGGTATCAGC AGAAACCGGGTCAGGCCCCGCGCCTGCTGATCTATGGGGCGAGCACGGGGGCTACC GGCGTTCCGGCGCGCTTTAGTGGCAGTCGCAGCGGCACCGATTTTACCCTGACCATT ACAAGTCTGCAGCCGGAAGATTTTGCGACCTATTATTGCCAGCAATATTATAGCTTC CTGGCGAAAACCTTTGGTCAGGGCACCCAGCTGGAAATTAAACGCACCGTGGCGGC ACCCAGCGTGACGGTGGCGGCACCCAGCGTGTTTATTTTTCCTCCCAGTGATGAACA GCTGAAAAGCGGGACCGCGAGTGTTGTGTGCCTGTTGAACAACTTCTATCCTCGCG AAGCGAAAGTGCAGTGGAAAGTGGATAACGCATTGCAGAGCGGCAACAGTCAGGA AAGCGTTACTGAACAGGATAGCAAAGATAGTACGTACAGCTTGAGCAACACTCTGA CCCTGAGTAAAGCGGATTATGAAAAACATAAAGTGTATGCATGCGAAGTTACGCAT CAGGGGCTGAGCAGTCCGGTGACAAAGAGCTTTAACCGCGGCGAA**TGC**
	Amino acid	DIVLTQSPGTLSLSPGETATLSC**RASQSVGSNLA**WYQQKPGQAPRLLIY**GASTGAT**GVP ARFSGSRSGTDFTLTITSLQPEDFATYYC**QQYYSFLAKT**FGQGTQLEIKRTVAAPSVTVA APSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKD STYSLSNTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC

*The linker and the effector domains were underlined and CDRs were written in bold.

[Fig. 22b]

2) SL335$_{\Delta ds}$-hGH

Heavy chain (Hser + hGH format)	**DNA**	CAAGTTCAGCTGGTTCAGAGCGGTGGCGGCCCGGTGAAACCAGGTGGCAGCCTGCGT CTGTCCTGCGCGGCGAGCGGTTTTATGTTTCGTGCGTATAGCATGAACTGGGTGCGCC AGGCGCCGGGCAAAGGCCTGGAATGGGTGAGCAGCATTAGCAGCAGTGGCCGCTAT ATTCATTATGCCGACAGTGTTAAAGGTCGTTTTACCATTTCTCGTGACAATGCGAAAA ACAGCCTGTATCTGCAAATGAATAGCCTGCGCGCGGAAGACACCGCGGTGTACTACT GTGCGCGCGAAACCGTGATGGCGGGCAAAGCACTGGATTATTGGGGTCAGGGCACCC TGGTGACCGTGAGCAGCGCGAGCACCAAAGGCCCGAGCGCGAGCACCAAAGGCCCG AGCGTGTTTCCGCTGGCACCTAGTTCGAAATCAACGAGCGGTGGCACCGCGGCTCTG GGCTGCCTGGTGAAAGATTATTTCCCGGAACCTGTTACCGTGAGCTGGAACAGCGGT GCGTTGACGAGTGGTGTGCATACCTTTCCCGCAGTTCTGCAATCGAGCGGCCTGTACT CACTGAGCAGCGTGGTTACGGTCCCGAGCAGTAGCCTGGGTACACAGACCTATATTT GTAACGTGAACCACAAGCCTTCGAACACGAAAGTTGACAAACGCGTGGAACCGAAG AGC**AGC**GGTTCTGCACCAGCTCCTGGATCT**TTTCCGACC**ATTCCGCTGAGCCGCCTGT TCGATAACGCGATGCTGCGCGCCCACCGCCTGCATCAACTGGCCTTTGATACCTATCA GGAGTTTGAGGAAGCGTACATCCCGAAGGAACAGAAATATTCTTTTCTGCAGAACCC ACAGACGAGCCTGTGCTTTAGCGAATCTATCCCGACCCCGTCCAACCGCGAAGAAAC CCAACAGAAGTCTAACCTGGAACTGCTGCGTATCTCTCTGCTGCTGATTCAATCCTGG CTGGAACCGGTTCAATTTCTGCGTAGCGTGTTTGCGAACTCTCTGGTGTATGGCGCGT CTGACTCTAACGTGTATGACCTGCTGAAAGATCTGGAAGAAGGCATCCAAACTCTGA TGGGCCGTCTGGAGGACGGCTCTCCACGTACCGGCCAGATCTTTAAACAGACCTATA GCAAATTTGACACCAATTCTCACAACGATGATGCGCTGCTGAAAAACTATGGCCTGCT GTATTGCTTCCGTAAAGACATGGATAAAGTTGAAACGTTCCTGCGCATTGTTCAGTGC CGTTCCGTGGAGGGCTCCTGCGGCTTC
	Amino acid	QVQLVQSGGGPVKPGGSLRLSCAASGFMFR**AYSMN**WVRQAPGKGLEWVS**SISSSGRY** **IHYADSVKG**RFTISRDNAKNSLYLQMNSLRAEDTAVYYCAR**ETVMAGKALDY**WGQG TLVTVSSASTKGPSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALT SGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKRVEPKSSGSAPA PGSFPTIPLSRLFDNAMLRAHRLHQLAFDTYQEFEEAYIPKEQKYSFLQNPQTSLCFSESIPT PSNREETQQKSNLELLRISLLLIQSWLEPVQFLRSVFANSLVYGASDSNVYDLLKDLEEGI QTLMGRLEDGSPRTGQIFKQTYSKFDTNSHNDDALLKNYGLLYCFRKDMDKVETFLRIV QCRSVEGSCGF
Light chain (Lser format)	**DNA**	GATATCGTTCTGACCCAATCTCCGGGTACGCTGAGCCTGAGCCCGGGCGAAACCGCG ACCCTGAGCTGCCGCGCGAGCCAAAGCGTGGGTTCTAATCTGGCTTGGTATCAGCAG AAACCGGGTCAGGCCCCGCGCCTGCTGATCTATGGGGCGAGCACGGGGGCTACCGGC GTTCCGGCGCGCTTTAGTGGCAGTCGCAGCGGCACCGATTTTACCCTGACCATTACAA GTCTGCAGCCGGAAGATTTTGCGACCTATTATTGCCAGCAATATTATAGCTTCCTGGC GAAAACCTTTGGTCAGGGCACCCAGCTGGAAATTAAACGCACCGTGGCGGCACCCAG CGTGACGGTGGCGGCACCCAGCGTGTTTATTTTTCCTCCCAGTGATGAACAGCTGAAA AGCGGGACCGCGAGTGTTGTGTGCCTGTTGAACAACTTCTATCCTCGCGAAGCGAAA GTGCAGTGGAAAGTGGATAACGCATTGCAGAGCGGCAACAGTCAGGAAAGCGTTACT GAACAGGATAGCAAAGATAGTACGTACAGCTTGAGCAACACTCTGACCCTGAGTAAA GCGGATTATGAAAAACATAAAGTGTATGCATGCGAAGTTACGCATCAGGGGCTGAGC AGTCCGGTGACAAAGAGCTTTAACCGCGGCGAA**AGC**
	Amino acid	DIVLTQSPGTLSLSPGETATLSC**RASQSVGSNLA**WYQQKPGQAPRLLIY**GASTGAT**GVPA RFSGSRSGTDFTLTITSLQPEDFATYYC**QQYYSFLA**KTFGQGTQLEIKRTVAAPSVTVAAP SVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTY SLSNTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGES

*The linker and the effector domains were underlined and CDRs were written in bold.

