



(86) Date de dépôt PCT/PCT Filing Date: 2014/03/10

(87) Date publication PCT/PCT Publication Date: 2014/10/09

(45) Date de délivrance/Issue Date: 2023/09/19

(85) Entrée phase nationale/National Entry: 2015/08/27

(86) N° demande PCT/PCT Application No.: US 2014/022404

(87) N° publication PCT/PCT Publication No.: 2014/164427

(30) Priorité/Priority: 2013/03/12 (US61/777,016)

(51) Cl.Int./Int.Cl. *C07K 19/00* (2006.01),
A61K 47/68 (2017.01), *A61P 35/00* (2006.01),
C07K 14/705 (2006.01), *C07K 14/71* (2006.01),
C07K 16/28 (2006.01), *C12N 15/62* (2006.01),
C12N 15/85 (2006.01), *C12P 21/02* (2006.01)

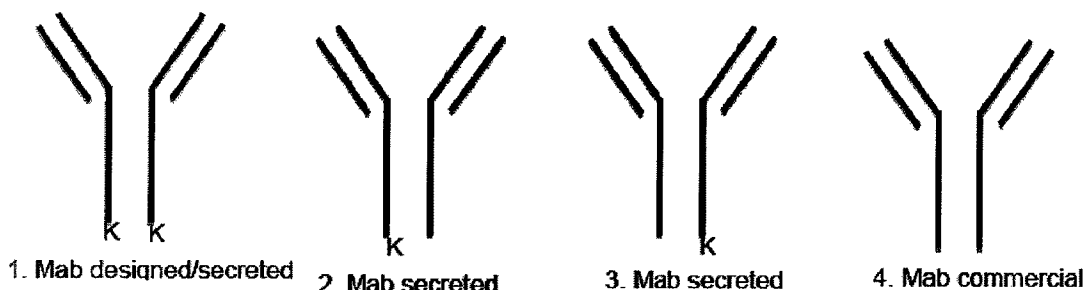
(72) Inventeurs/Inventors:
GOVINDAPPA, NAGARAJ, IN;
SOARES, MARIA MELINA, IN;
SASTRY, KEDARNATH, IN

(73) Propriétaire/Owner:
BIOCON LTD., IN

(74) Agent: KIRBY EADES GALE BAKER

(54) Titre : PROTEINES DE FUSION IMMUNOMODULATRICES ET LEURS PROCEDES DE FABRICATION

(54) Title: FUSION IMMUNOMODULATORY PROTEINS AND METHODS FOR MAKING SAME



(57) **Abrégé/Abstract:**

The present invention relates generally to the field of generating recombinant chimeric fusion proteins to be used in the cancer therapy, and more specifically, to fusion molecules of Anti- EGFR1-TGF β R11, Anti-EGFR1-PD1 and Anti-CTLA4-PD1 and methods of generating same, wherein the methods reduce production costs and increase homogeneity of the recombinant chimeric fusion proteins.

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property
Organization
International Bureau



(10) International Publication Number
WO 2014/164427 A1

(43) International Publication Date
9 October 2014 (09.10.2014)

(51) International Patent Classification:

C07K 19/00 (2006.01) *A61K 38/17* (2006.01)
C12N 15/62 (2006.01)

(21) International Application Number:

PCT/US2014/022404

(22) International Filing Date:

10 March 2014 (10.03.2014)

(25) Filing Language:

English

(26) Publication Language:

English

(30) Priority Data:

61/777,016 12 March 2013 (12.03.2013) US

(71) Applicant: BIOCON LTD. [IN/IN]; 20th K.M Hosur Rd., Electronic City PO, Bangalore 560100 (IN).

(72) Inventors; and

(71) Applicants : **GOVINDAPPA, Nagaraj** [IN/IN]; Nakshatra, #40, 3rd Cross, Santosh Residency, Kadabere, Bangalore, Karnataka 562130 (IN). **SOARES, Maria Melina** [US/IN]; L2, Chaitanya Samarpan, White-field-Hoskote, Main Road, Kannamangala, KadugodiPO, Bangalore, Karnataka 560067 (IN). **SASTRY, Kedarnath** [IN/IN]; #77 A, 2nd Cross, Bannashankari 3rd Stage, 3rd Phase 4th Block, Bangalore, Karnataka 560085 (IN).

(74) Agents: **FUIERER, Tristan** et al.; Moore & Van Allen, PLLC, P.O. Box 13706, Research Triangle Park, NC 27709 (US).