[Fig. 22c]

3) SL335$_{wt}$-GCSF

Heavy chain (Hcys + GCSF format)	**DNA**	CAAGTTCAGCTGGTTCAGAGCGGTGGCGGCCCGGTGAAACCAGGTGGCAGCCTGCGT CTGTCCTGCGCGGCGAGCGGTTTTATGTTTCGTGCGTATAGCATGAACTGGGTGCGCC AGGCGCCGGGCAAAGGCCTGGAATGGGTGAGCAGCATTAGCAGCAGTGGCCGCTAT ATTCATTATGCCGACAGTGTTAAAGGTCGTTTTACCATTTCTCGTGACAATGCGAAAA ACAGCCTGTATCTGCAAATGAATAGCCTGCGCGCGGAAGACACCGCGGTGTACTACT GTGCGCGCGAAACCGTGATGGCGGGCAAAGCACTGGATTATTGGGGTCAGGGCACCC TGGTGACCGTGAGCAGCGCGAGCACCAAAGGCCCGAGCGCGAGCACCAAAGGCCCG AGCGTGTTTCCGCTGGCACCTAGTTCGAAATCAACGAGCGGTGGCACCGCGGCTCTG GGCTGCCTGGTGAAAGATTATTTCCCGGAACCTGTTACCGTGAGCTGGAACAGCGGT GCGTTGACGAGTGGTGTGCATACCTTTCCCGCAGTTCTGCAATCGAGCGGCCTGTACT CACTGAGCAGCGTGGTTACGGTCCCGAGCAGTAGCCTGGGTACACAGACCTATATTT GTAACGTGAACCACAAGCCTTCGAACACGAAAGTTGACAAACGCGTGGAACCGAAG AGC**TGC**GGTTCTGCACCAGCTCCTGGATCTGCGCCTACCTATCGCGCGAGCAGCCTGC CGCAGTCGTTTCTGCTGAAAAGCCTGGAACAGGTGCGCAAGATTCAGGGTGACGGCG CAGCTCTGCAAGAAAAACTGTGCGCGACCTACAAATTGTGCCACCCTGAGGAACTGG TTCTGCTGGGCCATAGTCTGGGCATTCCGTGGGCGCCGCTGAGCAGCTGCCCGTCGCA GGCATTGCAGCTGGCTGGCTGTCTGAGCCAGTTACATAGCGGTCTGTTTCTGTATCAG GGCCTGCTGCAAGCGCTGGAAGGCATCAGTCCTGAGTTGGGTCCGACCCTGGATACC TTACAGCTGGATGTGGCGGATTTCGCAACCACCATTTGGCAGCAGATGGAAGAATTG GGCATGGCTCCGGCGTTGCAGCCGACCCAGGGCGCGATGCCTGCGTTTGCAAGCGCT TTTCAGCGCCGCGCGGGTGGGGTGCTGGTGGCGTCGCACTTGCAGAGCTTCCTGGAA GTGAGCTACCGTGTCCTGCGCCATCTGGCACAGCCT
	Amino acid	QVQLVQSGGGPVKPGGSLRLSCAASGFMFR**AYSMN**WVRQAPGKGLEWVS**SISSSG** **RYIHYADSVKG**RFTISRDNAKNSLYLQMNSLRAEDTAVYYCAR**ETVMAGKALDY** WGQGTLVTVSSASTKGPSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWN SGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKRVEPKSC GSAPAPGSAPTYRASSLPQSFLLKSLEQVRKIQGDGAALQEKLCATYKLCHPEELVLLGHS LGIPWAPLSSCPSQALQLAGCLSQLHSGLFLYQGLLQALEGISPELGPTLDTLQLDVADFA TTIWQQMEELGMAPALQPTQGAMPAFASAFQRRAGGVLVASHLQSFLEVSYRVLRHLA QP
Light chain (Lcys format)	**DNA**	GATATCGTTCTGACCCAATCTCCGGGTACGCTGAGCCTGAGCCCGGGCGAAACCGCG ACCCTGAGCTGCCGCGCGAGCCAAAGCGTGGGTTCTAATCTGGCTTGGTATCAGCAG AAACCGGGTCAGGCCCCGCGCCTGCTGATCTATGGGGCGAGCACGGGGGCTACCGGC GTTCCGGCGCGCTTTAGTGGCAGTCGCAGCGGCACCGATTTTACCCTGACCATTACAA GTCTGCAGCCGGAAGATTTTGCGACCTATTATTGCCAGCAATATTATAGCTTCCTGGC GAAAACCTTTGGTCAGGGCACCCAGCTGGAAATTAAACGCACCGTGGCGGCACCCAG CGTGACGGTGGCGGCACCCAGCGTGTTTATTTTTCCTCCCAGTGATGAACAGCTGAAA AGCGGGACCGCGAGTGTTGTGTGCCTGTTGAACAACTTCTATCCTCGCGAAGCGAAA GTGCAGTGGAAAGTGGATAACGCATTGCAGAGCGGCAACAGTCAGGAAAGCGTTACT GAACAGGATAGCAAAGATAGTACGTACAGCTTGAGCAACACTCTGACCCTGAGTAAA GCGGATTATGAAAAACATAAAGTGTATGCATGCGAAGTTACGCATCAGGGGCTGAGC AGTCCGGTGACAAAGAGCTTTAACCGCGGCGAA**TGC**
	Amino acid	DIVLTQSPGTLSLSPGETATLSC**RASQSVGSNLA**WYQQKPGQAPRLLIY**GASTGAT**GVPA RFSGSRSGTDFTLTITSLQPEDFATYYC**QQYYSFLA**KTFGQGTQLEIKRTVAAPSVTVAAP SVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTY SLSNTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC

*The linker and the effector domains were underlined and CDRs were written in bold.

[Fig. 22d]

4) SL335$_{\triangle ds}$-GCSF

Heavy chain (Hser + GCSF format)	**DNA**	CAAGTTCAGCTGGTTCAGAGCGGTGGCGGCCCGGTGAAACCAGGTGGCAGCCTGCGT CTGTCCTGCGCGGCGAGCGGTTTTATGTTCGTGCGTATAGCATGAACTGGGTGCGCC AGGCGCCGGGCAAAGGCCTGGAATGGGTGAGCAGCATTAGCAGCAGTGGCCGCTAT ATTCATTATGCCGACAGTGTTAAAGGTCGTTTTACCATTTCTCGTGACAATGCGAAAA ACAGCCTGTATCTGCAAATGAATAGCCTGCGCGCGGAAGACACCGCGGTGTACTACT GTGCGCGCGAAACCGTGATGGCGGGCAAAGCACTGGATTATTGGGGTCAGGGCACCC TGGTGACCGTGAGCAGCGCGAGCACCAAAGGCCCGAGCGCGAGCACCAAAGGCCCG AGCGTGTTTCCGCTGGCACCTAGTTCGAAATCAACGAGCGGTGGCACCGCGGCTCTG GGCTGCCTGGTGAAAGATTATTTCCCGGAACCTGTTACCGTGAGCTGGAACAGCGGT GCGTTGACGAGTGGTGTGCATACCTTTCCCGCAGTTCTGCAATCGAGCGGCCTGTACT CACTGAGCAGCGTGGTTACGGTCCCGAGCAGTAGCCTGGGTACACAGACCTATATTT GTAACGTGAACCACAAGCCTTCGAACACGAAAGTTGACAAACGCGTGGAACCGAAG AGC**AGC**GGTTCTGCACCAGCTCCTGGATCTGCGCCTACCTATCGCGCGAGCAGCCTGC CGCAGTCGTTTCTGCTGAAAAGCCTGGAACAGGTGCGCAAGATTCAGGGTGACGGCG CAGCTCTGCAAGAAAAACTGTGCGCGACCTACAAATTGTGCCACCCTGAGGAACTGG TTCTGCTGGGCCATAGTCTGGGCATTCCGTGGGCGCCGCTGAGCAGCTGCCCGTCGCA GGCATTGCAGCTGGCTGGCTGTCTGAGCCAGTTACATAGCGGTCTGTTTCTGTATCAG GGCCTGCTGCAAGCGCTGGAAGGCATCAGTCCTGAGTTGGGTCCGACCCTGGATACC TTACAGCTGGATGTGGCGGATTTCGCAACCACCATTTGGCAGCAGATGGAAGAATTG GGCATGGCTCCGGCGTTGCAGCCGACCCAGGGCGCGATGCCTGCGTTTGCAAGCGCT TTTCAGCGCCGCGCGGGTGGGGTGCTGGTGGCGTCGCACTTGCAGAGCTTCCTGGAA GTGAGCTACCGTGTCCTGCGCCATCTGGCACAGCCT
	Amino acid	QVQLVQSGGGPVKPGGSLRLSCAASGFMFR**AYSMN**WVRQAPGKGLEWVS**SISSSGRY** **IHYADSVKG**RFTISRDNAKNSLYLQMNSLRAEDTAVYYCAR**ETVMAGKALDY**WGQGTL VTVSSASTKGPSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSG VHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKRVEPKSSGSAPAPG SAPTYRASSLPQSFLLKSLEQVRKIQGDGAALQEKLCATYKLCHPEELVLLGHSLGIPWAP LSSCPSQALQLAGCLSQLHSGLFLYQGLLQALEGISPELGPTLDTLQLDVADFATTIWQQM EELGMAPALQPTQGAMPAFASAFQRRAGGVLVASHLQSFLEVSYRVLRHLAQP
Light chain (Lser format)	**DNA**	GATATCGTTCTGACCCAATCTCCGGGTACGCTGAGCCTGAGCCCGGGCGAAACCGCG ACCCTGAGCTGCCGCGCGAGCCAAAGCGTGGGTTCTAATCTGGCTTGGTATCAGCAG AAACCGGGTCAGGCCCCGCGCCTGCTGATCTATGGGGCGAGCACGGGGGCTACCGGC GTTCCGGCGCGCTTTAGTGGCAGTCGCAGCGGCACCGATTTTACCCTGACCATTACAA GTCTGCAGCCGGAAGATTTTGCGACCTATTATTGCCAGCAATATTATAGCTTCCTGGC GAAAACCTTTGGTCAGGGCACCCAGCTGGAAATTAAACGCACCGTGGCGGCACCCAG CGTGACGGTGGCGGCACCCAGCGTGTTTATTTTTCCTCCCAGTGATGAACAGCTGAAA AGCGGGACCGCGAGTGTTGTGTGCCTGTTGAACAACTTCTATCCTCGCGAAGCGAAA GTGCAGTGGAAAGTGGATAACGCATTGCAGAGCGGCAACAGTCAGGAAAGCGTTACT GAACAGGATAGCAAAGATAGTACGTACAGCTTGAGCAACACTCTGACCCTGAGTAAA GCGGATTATGAAAAACATAAAGTGTATGCATGCGAAGTTACGCATCAGGGGCTGAGC AGTCCGGTGACAAAGAGCTTTAACCGCGGCGAA**AGC**
	Amino acid	DIVLTQSPGTLSLSPGETATLSC**RASQSVGSNLA**WYQQKPGQAPRLLI**YGASTGAT**GVPA RFSGSRSGTDFTLTITSLQPEDFATYYC**QQYYSFLA**KTFGQGTQLEIKRTVAAPSVTVAAP SVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTY SLSNTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGES

*The linker and the effector domains were underlined and CDRs were written in bold.

[Fig. 22e]