(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

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

Published:

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

(54) Title: FUSION IMMUNOMODULATORY PROTEINS AND METHODS FOR MAKING SAME

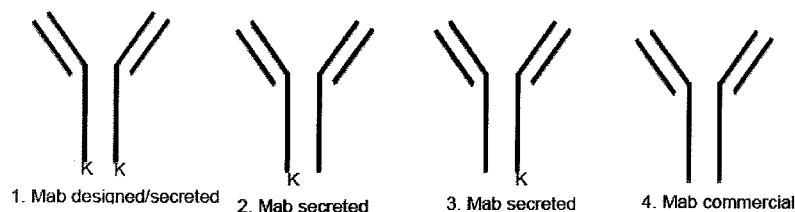


Figure 1

(57) Abstract: The present invention relates generally to the field of generating recombinant chimeric fusion proteins to be used in the cancer therapy, and more specifically, to fusion molecules of Anti-EGFR1-TGFβRII, Anti-EGFR1-PD1 and Anti-CTLA4-PD1 and methods of generating same, wherein the methods reduce production costs and increase homogeneity of the recombinant chimeric fusion proteins.

FUSION IMMUNOMODULATORY PROTEINS AND METHODS FOR MAKING SAME

[001]

BACKGROUND

[002] Technical Field

[003] The present disclosure relates generally to the field of generating recombinant chimeric fusion proteins to be used in the cancer therapy, and more specifically, to fusion molecules of Anti-EGFR1-TGF β RII, Anti-EGFR1-PD1 and Anti-CTLA4-PD1 and methods of generating same, wherein the methods reduce production costs and increase homogeneity of the recombinant chimeric fusion proteins.

[004] Related Art

[005] In spite of numerous advances in medical research, cancer remains the second leading cause of death in the United States. Traditional modes of clinical care, such as surgical resection, radiotherapy and chemotherapy, have a significant failure rate, especially for solid tumors. Failure occurs either because the initial tumor is unresponsive, or because of recurrence due to regrowth at the original site or metastasis. Cancer remains a central focus for medical research and development.

[006] Immunotherapy of cancer has been explored for over a century, but it is only in the last decade that various antibody-based products have been introduced into the management of patients with diverse forms of cancer. At present, this is one of the most active areas of clinical research, with numerous antibody therapeutic products already approved in oncology.

[007] Using specific antibodies as therapeutic agents offers advantages over the non-targeted therapies such as systemic chemotherapy via oral or intravenous administration of

drugs or radiation therapy. There are two types of antibody-based therapies. The more common type is to identify a tumor antigen (i.e., a protein expressed on tumors and cancer cells and not in normal tissues) and develop an antibody, preferably a monoclonal antibody (mAb) directed to the tumor antigen. One can then conjugate any therapeutic agent, such as a chemotherapeutic agent, a radionuclide, modified toxin, etc., to this antibody to achieve targeted therapy by the therapeutic agent to the tumor. The other kind of antibody based therapy is by providing an antibody which in itself has therapeutic properties against the tumor/cancer cells it targets. The added advantage of this second form of antibody-based therapy is that one may additionally conjugate another therapeutic agent to the therapeutic antibody to achieve a more effective treatment. The major advantage with any antibody-directed therapy and of therapy using monoclonal antibodies (mAbs) in particular, is the ability to deliver increased doses of a therapeutic agent to a tumor, with greater sparing of normal tissue from the side effects of the therapeutic agent.

[008] Despite the identification of several antibodies for cancer therapies, there is still a need to identify new and more effective therapeutics to overcome immune tolerance and activate T cell responses. Further, even though molecular engineering has improved the prospects for such antibody-based therapeutics issues still remain regarding continuity in the generated recombinant products.

SUMMARY

[008a] Selected exemplary embodiments provide a method for preparing a therapeutically active fusion protein having bispecific binding activity, wherein the fusion protein comprises a tumor targeting moiety and at least one immunomodulatory moiety that counteracts immune tolerance of a cancer cell, wherein the tumor targeting moiety is an antibody that binds to EGFR1 and wherein the fusion protein is prepared by the following steps: preparing a codon optimized nucleotide sequence encoding the fusion protein for expression in host CHO cells, wherein the codon optimized nucleotide sequence comprises SEQ ID NO: 1 and SEQ ID NO: 2 for encoding the antibody that binds to EGFR1, wherein the codon optimized nucleotide sequence comprises SEQ ID NO: 3 or 7 for encoding the at least one immunomodulatory

moiety, wherein the at least one immunomodulatory moiety is selected from the group consisting of TGFβRII and PD1 respectively, wherein the immunomodulatory moiety is linked to the antibody by the entire amino acid sequence expressed by codon optimized nucleotide sequence SEQ ID NO: 4, and wherein the codon optimized nucleotide sequence SEQ ID NO: 1 for encoding the heavy chain of the antibody that binds to EGFR1 is lacking nucleotides for expression of a lysine at the C-terminal end of the heavy chain of the antibody; cloning the codon optimized nucleotide sequence of said fusion protein in the host CHO cells capable of transient or stable expression; growing the host CHO cells in a media under suitable conditions for growing and allowing the host cell to express the fusion protein; and collecting secreted fusion proteins.

[009] The present disclosure provides for a novel and consistent synthesis method for generating homogeneous recombinant fusion immunomodulatory molecules, and more specifically, recombinant chimeric polypeptides including targeting antibodies linked to immunomodulatory proteins.

[0010] To mediate an immune response against cancer, T cell activation and co-stimulation are both important. Co-stimulation of T cells is mainly mediated through engaging of CD28 with its ligands of B7 family on antigen presenting cells (APCs). However, after activation, T cells express a molecule called Cytotoxic T-Lymphocyte Antigen 4 (CTLA-4), which binds to B7 ligands with much more affinity than CD28 and such binding down-modulates T cell activity. Thus, including an antibody that binds to the CTLA-4 receptor would block interaction with ligands of the B7 family and enhance anti-tumor response.

[0011] Programmed Death Ligand-1 (PDL1), one of the B7 ligands discussed above, obstructs anti-tumor immunity by (i) tolerizing tumor-reactive T cells by binding to its receptor PD1 (CD279) on T cells; (ii) rendering tumor cells resistant to CD8+ T cell and FasL-mediated lysis by PD-1 signaling through tumor cell-expressed PDL1; and (iii) promoting the development and maintenance of induced T regulatory cells. Therefore, PDL1 is a major obstacle to natural anti-tumor immunity and to cancer immunotherapies requiring activation of host T cell-mediated anti-tumor immunity. This concept is supported by studies demonstrating that antibody blocking of PDL1-PD1 interactions improves T cell activation and reduces tumor progression. Although antibodies to PDL1 or PD1 have shown therapeutic efficacy in a subset of cancer patients, the majority of patients do not benefit from antibody treatment. Thus, there is needed a mechanism for regulating PD-L1 function that will lead to a new universally applicable treatment for minimizing PD-L1-mediated immune suppression in cancer patients and that is more effective than currently available mAbs to PD-1 or PD-L1.

[0012] A characteristic of many epithelial cancers, such as, cancers of the colon, head and neck, breast, ovary, non-small cell lung (NSCL), and pancreas, is abnormally high levels of epidermal growth factor receptor (EGFR) on the surface of cancer cells. The family of epidermal growth factor receptors (EGFR; HER1, HER2/*neu*, HER3, and HER4) includes cell membrane receptors with intrinsic tyrosine kinase activity that trigger a cascade of biophysiological signaling reactions in response to the binding of different ligands. These receptors play a key role in the behavior of malignant cells in a variety of human tumors, inducing increased proliferation, decreasing apoptosis, and enhancing tumor cell motility and angiogenesis. Thus, the present disclosure includes antibodies targeting EGFR family members.

[0013] Further provided are methods of reducing growth of cancer cells by counteracting immune tolerance of cancer cells, wherein T cells remain active and inhibit the recruitment of T-regulatory that are known to suppress the immune system's response to the tumor. Thus, the chimeric polypeptides generated by the polynucleotides sequences of selected embodiments are useful for treating cancer because of the expressed fusion or chimeric polypeptides.