5) $SL335_{wt}$-IFNβ

Heavy chain (Hcys + IFNβ formt)	**DNA**	CAAGTTCAGCTGGTTCAGAGCGGTGGCGGCCCGGTGAAACCAGGTGGCAGCCTGCGT CTGTCCTGCGCGGCGAGCGGTTTTATGTTTCGTGCGTATAGCATGAACTGGGTGCGCC AGGCGCCGGGCAAAGGCCTGGAATGGGTGAGCAGCATTAGCAGCAGTGGCCGCTAT ATTCATTATGCCGACAGTGTTAAAGGTCGTTTTACCATTTCTCGTGACAATGCGAAAA ACAGCCTGTATCTGCAAATGAATAGCCTGCGCGCGGAAGACACCGCGGTGTACTACT GTGCGCGCGAAACCGTGATGGCGGGCAAAGCACTGGATTATTGGGGTCAGGGCACCC TGGTGACCGTGAGCAGCGCGAGCACCAAAGGCCCGAGCGCGAGCACCAAAGGCCCG AGCGTGTTTCCGCTGGCACCTAGTTCGAAATCAACGAGCGGTGGCACCGCGGCTCTG GGCTGCCTGGTGAAAGATTATTTCCCGGAACCTGTTACCGTGAGCTGGAACAGCGGT GCGTTGACGAGTGGTGTGCATACCTTTCCCGCAGTTCTGCAATCGAGCGGCCTGTACT CACTGAGCAGCGTGGTTACGGTCCCGAGCAGTAGCCTGGGTACACAGACCTATATTT GTAACGTGAACCACAAGCCTTCGAACACGAAAGTTGACAAACGCGTGGAACCGAAG AGCTGCGGTTCTGCACCAGCTCCTGGATCTTCATACAACCTGCTGGGCTTCCTGCAAC GTAGCAGTAACTTTCAGAGCCAGAAGCTGTTATGGCAACTGAACGGCCGCCTGGAGT ACTGCCTGAAGGATCGCATGAACTTTGATATTCCGGAAGAAATTAAACAGCTGCAAC AGTTCCAGAAAGAAGATGCGGCGCTGACCATTTATGAAATGCTGCAAAACATTTTTG CGATTTTTCGCCAAGATAGTAGTAGCACCGGCTGGAACGAAACCATTGTGGAAAACC TGCTCGCCAACGTGTACCATCAGATTAACCACCTGAAGACCGTGCTGGAAGAAAAAC TGGAAAAAGAAGATTTTACCCGCGGCAAACTGATGAGCAGCCTGCATCTGAAACGCT ATTATGGCCGCATTCTCCATTATCTGAAAGCCAAAGAGTATTCCCACTGTGCTTGGAC CATTGTTCGCGTGGAAATTCTGCGCAACTTTTATTTTATTAACCGCCTGACCGGCTATC TGCGCAAC
	Amino acid	QVQLVQSGGGPVKPGGSLRLSCAASGFMFR**AYSMN**WVRQAPGKGLEWVS**SISSSGRYIH** **YADSVKG**RFTISRDNAKNSLYLQMNSLRAEDTAVYYCAR**ETVMAGKALDY**WGQGTLV TVSSASTKGPSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGV HTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKRVEPKSCGSAPAPGS SYNLLGFLQRSSNFQSQKLLWQLNGRLEYCLKDRMNFDIPEEIKQLQQFQKEDAALTIYE MLQNIFAIFRQDSSSTGWNETIVENLLANVYHQINHLKTVLEEKLEKEDFTRGKLMSSLHL KRYYGRILHYLKAKEYSHCAWTIVRVEILRNFYFINRLTGYLRN
Light chain (Lcys format)	**DNA**	GATATCGTTCTGACCCAATCTCCGGGTACGCTGAGCCTGAGCCCGGGCGAAACCGCG ACCCTGAGC**T**GCCGCGCGAGCCAAAGCGTGGGTTCTAATCTGGC**TT**GGTATCAGCAG AAACCGGGTCAGGCCCCGCGCCTGCTGATCTATGGGGCGAGCACGGGGGCTACCGGC GTTCCGGCGCGCTTTAGTGGCAGTCGCAGCGGCACCGATTTTACCCTGACCATTACAA GTCTGCAGCCGGAAGATTTTGCGACCTATTATTGCCAGCAATATTATAGCTTCCTGGC GAAAACCTTTGGTCAGGGCACCCAGCTGGAAATTAAACGCACCGTGGCGGCACCCAG CGTGACGGTGGCGGCACCCAGCGTGTTTATTTTTCCTCCCAGTGATGAACAGCTGAAA AGCGGGACCGCGAGTGTTGTGTGCCTGTTGAACAACTTCTATCCTCGCGAAGCGAAA GTGCAGTGGAAAGTGGATAACGCATTGCAGAGCGGCAACAGTCAGGAAAGCGTTACT GAACAGGATAGCAAAGATAGTACGTACAGCTTGAGCAACACTCTGACCCTGAGTAAA GCGGATTATGAAAAACATAAAGTGTATGCATGCGAAGTTACGCATCAGGGGCTGAGC AGTCCGGTGACAAAGAGCTTTAACCGCGGCGAA**TGC**
	Amino acid	DIVLTQSPGTLSLSPGETATLSC**RASQSVGSNLA**WYQQKPGQAPRLLI**YGASTGAT**GVPA RFSGSRSGTDFTLTITSLQPEDFATYYC**QQYYSFLAK**TFGQGTQLEIKRTVAAPSVTVAAP SVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTY SLSNTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC

*The linker and the effector domains were underlined and CDRs were written in bold.

[Fig. 22f]

6) SL335$_{\triangle ds}$-IFNβ

Heavy chain (Hser + IFNβ formt)	**DNA**	CAAGTTCAGCTGGTTCAGAGCGGTGGCGGCCCGGTGAAACCAGGTGGCAGCCTGCGT CTGTCCTGCGCGGCGAGCGGTTTTATGTTTCGTGCGTATAGCATGAACTGGGTGCGCC AGGCGCCGGGCAAAGGCCTGGAATGGGTGAGCAGCATTAGCAGCAGTGGCCGCTAT ATTCATTATGCCGACAGTGTTAAAGGTCGTTTTACCATTTCTCGTGACAATGCGAAAA ACAGCCTGTATCTGCAAATGAATAGCCTGCGCGCGGAAGACACCGCGGTGTACTACT GTGCGCGCGAACCGTGATGGCGGGCAAAGCACTGGATTATTGGGGTCAGGGCACCC TGGTGACCGTGAGCAGCGCGAGCACCAAAGGCCCGAGCGCGAGCACCAAAGGCCCG AGCGTGTTTCCGCTGGCACCTAGTTCGAAATCAACGAGCGGTGGCACCGCGGCTCTG GGCTGCCTGGTGAAAGATTATTTCCCGGAACCTGTTACCGTGAGCTGGAACAGCGGT GCGTTGACGAGTGGTGTGCATACCTTTCCCGCAGTTCTGCAATCGAGCGGCCTGTACT CACTGAGCAGCGTGGTTACGGTCCCGAGCAGTAGCCTGGGTACACAGACCTATATTT GTAACGTGAACCACAAGCCTTCGAACACGAAAGTTGACAAACGCGTGGAACCGAAG AGCAGCGGTTCTGCACCAGCTCCTGGATCTTCATACAACCTGCTGGGCTTCCTGCAAC GTAGCAGTAACTTTCAGAGCCAGAAGCTGTTATGGCAACTGAACGGCCGCCTGGAGT ACTGCCTGAAGGATCGCATGAACTTTGATATTCCGGAAGAAATTAAACAGCTGCAAC AGTTCCAGAAAGAAGATGCGGCGCTGACCATTTATGAAATGCTGCAAAACATTTTTG CGATTTTTCGCCAAGATAGTAGTAGCACCGGCTGGAACGAAACCATTGTGGAAAACC TGCTCGCCAACGTGTACCATCAGATTAACCACCTGAAGACCGTGCTGGAAGAAAAAC TGGAAAAAGAAGATTTTACCCGCGGCAAACTGATGAGCAGCCTGCATCTGAAACGCT ATTATGGCCGCATTCTCCATTATCTGAAAGCCAAAGAGTATTCCCACTGTGCTTGGAC CATTGTTCGCGTGGAAATTCTGCGCAACTTTTATTTTATTAACCGCCTGACCGGCTATC TGCGCAAC
	Amino acid	QVQLVQSGGGPVKPGGSLRLSCAASGFMFR**AYSMN**WVRQAPGKGLEWVS**SISSSGRYIH** **YADSVKG**RFTISRDNAKNSLYLQMNSLRAEDTAVYYCAR**ETVMAGKALDY**WGQGTLV TVSSASTKGPSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGV HTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKRVEPKSSGSAPAPGS SYNLLGFLQRSSNFQSQKLLWQLNGRLEYCLKDRMNFDIPEEIKQLQQFQKEDAALTIYE MLQNIFAIFRQDSSSTGWNETIVENLLANVYHQINHLKTVLEEKLEKEDFTRGKLMSSLHL KRYYGRILHYLKAKEYSHCAWTIVRVEILRNFYFINRLTGYLRN
Light chain (Lser format)	**DNA**	GATATCGTTCTGACCCAATCTCCGGGTACGCTGAGCCTGAGCCCGGGCGAAACCGCG ACCCTGAGCTGCCGCGCGAGCCAAAGCGTGGGTTCTAATCTGGCTTGGTATCAGCAG AAACCGGGTCAGGCCCCGCGCCTGCTGATCTATGGGGCGAGCACGGGGGCTACCGGC GTTCCGGCGCGCTTTAGTGGCAGTCGCAGCGGCACCGATTTTACCCTGACCATTACAA GTCTGCAGCCGGAAGATTTTGCGACCTATTATTGCCAGCAATATTATAGCTTCCTGGC GAAAACCTTTGGTCAGGGCACCCAGCTGGAAATTAAACGCACCGTGGCGGCACCCAG CGTGACGGTGGCGGCACCCAGCGTGTTTATTTTCCTCCCAGTGATGAACAGCTGAAA AGCGGGACCGCGAGTGTTGTGTGCCTGTTGAACAACTTCTATCCTCGCGAAGCGAAA GTGCAGTGGAAAGTGGATAACGCATTGCAGAGCGGCAACAGTCAGGAAAGCGTTACT GAACAGGATAGCAAAGATAGTACGTACAGCTTGAGCAACACTCTGACCCTGAGTAAA GCGGATTATGAAAAACATAAAGTGTATGCATGCGAAGTTACGCATCAGGGGCTGAGC AGTCCGGTGACAAAGAGCTTTAACCGCGGCGAA**AGC**
	Amino acid	DIVLTQSPGTLSLSPGETATLSC**RASQSVGSNLA**WYQQKPGQAPRLLIY**GASTGAT**GVPA RFSGSRSGTDFTLTITSLQPEDFATYYC**QQYYSFLA**KTFGQGTQLEIKRTVAAPSVTVAAP SVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTY SLSNTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGES

*The linker and the effector domains were underlined and CDRs were written in bold.

[Fig. 22g]

7) EGL4$_{wt}$-hGH

Heavy chain (Hcys _ hGH format)	**DNA**	GAGGTGCAGCTGGTGCAGTCTGGGGGAGGCTTGGTACAGCCTGGCAGGTCCCTGAGA CTCTCCTGCACAGCCTCTGGATTCACCTTTGATGATTATGCCATGCACTGGGTCCGGC AAGCTCCAGGGAAGGGCCTGGAGTGGGTCTCAGGTATTAGTTGGAATGGTGGTAGCG TAGTCTATGCGGACTCTGTCAGGGGCCGATTCACCATCTCCAGAGACAACGCCAAGA ACTCCCTGTATCTGCAAATGAACAGTCTGAGAACTGAGGACACGGCCGTCTATTACTG TGCGAGAGATTACGGTTACTACGGTATGGACGTCTGGGGCCAAGGAACCCTGGTCAC CGTCTCCTCATCGGCCACATTGGCCGCCTCCACCAAGGGCCCATCGGTCTTCCCCCTG GCACCCTCCTCCAAGAGCACCTCTGAGGGCACAGCGGCCCTGGGCTGCCTGGTCAAG GACTACTTCCCCGAACCGGTGACGGTGTCGTGGAACTCAGGCGCCCTGACCAGCGGC GTGCACACCTTCCCGGCTGTCCTACAGTCCTCAGGACTCTACTCCCTCAGCAGCGTGG TGACCGTGCCCTCCAGCAGCTTGGGCACCCAGACCTACATCTGCAACGTGAATCACA AGCCCAGCAACACCAAGGTGGACAAGAGAGTTGAGCCCAAATCTTGTGGTTCTGCAC CAGCTCCTGGATCTTTTCCGACCATTCCGCTGAGCCGCCTGTTCGATAACGCGATGCT GCGCGCCCACCGCCTGCATCAACTGGCCTTTGATACCTATCAGGAGTTTGAGGAAGC GTACATCCCGAAGGAACAGAAATATTCTTTTCTGCAGAACCCACAGACGAGCCTGTG CTTTAGCGAATCTATCCCGACCCCGTCCAACCGCGAAGAAACCCAACAGAAGTCTAA CCTGGAACTGCTGCGTATCTCTCTGCTGCTGATTCAATCCTGGCTGGAACCGGTTCAA TTTCTGCGTAGCGTGTTTGCGAACTCTCTGGTGTATGGCGCGTCTGACTCTAACGTGT ATGACCTGCTGAAAGATCTGGAAGAAGGCATCCAAACTCTGATGGGCCGTCTGGAGG ACGGCTCTCCACGTACCGGCCAGATCTTTAAACAGACCTATAGCAAATTTGACACCA ATTCTCACAACGATGATGCGCTGCTGAAAAACTATGGCCTGCTGTATTGCTTCCGTAA AGACATGGATAAAGTTGAAACGTTCCTGCGCATTGTTCAGTGCCGTTCCGTGGAGGG CTCCTGCGGCTTC
	Amino acid	EVQLVQSGGGLVQPGRSLRLSCTASGFTFD**DYAMH**WVRQAPGKGLEWVS**GISWNGGSV** **VYADSVRG**RFTISRDNAKNSLYLQMNSLRTEDTAVYYCAR**DYGYYGMDV**WGQGTLVT VSSSATLAASTKGPSVFPLAPSSKSTSEGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTF PAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKRVEPKSCGSAPAPGSFPTI PLSRLFDNAMLRAHRLHQLAFDTYQEFEEAYIPKEQKYSFLQNPQTSLCFSESIPTPSNREE TQQKSNLELLRISLLLIQSWLEPVQFLRSVFANSLVYGASDSNVYDLLKDLEEGIQTLMGR LEDGSPRTGQIFKQTYSKFDTNSHNDDALLKNYGLLYCFRKDMDKVETFLRIVQCRSVEG SCGF
Light chain (Lcys format)	**DNA**	GATATTGTGATGACCCAGTCTCCATCTTCCGTGTCTGCATCTGTTGGAGACAGAGTCA CCATCACTTGTCGGGCGAGTCAGAATATTGGCAGCTGGTTAGCCTGGTATCAGCAGA AACCAGGTAACGCCCCTAAGTTGTTGATCTATAGAGCATCCAATTTGCGAAGTGGGG TCCCATCAAGGTTCAGCGGCAGTGGCTCTGGGACAGATTTCACTCTTACCATCAGCAG CCTGCAGCCTGAAGATTTCGCAACTTACTTTTGTCAACAGGCTACCATTTTCCCTCTCA CTTTCGGCGGAGGGACCCGGGTGGATATCAAACGTTCTAGAGCTGTGGCTGCACCAT CTGTCTTCATCTTCCCGCCATCTGATGAGCAGTTGAAATCTGGAACTGCCTCTGTTGTG TGCCTGCTGAATAACTTCTATCCCAGAGAGGCCAAAGTACAGTGGAAGGTGGATAAC GCCCTCCAATCGGGTAACTCCCAGGAGAGTGTCACAGAGCAGGACAGCAAGGACAG CACCTACAGCCTCAGCAACACCCTGACGCTGAGCAAAGCAGACTACGAGAAACACAA AGTCTACGCCTGCGAAGTCACCCATCAGGGCCTGAGTTCGCCCGTCACAAAGAGCTT CAACAGGGGAGAGTGT
	Amino acid	DIVMTQSPSSVSASVGDRVTITC**RASQNIGSWLA**WYQQKPGNAPKLLIY**RASNLR**SGVPS RFSGSGSGTDFTLTISSLQPEDFATYFC**QQATIFPLT**FGGGTRVDIKRSRTVAAPSVFIFPPS DEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSNTLT LSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC

*The linker and the effector domains were underlined and CDRs were written in bold.

[Fig. 22h]

8) EGL4$_{\triangle ds}$-hGH

Heavy chain (Hser _ hGH format)	**DNA**	GAGGTGCAGCTGGTGCAGTCTGGGGGAGGCTTGGTACAGCCTGGCAGGTCCCTGAGA CTCTCCTGCACAGCCTCTGGATTCACCTTTGATGATTATGCCATGCACTGGGTCCGGCA AGCTCCAGGGAAGGGCCTGGAGTGGGTCTCAGGTATTAGTTGGAATGGTGGTAGCGT AGTCTATGCGGACTCTGTCAGGGGCCGATTCACCATCTCCAGAGACAACGCCAAGAA CTCCCTGTATCTGCAAATGAACAGTCTGAGAACTGAGGACACGGCCGTCTATTACTGT GCGAGAGATTACGGTTACTACGGTATGGACGTCTGGGGCCAAGGAACCCTGGTCACC GTCTCCTCATCGGCCACATTGGCCGCCTCCACCAAGGGCCCATCGGTCTTCCCCCTGG CACCCTCCTCCAAGAGCACCTCTGAGGGCACAGCGGCCCTGGGCTGCCTGGTCAAGG ACTACTTCCCCGAACCGGTGACGGTGTCGTGGAACTCAGGCGCCCTGACCAGCGGCGT GCACACCTTCCCGGCTGTCCTACAGTCCTCAGGACTCTACTCCCTCAGCAGCGTGGTG ACCGTGCCCTCCAGCAGCTTGGGCACCCAGACCTACATCTGCAACGTGAATCACAAGC CCAGCAACACCAAGGTGGACAAGAGAGTTGAGCCCAAATCTAGTGGTTCTGCACCAG CTCCTGGATCTTTTCCGACCATTCCGCTGAGCCGCCTGTTCGATAACGCGATGCTGCGC GCCCACCGCCTGCATCAACTGGCCTTTGATACCTATCAGGAGTTTGAGGAAGCGTACA TCCCGAAGGAACAGAAATATTCTTTTCTGCAGAACCCACAGACGAGCCTGTGCTTTAG CGAATCTATCCCGACCCCGTCCAACCGCGAAGAAACCCAACAGAAGTCTAACCTGGA ACTGCTGCGTATCTCTCTGCTGCTGATTCAATCCTGGCTGGAACCGGTTCAATTTCTGC GTAGCGTGTTTGCGAACTCTCTGGTGTATGGCGCGTCTGACTCTAACGTGTATGACCT GCTGAAAGATCTGGAAGAAGGCATCCAAACTCTGATGGGCCGTCTGGAGGACGGCTC TCCACGTACCGGCCAGATCTTTAAACAGACCTATAGCAAATTTGACACCAATTCTCAC AACGATGATGCGCTGCTGAAAAACTATGGCCTGCTGTATTGCTTCCGTAAAGACATGG ATAAAGTTGAAACGTTCCTGCGCATTGTTCAGTGCCGTTCCGTGGAGGGCTCCTGCGG CTTC___
	Amino acid	EVQLVQSGGGLVQPGRSLRLSCTASGFTFD**DYAMH**WVRQAPGKGLEWV**SGISWNGGSV** **VYADSVRG**RFTISRDNAKNSLYLQMNSLRTEDTAVYYCAR**DYGYYGMDV**WGQGTLVT VSSSATLAASTKGPSVFPLAPSSKSTSEGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTF PAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKRVEPKSSGSAPAPGSFPTI PLSRLFDNAMLRAHRLHQLAFDTYQEFEEAYIPKEQKYSFLQNPQTSLCFSESIPTPSNREE TQQKSNLELLRISLLLIQSWLEPVQFLRSVFANSLVYGASDSNVYDLLKDLEEGIQTLMGR LEDGSPRTGQIFKQTYSKFDTNSHNDDALLKNYGLLYCFRKDMDKVETFLRIVQCRSVEG SCGF
Light chain (Lser format)	**DNA**	GATATTGTGATGACCCAGTCTCCATCTTCCGTGTCTGCATCTGTTGGAGACAGAGTCA CCATCACTTGTCGGGCGAGTCAGAATATTGGCAGCTGGTTAGCCTGGTATCAGCAGAA ACCAGGTAACGCCCCTAAGTTGTTGATCTATAGAGCATCCAATTTGCGAAGTGGGGTC CCATCAAGGTTCAGCGGCAGTGGCTCTGGGACAGATTTCACTCTTACCATCAGCAGCC TGCAGCCTGAAGATTTCGCAACTTACTTTTGTCAACAGGCTACCATTTTCCCTCTCACT TTCGGCGGAGGGACCCGGGTGGATATCAAACGTTCTAGAGCTGTGGCTGCACCATCTG TCTTCATCTTCCCGCCATCTGATGAGCAGTTGAAATCTGGAACTGCCTCTGTTGTGTGC CTGCTGAATAACTTCTATCCCAGAGAGGCCAAAGTACAGTGGAAGGTGGATAACGCC CTCCAATCGGGTAACTCCCAGGAGAGTGTCACAGAGCAGGACAGCAAGGACAGCACC TACAGCCTCAGCAACACCCTGACGCTGAGCAAAGCAGACTACGAGAAACACAAAGTC TACGCCTGCGAAGTCACCCATCAGGGCCTGAGTTCGCCCGTCACAAAGAGCTTCAACA GGGGAGAGAGT
	Amino acid	DIVMTQSPSSVSASVGDRVTITC**RASQNIGSWLA**WYQQKPGNAPKLLIY**RASNLRS**GVPSR FSGSGSGTDFTLTISSLQPEDFATYFC**QQATIFPLT**FGGGTRVDIKRSRTVAAPSVFIFPPSDE QLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSNTLTLS KADYEKHKVYACEVTHQGLSSPVTKSFNRGES

*The linker and the effector domains were underlined and CDRs were written in bold.