[0013a] Certain exemplary embodiments provide a method for preparing therapeutically active fusion protein having bispecific binding activity, wherein the fusion protein comprises a tumor targeting moiety and at least one immunomodulatory molecule that counteracts immune tolerance of a cancer cell, wherein the tumor targeting moiety is an antibody that binds to CTLA-4 or EGFR1 and wherein the fusion protein is prepared by the following steps: preparing a codon optimized nucleotide sequence encoding the fusion protein for expression in CHO cells, wherein the codon optimized nucleotide sequence is modified to increase CG sequences, and wherein the codon optimized nucleotide sequence is SEQ ID NO: 1 and SEQ ID NO: 2 for the antibody that binds to CTLA-4 or SEQ ID NO: 5 and SEQ ID NO: 6 for the antibody that binds to EGFR1, and SEQ ID NO: 3 or 7 for the at least one immunomodulatory molecule, wherein the codon optimized nucleotide sequence SEQ ID NO: 1 or 5 for the heavy chain of the antibody that binds to CTLA-4 or EGFR1 respectively is lacking nucleotides for expression of a lysine at the C-terminal end of the heavy chain of the antibody; cloning the optimized sequence of said fusion protein in the CHO cells capable of transient or stable expression; growing the CHO cells in a media under suitable conditions for growing and allowing the host cell to express the fusion protein; and collecting secreted fusion proteins and optionally for further purification.

[0014] In one aspect, selected embodiments provide for chimeric polypeptides containing at least one targeting moiety to target a cancer cell and at least one immunomodulating moiety that counteracts immune tolerance of cancer cell, wherein the targeting moiety and the immunomodulating moiety are linked by an amino acid spacer of sufficient length of amino acid residues so that both moieties can successfully bond to their individual target. In the alternative, the targeting moiety and the immunomodulating moiety that counteract immune tolerance of cancer cell may be bound directly to each other. The chimeric/fusion polypeptides of selected embodiments are useful for binding to a cancer cell receptor and reducing the ability of cancer cells to avoid an immune response.

[0015] Preferably the targeting moiety is an antibody having binding affinity for CTLA-4 or EGFR1, wherein the antibody is transcribed from a polynucleotide sequence lacking nucleotides for expression of the C-terminal lysine of the heavy chain of the expressed antibody. It has been discovered that by removing the C-terminal lysine of the heavy chain of an antibody during transcription that the end product exhibits increased homogeneity, thereby reducing the need and costs for further purification.

[0016] It is known that during the process of transcription and translation of an IgG molecule in CHO cells, the lysine (K) at the C-terminal of the heavy chain will be expressed. In the commercial product such expressed lysines have to be removed to increase purity. There is much heterogeneity in the produced product, as shown in Figure 1. This occurs because the CHO cell has an endogenous enzyme Carboxypeptidase B (CPB) which will cleave the C-terminal lysine as long as the expressed antibody is still available intracellularly. However, this enzyme will not cleave the lysine once the antibody is secreted into the medium. Thus, the cleavage efficiency of this endogenous CPB is based on the availability within the cell. As such, some of the antibodies will be secreted with the lysine and some will not, and such combination will cause significant heterogeneity in the secreted product, that being some antibodies with the C-terminal lysine and some without. As the recombinant product is being used for the therapeutic use, one needs to purify to homogeneity. Thus, the recombinant products of the prior art requires additional purification steps wherein the recombinant product need to be treated with the enzyme CPB first and purified once again using an additional step to remove any lysine and the enzyme CPB from the final product. These additional steps add a significant cost to the manufacturing process.

[0017] Selected embodiments avoid the shortcomings of previous methods of synthesizing recombinant anti-CTLA-4 and anti-EGFR1 antibodies by transcribing an expressed protein from a polynucleotide sequence lacking nucleotides for expression of the C-terminal lysine at the heavy chain of the expressed antibody.

[0018] Selected embodiments are based on preparing chimeric/fusion proteins by expression of polynucleotides encoding the fusion proteins that counteract or reverse immune tolerance of cancer cells. Cancer cells are able to escape elimination by chemotherapeutic agents or tumor-targeted antibodies via specific immunosuppressive mechanisms in the tumor microenvironment and such ability of cancer cells is recognized as immune tolerance. Such immunosuppressive mechanisms include immunosuppressive cytokines (for example, Transforming growth factor beta (TGF- β)) and regulatory T cells and/or immunosuppressive myeloid dendritic cells (DCs). By counteracting tumor-induced immune tolerance, the present invention provides effective compositions and methods for cancer treatment, optional in combination with another existing cancer treatment. Selected embodiments provide strategies to counteract tumor-induced immune tolerance and enhance the antitumor efficacy of chemotherapy by activating and leveraging T cell-mediated adaptive antitumor against resistant or disseminated cancer cells.