[Fig. 22i]

9) 1β28$_{wt}$-hGH

Heavy chain (Hcys _ hGH format)	**DNA**	CAGGTGCAGCTGGTGCAGTCAGGGGGAGGCCTGGTCAGGCCGGGGGGGTCCCTGAG ACTCTCCTGTGCAGCCTCTGGACTCATATTCAGTAATTATAGCATGAACTGGGTCCGC CAGGCTCCGGGGAAGGGGCTGGAGTGGGTCTCATCAATAAGTAGTGCTGGTAGTTAC AAATACTACACAGACTCAGTGAAGGGCCGATTCACCATCTCCAGAGACAACGCCAAG AAGTCACTGTATCTGCAAATGAACAGCCTGAGAGTCGACGACACGGCCGTCTATTAC TGTGCAAGAGGGGACTATGATACGGGCATGGAGCCCTGGGGCCAAGGCACCATGGTC ACCGTCTCCTCATCGGCCACATTGGCCGCCTCCACCAAGGGCCCATCGGTCTTCCCCC TGGCACCCTCCTCCAAGAGCACCTCTGGGGGCACAGCGGCCCTGGGCTGCCTGGTCA AGGACTACTTCCCCGAACCGGTGACGGTGTCGTGGAACTCAGGCGCCCTGACCAGCG GCGTGCACACCTTCCCGGCTGTCCTACAGTCCTCAGGACTCTACTCCCTCAGCAGCGT GGTGACCGTGCCCTCCAGCAGCTTGGGCACCCAGACCTACATCTGCAACGTGAATCA CAAGCCCAGCAACACCAAGGTGGACAAGAGAGTTGAGCCCAAATCTTGT**GGTTCTGC** **ACCAGCT**CCTGGATC**TTTTCCGACC**ATTCCGCTGAGCCGCCTGTTCGATAACGCGATG CTGCGCGCCCACCGCCTGCATCAACTGGCCTTTGATACCTATCAGGAGTTTGAGGAAG CGTACATCCCGAAGGAACAGAAATATTCTTTTCTGCAGAACCCACAGACGAGCCTGT GCTTTAGCGAATCTATCCCGACCCCGTCCAACCGCGAAGAAACCCAACAGAAGTCTA ACCTGGAACTGCTGCGTATCTCTCTGCTGCTGATTCAATCCTGGCTGGAACCGGTTCA ATTTCTGCGTAGCGTGTTTGCGAACTCTCTGGTGTATGGCGCGTCTGACTCTAACGTG TATGACCTGCTGAAAGATCTGGAAGAAGGCATCCAAACTCTGATGGGCCGTCTGGAG GACGGCTCTCCACGTACCGGCCAGATCTTTAAACAGACCTATAGCAAATTTGACACC AATTCTCACAACGATGATGCGCTGCTGAAAAACTATGGCCTGCTGTATTGCTTCCGTA AAGACATGGATAAAGTTGAAACGTTCCTGCGCATTGTTCAGTGCCGTTCCGTGGAGG GCTCCTGCGGCTTC
	Amino acid	QVQLVQSGGGLVRPGGSLRLSCAASGLIFS**NYSMN**WVRQAPGKGLEWVS**SISSAGSYKY** **YTDSVKG**RFTISRDNAKKSLYLQMNSLRVDDTAVYYCAR**GDYDTGMEP**WGQGTMVTV SSSATLAASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFP AVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKRVEPKSCGSAPAPGSFPTIP LSRLFDNAMLRAHRLHQLAFDTYQEFEEAYIPKEQKYSFLQNPQTSLCFSESIPTPSNREET QQKSNLELLRISLLLIQSWLEPVQFLRSVFANSLVYGASDSNVYDLLKDLEEGIQTLMGRL EDGSPRTGQIFKQTYSKFDTNSHNDDALLKNYGLLYCFRKDMDKVETFLRIVQCRSVEGS CGF
Light chain (Lcys format)	**DNA**	GAGCTCGAGCTCGTGTCGACGCAGTCTCCATCCTCCCTGTCTGCATCTGTGGGAGACA GAGTCACCATTACTTGCCGGGCAAGTCAGAGCATTAGCAGGTATTTAAATTGGTATCA GCAGAAACCAGGGAAAGCCCCTAAGCTCCTGATCTATGGTGCATCCAGATTAGAAAG TGGGGTCCCATCAAGGTTCAGTGGCAGTGGTTCTGGGACAGACTTCACTCTCACCATC AACAGCCTGCAACCTGAAGATTTTGCAACTTACTACTGTCAACAGAGTTACAGTACCC CTCTAACTTTTGGCCAGGGGACCCGAGTCGAAATTAAACGTGCTGTGGCTGCACCATC TGTCTTCATCTTCCCGCCATCTGATGAGCAGTTGAAATCTGGAACTGCCTCTGTTGTGT GCCTGCTGAATAACTTCTATCCCAGAGAGGCCAAAGTACAGTGGAAGGTGGATAACG CCCTCCAATCGGGTAACTCCCAGGAGAGTGTCACAGAGCAGGACAGCAAGGACAGC ACCTACAGCCTCAGCAACACCCTGACGCTGAGCAAAGCAGACTACGAGAAACACAA AGTCTACGCCTGCGAAGTCACCCATCAGGGCCTGAGTTCGCCCGTCACAAAGAGCTT CAACAGGGGAGAGTGT
	Amino acid	ELVSTQSPSSLSASVGDRVTITC**RASQSISRYLN**WYQQKPGKAPKLLIY**GASRLES**GVPSRF SGSGSGTDFTLTINSLQPEDFATYYC**QQSYSTPLT**FGQGTRVEIKRSRTVAAPSVFIFPPSDE QLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSNTLTLS KADYEKHKVYACEVTHQGLSSPVTKSFNRGEC

*The linker and the effector domains were underlined and CDRs were written in bold.

[Fig. 22j]

10) 1β28$_{\triangle ds}$-hGH

Heavy chain (Hser_hGH format)	**DNA**	CAGGTGCAGCTGGTGCAGTCAGGGGGAGGCCTGGTCAGGCCGGGGGGGTCCCTGAG ACTCTCCTGTGCAGCCTCTGGACTCATATTCAGTAATTATAGCATGAACTGGGTCCGC CAGGCTCCGGGGAAGGGGCTGGAGTGGGTCTCATCAATAAGTAGTGCTGGTAGTTAC AAATACTACACAGACTCAGTGAAGGGCCGATTCACCATCTCCAGAGACAACGCCAAG AAGTCACTGTATCTGCAAATGAACAGCCTGAGAGTCGACGACACGGCCGTCTATTAC TGTGCAAGAGGGGACTATGATACGGGCATGGAGCCCTGGGGCCAAGGCACCATGGTC ACCGTCTCCTCATCGGCCACATTGGCCGCCTCCACCAAGGGCCCATCGGTCTTCCCCC TGGCACCCTCCTCCAAGAGCACCTCTGGGGGCACAGCGGCCCTGGGCTGCCTGGTCA AGGACTACTTCCCCGAACCGGTGACGGTGTCGTGGAACTCAGGCGCCCTGACCAGCG GCGTGCACACCTTCCCGGCTGTCCTACAGTCCTCAGGACTCTACTCCCTCAGCAGCGT GGTGACCGTGCCCTCCAGCAGCTTGGGCACCCAGACCTACATCTGCAACGTGAATCA CAAGCCCAGCAACACCAAGGTGGACAAGAGAGTTGAGCCCAAATCTAGT**GGTTCTGC** **ACCAGCT**CCTGGATCT**TTTCCGACC**ATTCCGCTGAGCCGCCTGTTCGATAACGCGATG CTGCGCGCCCACCGCCTGCATCAACTGGCCTTTGATACCTATCAGGAGTTTGAGGAAG CGTACATCCCGAAGGAACAGAAATATTCTTTTCTGCAGAACCCACAGACGAGCCTGT GCTTTAGCGAATCTATCCCGACCCCGTCCAACCGCGAAGAAACCCAACAGAAGTCTA ACCTGGAACTGCTGCGTATCTCTCTGCTGCTGATTCAATCCTGGCTGGAACCGGTTCA ATTTCTGCGTAGCGTGTTTGCGAACTCTCTGGTGTATGGCGCGTCTGACTCTAACGTG TATGACCTGCTGAAAGATCTGGAAGAAGGCATCCAAACTCTGATGGGCCGTCTGGAG GACGGCTCTCCACGTACCGGCCAGATCTTTAAACAGACCTATAGCAAATTTGACACC AATTCTCACAACGATGATGCGCTGCTGAAAAACTATGGCCTGCTGTATTGCTTCCGTA AAGACATGGATAAAGTTGAAACGTTCCTGCGCATTGTTCAGTGCCGTTCCGTGGAGG GCTCCTGCGGCTTC
	Amino acid	QVQLVQSGGGLVRPGGSLRLSCAASGLIFS**NYSMN**WVRQAPGKGLEWVS**SISSAGSYKY** **YTDSVKG**RFTISRDNAKKSLYLQMNSLRVDDTAVYYCAR**GDYDTGMEP**WGQGTMVTV SSSATLAASTKGPSVFPLAPSSKSTSEGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFP AVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKRVEPKSSGSAPAPGSFPTIP LSRLFDNAMLRAHRLHQLAFDTYQEFEEAYIPKEQKYSFLQNPQTSLCFSESIPTPSNREET QQKSNLELLRISLLLIQSWLEPVQFLRSVFANSLVYGASDSNVYDLLKDLEEGIQTLMGRL EDGSPRTGQIFKQTYSKFDTNSHNDDALLKNYGLLYCFRKDMDKVETFLRIVQCRSVEGS CGF
Light chain (Lser format)	**DNA**	GAGCTCGAGCTCGTGTCGACGCAGTCTCCATCCTCCCTGTCTGCATCTGTGGGAGACA GAGTCACCATTACTTGCCGGGCAAGTCAGAGCATTAGCAGGTATTTAAATTGGTATCA GCAGAAACCAGGGAAAGCCCCTAAGCTCCTGATCTATGGTGCATCCAGATTAGAAAG TGGGGTCCCATCAAGGTTCAGTGGCAGTGGTTCTGGGACAGACTTCACTCTCACCATC AACAGCCTGCAACCTGAAGATTTTGCAACTTACTACTGTCAACAGAGTTACAGTACCC CTCTAACTTTTGGCCAGGGGACCCGAGTCGAAATTAAACGTGCTGTGGCTGCACCATC TGTCTTCATCTTCCCGCCATCTGATGAGCAGTTGAAATCTGGAACTGCCTCTGTTGTGT GCCTGCTGAATAACTTCTATCCCAGAGAGGCCAAAGTACAGTGGAAGGTGGATAACG CCCTCCAATCGGGTAACTCCCAGGAGAGTGTCACAGAGCAGGACAGCAAGGACAGC ACCTACAGCCTCAGCAACACCCTGACGCTGAGCAAAGCAGACTACGAGAAACACAA AGTCTACGCCTGCGAAGTCACCCATCAGGGCCTGAGTTCGCCCGTCACAAAGAGCTT CAACAGGGGAGAGAGT
	Amino acid	ELVSTQSPSSLSASVGDRVTITC**RASQSISRYLN**WYQQKPGKAPKLLIY**GASRLES**GVPSRF SGSGSGTDFTLTINSLQPEDFATYYC**QQSYSTPLT**FGQGTRVEIKRSRTVAAPSVFIFPPSDE QLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSNTLTLS KADYEKHKVYACEVTHQGLSSPVTKSFNRGES

*The linker and the effector domains were underlined and CDRs were written in bold.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR2014/008106

A. CLASSIFICATION OF SUBJECT MATTER

C07K 16/18(2006.01)i, C07K 16/46(2006.01)i, A61K 39/395(2006.01)i

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C07K 16/18; A61K 39/395; C07K 19/00; A61K 51/00; A61K 38/04; C07K 16/00; C09K 19/00; A61K 47/48; C07K 16/46

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
Korean utility models and applications for utility models
Japanese utility models and applications for utility models

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
eKOMPASS(KIPO internal) & Keywords:antigen binding fragment(Fab), serum albumin, fusion construct, half-life

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	KR 10-2012-0133403 A (Domantis, Ltd) 10 Dec. 2012 See abstract, page 23	7,18
A		1-6,19
X	KR 10-2007-0073886 A (Genentech, Inc.) 10 Jul. 2007 See abstract, claims 1, 10	7,18
A		1-6,19
A	KR 10-2007-0041781 A (Trubion Pharmaceuticals, Inc.) 19 Apr.2007 See abstract, claim 1	1-7,18,19
A	KR 10-2011-0008086 A (Merrimack Pharmaceuticals, Inc.) 25 Jan.2011 See abstract	1-7,18,19
A	Smith, B.J. et al., Prolonged in vivo residence times of antibody fragments associated with albumin. Bioconjug. Chem. 2001 Sep.-Oct.; 12(5): 750-756. See the whole document	1-7,18,19

☐ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents,such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
01 December 2014 (01.12.2014)	**02 December 2014 (02.12.2014)**

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Form PCT/ISA/210 (second sheet) (July 2009)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR2014/008106

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 12, 15-17, 20-23, 25
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
Claims 12, 15, 16, 20-23, 25 ultimately refer to one of claims 11, 13, 14, 24, which are not drafted in accordance with third sentence of Rule 6.4(a), rendering said claims unclear.
Claim 17 is unclear, since it refers to claim 17 itself.

3. ☒ Claims Nos.: 8-11, 13, 14, 24, 26, 27
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required addtional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of any additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
☐ No protest accompanied the payment of additional search fees.

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