[0019] In another aspect, selected embodiments provide a molecule including at least one targeting moiety fused with at least one immunomodulatory moiety. The targeting moiety specifically binds a target molecule, and the immunomodulatory moiety specifically binds one of the following molecules: (i) Transforming growth factor-beta (TGF- β) and or (ii) Programmed death- 1 ligand 1 (PD-L1).

[0020] In a further aspect, the targeting moiety includes an antibody, including both heavy chains and light chains, wherein the antibody specifically binds a component of a tumor cell, tumor antigen, tumor vasculature, tumor microenvironment, or tumor-infiltrating immune cell. Notably the heavy chain and/or light chain may individually be linked to a same type immunomodulatory moiety or a separate and distinct immunomodulatory moiety. Further, a heavy or light chain of an antibody targeting moiety may be linked to an immunomodulatory moiety which in turn can be further linked to a second immunomodulatory moiety wherein there is a linker between the two immunomodulatory moieties.

[0021] In a still further aspect, there is provided a chimeric polypeptide that comprised a tumor targeting moiety and an immunomodulatory moiety comprising a molecule that binds transforming growth factor beta (TGF- β), wherein the tumor targeting moiety is an antibody that binds to EGFR1, where in the antibody can be the full antibody, heavy chain or light chain.

[0022] The tumor targeting moiety may include monoclonal antibodies that target a cancer cell, including but not limited to cetuximab, trastuzumab, rituximab, ipilimumab, tremelimumab, muromonab-CD3, abciximab, daclizumab, basiliximab, palivizumab, infliximab, gemtuzumab, ozogamicin, alemtuzumab, ibritumomab tiuxetan, adalimumab, omalizumab, tositumomab, I-131 tositumomab, efalizumab, bevacizumab, panitumumab, pertuzumab, natalizumab, etanercept, IGN101 (Aphtron), volociximab (Biogen Idec and PDL BioPharm), Anti-CD80 mAb (Biogen Idec), Anti-CD23 mAb (Biogen Idec), CAT-3888 (Cambridge Antibody Technology), CDP-791 (Imclone), eraptuzumab (Immunomedics), MDX-010 (Medarex and BMS), MDX-060 (Medarex), MDX-070 (Medarex), matuzumab (Merck), CP-675,206 (Pfizer), CAL (Roche), SGN-30 (Seattle Genetics), zanolimumab (Serono and Genmab), adecatumumab (Serono), oregovomab (United Therapeutics), nimotuzumab (YM Bioscience), ABT-874 (Abbott Laboratories), denosumab (Amgen), AM 108 (Amgen), AMG 714 (Amgen), fontolizumab (Biogen Idec and PDL BioPharm), daclizumab (Biogen Idec and PDL BioPharm), golimumab (Centocor and Schering-Plough), CNTO 1275 (Centocor), ocrelizumab (Genentech and Roche), HuMax-CD20 (Genmab), belimumab (HGS and GSK), epratuzumab (Immunomedics), MLN1202 (Millennium Pharmaceuticals), visilizumab (PDL BioPharm), tocilizumab (Roche), ocrerlizumab (Roche), certolizumab pegol (UCB, formerly Celltech), eculizumab (Alexion Pharmaceuticals), pexelizumab (Alexion Pharmaceuticals and Procter & Gamble), abciximab (Centocor), ranibizumab (Genentech), mepolizumab (GSK), TNX-355 (Tanox), or MYO-029 (Wyeth).

[0023] In a preferred embodiment, the tumor targeting moiety is a monoclonal antibody that binds to CTLA-4 or EGFR1 generated by the methods disclosed herein, wherein the method comprises the following steps:

- a. preparing a codon optimized nucleotide sequence encoding the fusion protein, wherein the codon optimized sequence for the antibody is lacking nucleotides for expression of a lysine at the C-terminal end of the heavy chains of the antibody;

- b. cloning the optimized sequence of said fusion protein in a host cell capable of transient or continued expression;
- c. growing the host cell in a media under suitable conditions for growing and allowing the host cell to express the fusion protein; and
- d. collecting secreted fusion proteins.

[0024] In yet another aspect, the immunomodulatory moiety includes a molecule that binds TGF- β and inhibits the function thereof. Specifically the immunomodulatory moiety includes an extracellular ligand-binding domain of Transforming growth factor-beta receptor TGF- β RII, TGF- β RIIb or TGF- β RIII. In another aspect the immunomodulatory moiety includes an extracellular ligand-binding domain (ECD) of TGF- β RII

[0025] In a still further aspect, the targeting moiety includes an antibody that specifically binds to HER2/neu, EGFR1, CD20, or cytotoxic T-lymphocyte antigen-4 (CTLA-4) and wherein the immunomodulatory moiety includes an extracellular ligand-binding domain of TGF- β RII.

[0026] In yet another aspect, the immunomodulatory moiety includes a molecule that specifically binds to and inhibits the activity of Programmed death- 1 ligand 1 (PD-L 1).

[0027] In a further aspect, the targeting moiety includes an antibody, antibody fragment, or polypeptide that specifically binds to HER2/neu, EGFR1, CD20, cytotoxic T-lymphocyte antigen-4 (CTLA-4), CD25 (IL-2a receptor; IL-2aR), or CD4 and wherein, the immunomodulatory moiety includes an extracellular ligand-binding domain or ectodomain of Programmed Death- 1 (PD-1).

[0028] In a still further aspect, the targeting moiety includes an antibody that specifically binds to EGFR1 and CTLA-4, and the immunomodulatory moiety includes a sequence from interacts with transforming growth factor- β (TGF- β).

[0029] In one aspect, embodiments provide for optimized genes encoding for a fusion polypeptide comprising at least one targeting moiety and at least one immunomodulatory moiety for treating cancer in a human subject wherein the genes have been optimized to increase expression in a human subject and/or cells.

[0030] In another aspect, selected embodiments provide for a vector comprising optimized genes for treating cancer in a human subject wherein the optimized genes have been modified to increase CG sequences. Preferably, the vector includes nucleotide sequences for encoding at least one targeting moiety, at least one immunomodulatory moiety and a linking moiety, wherein the optimized nucleotide sequences are selected from SEQ ID NOs: 1 to 7, as set forth in Figure 2.

[0031] In yet another aspect, embodiments provide for a method of treating cancer in a subject, the method comprising:

providing at least one recombinant vector comprising nucleotide sequences that encode at least one targeting moiety, at least one immunomodulatory moiety and a linking moiety positioned between the targeting moiety and immunomodulatory moiety, wherein the nucleotide sequences are selected from SEQ ID NOs: 1 to 7; and

administering the recombinant vector to the subject under conditions such that said nucleotide sequences are expressed at a level which produces a therapeutically effective amount of the encoded fusion proteins in the subject.

[0032] In yet another aspect, there is provided a recombinant host cell transfected with a polynucleotide sequence that encodes a fusion protein peptide of the present invention, wherein the polynucleotide sequences are selected from SEQ ID NOs: 1 to 7.

[0033] In a still further aspect, embodiments contemplate a process of preparing a chimeric fusion protein comprising:

transfecting a host cell with a polynucleotide sequence that encodes a chimeric fusion protein to produce a transformed host cell, wherein the polynucleotide sequence encodes at least one targeting moiety and at least one immunomodulatory moiety, wherein the polynucleotide sequence comprises a combination of sequences selected from SEQ ID NOs: 1 to 7; and

maintaining the transformed host cell under biological conditions sufficient for expression of the chimeric fusion protein.

[0034] In another aspect, embodiments relate to the use of a chimeric fusion protein, wherein the chimeric fusion protein comprises anti-EGFR1 linker PD1 (SEQ ID NOs: 8, 9, 10 and 11); anti-EGFR1-linker-TGF β RII (SEQ ID NOs: 8, 9, 10 and 12); Anti-CTLA-4-linker-PD1 (SEQ ID NOs: 13, 14, 10 and 11), as shown in Figures 3, 4 and 5 respectively, in the use of a medicament for the treatment of cancer. Preferably, the fusion protein is expressed in a host cell and such expressed proteins are administered in a therapeutic amount to reduce the effects of cancer in a subject in need thereof.

[0035] In a still further aspect, there is provided a method of treating a neoplastic disease. The method includes administration to a subject in need thereof one or more fusion proteins of the present invention, in various aspects, the subject is administered one or more fusion protein of the present invention in combination with another anticancer therapy. In one aspect, the anticancer therapy includes a chemotherapeutic molecule, antibody, small molecule kinase inhibitor, hormonal agent or cytotoxic agent. The anticancer therapy may also include ionizing radiation, ultraviolet radiation, cryoablation, thermal ablation, or radiofrequency ablation.

[0036] In a preferred embodiment the therapeutically active antibody-peptide fusion proteins is a targeting antibody fused to one or more immunomodulating moiety that counteracts immune tolerance of a cancer cell. In one aspect, the immunomodulating moiety may be linked by an amino acid spacer of sufficient length to allow bi-specific binding of the molecule. The immunomodulating moiety may be bound to either the N- terminus or C-terminus of the heavy chain or the N- terminus or C-terminus of the light chain of the antibody

[0037] The method can in selected embodiments provide nucleotide sequences that encode the therapeutically active antibody-peptide fusion proteins and such expression may be conducted in a transient cell line or a stable cell line. The transient expression is accomplished by transfecting or transforming the host cell with vectors carrying the encoded fusion proteins into mammalian host cells

[0038] Once the fusion peptides are expressed, they are preferably subjected to purification and *in-vitro* tests to check its bi-specificity, that being, having the ability to bind to both the target moiety and immunomodulating moiety. Such tests may include *in-vitro* tests such as

ELISA or NK/T-cell binding assays to validate bi-functional target binding or immune cell stimulation.

[0039] Notably once the specific fusion peptides demonstrate the desired bi-specificity, the polynucleotide sequences encoding such fusion peptides are selected for sub-cloning into a stable cell line for larger scale expression and purification. Such stable cell lines are previously disclosed, such as a mammalian cell line, including but not limited to HEK293, CHO or NSO.

[0040] In another aspect there is provided a method to inhibit and/or reduce binding of PDL1 to PD1 thereby increasing immune response against tumor cells, the method comprising:

- a. providing a chimeric polypeptide comprising PD1 and an anti-EGFR1 or anti-CTLA-4 antibody; and
- b. contacting a tumor cell with the chimeric polypeptide wherein the chimeric polypeptide binds with at least PDL1 of the tumor cell.

[0041] In yet another aspect, there is provided a method of preparing therapeutically active antibody-peptide fusion proteins, the method comprising;

- a. preparing a codon optimized sequence of the said fusion protein, wherein the codon optimized sequences for anti-EGFR1 and anti-CTLA-4 antibodies are lacking nucleotides for expression of a lysine at the C-terminal end of the heavy chains of the antibodies;
- b. cloning the optimized sequence of said fusion protein in a host cell capable of transient or continued expression;
- c. growing the host cell in a media under suitable conditions for growing and allowing the host cell to express the fusion protein; and
- d. collecting secreted fusion proteins.

[0042] In a still further aspect selected embodiments provide for a nucleic acid sequence encoding a chimeric fusion protein, wherein the chimeric fusion protein comprises at least one targeting moiety having affinity for a cancer cell and at least one immunomodulatory moiety that counteract immune tolerance of the cancer cell, wherein targeting moiety is an antibody and the nucleic acid sequence of the targeting moiety is lacking nucleotides for expression of a lysine at the C-terminal end of the heavy chains of the antibody. The nucleic

acid sequence encoding the heavy chain of the antibody preferably includes SEQ ID NO: 1 or SEQ ID NO:5. The nucleic acid sequence encoding the chimeric fusion proteins preferably comprises a sequence selected from the group consisting of SEQ ID NOs: 1, 2, 4 and 7; SEQ ID NOs: 1, 2, 3 and 4; and SEQ ID NOs: 5, 6, 3 and 4.

[0043] In yet another aspect there is provided a method of treating cancer in a subject, the method comprising:

a) preparing a preparing therapeutically active fusion protein, wherein the fusion protein comprises a tumor targeting moiety and at least one immunomodulatory molecule, wherein the tumor targeting moiety is an antibody that binds to CTLA-4 or EGFR1 and wherein the fusion protein is prepared by the following steps:

[0044] preparing a codon optimized nucleotide sequence encoding the fusion protein, wherein the codon optimized nucleotide sequence for the antibody is lacking nucleotides for expression of a lysine at the C-terminal end of the heavy chains of the antibody;

- a) ii) cloning the optimized sequence of said fusion protein in a host cell capable of transient or continued expression;
- b) iii) growing the host cell in a media under suitable conditions for growing and allowing the host cell to express the fusion protein; and
- c) iv) collecting secreted fusion proteins;
- b. b) administering a therapeutically active amount of the secreted fusion proteins to the subject.

[0045] The fusion protein is selected from the group of amino acid sequences consisting of SEQ ID NOs: 15 and 9; SEQ ID NOs: 8 and 16; SEQ ID NOs: 17 and 9; SEQ ID NOs: 8 and 18; SEQ ID NOs: 27 and 9; SEQ ID NOs: 8 and 28; SEQ ID NOs: 29 and 9; SEQ ID NOs: 8 and 30; SEQ ID NOs: 31 and 28; SEQ ID NOs: 31 and 30; SEQ ID NOs: 29 and 28; SEQ ID NOs: 29 and 30; SEQ ID NOs: 32 and 14; SEQ ID NOs: 13 and 33; SEQ ID NOs: 34 and 14; SEQ ID NOs: 13 and 35; SEQ ID NOs: 32 and 33; SEQ ID NOs: 32 and 35; SEQ ID NOs: 34 and 33 and SEQ ID NOs: 34 and 35.

[0046] In another aspect, there is provided a method of treating a neoplastic disease, the method comprising administration to a subject in need thereof one or more fusion proteins encoded by at least one polynucleotide sequence selected from the group consisting of SEQ ID NOs: 1, 2, 4 and 7; SEQ ID NOs: 1, 2, 3 and 4; and SEQ ID NOs: 5, 6, 3 and 4.

Notably by using the above defined polynucleotide sequences, the following combination of fusion proteins can be expressed including anti-EGFR1 linker PD1 (SEQ ID NOs: 8, 9, 10 and 11); anti-EGFR1-linker-TGF β RII (SEQ ID NOs: 8, 9, 10 and 12); and Anti-CTLA-4-linker-PD1 (SEQ ID NOs: 13, 14, 10 and 11).

[0047] Other features and advantages of the invention will be apparent from the following detailed description, drawings and claims.

BRIEF DESCRIPTION OF THE FIGURES

[0048] Figure 1 shows the different possibilities of lysine placement on a heavy chain and such heterogeneity causing the need to provide purification.

[0049] Figure 2 shows the optimized codon nucleotide sequences used for expression of the antibody-peptide fusion proteins of the present invention, including Anti-EGFR1 heavy chain (SEQ ID NO: 1); Anti-EGFR1 light chain (SEQ ID NO: 2); PD1 (SEQ ID NO: 7); Linker (SEQ ID NO: 4); Anti-CTLA-4 heavy chain (SEQ ID NO: 5); Anti-CTLA-4 light chain (SEQ ID NO: 6) and TGF β RII (SEQ ID NO: 3).

[0050] Figure 3 shows the amino acid residues for the anti-EGFR1 linker PD1 construct (SEQ ID NOs: 8, 9, 10 and 11).

[0051] Figure 4 shows the amino acid residues for anti-EGFR1-linker-TGF β RII construct (SEQ ID NOs: 8, 9, 10 and 12).

[0052] Figure 5 shows the amino acid residues for the anti-CTLA-4-linker-PD1 (SEQ ID NOs: 13, 14, 10 and 11).

[0053] Figure 6 shows the different possibilities for placement of the PD1 molecule on the anti-EGFR1 antibody for FMab5, FMab6, FMab7 and FMab8.

[0054] Figure 7 shows the amino acid sequences for Anti-EGFR1 HC-PD1 + Anti-EGFR1 LC wherein the PD1 molecule is connected to the C terminus of the heavy chain separated by a linker and including SEQ ID NOs: 15 and 9.

[0055] Figure 8 shows the amino acid sequences for Anti-EGFR1 HC + Anti-EGFR1 LC -PD1 wherein the PD1 molecule is connected to the C terminus of the light chain separated by a linker and including SEQ ID NOs: 8 and 16.

[0056] Figure 9 shows the amino acid sequences for Anti-EGFR1 HC + Anti-EGFR1 LC -PD1 wherein the PD1 molecule is connected to the N terminus of the heavy chain separated by a linker and including SEQ ID NOs: 17 and 9.

[0057] Figure 10 shows the amino acid sequences for Anti-EGFR1 HC + PD1-Anti-EGFR1 LC wherein the PD1 molecule is connected to the N terminus of the light chain separated by a linker and including SEQ ID NOs: 8 and 18.

[0058] Figure 11 shows expression constructs developed using the cDNAs as set forth in SEQ ID NOs: 1, 2 and 7.

[0059] Figure 12 shows the different possibilities for placement of the TGF β RII molecule on the anti-EGFR1 antibody, FMab1, FMab2, FMab3, FMab4, FMab9, FMab10, FMab11 and, FMab1 2.

[0060] Figure 13 shows the amino acid sequences for Anti-EGFR1 HC-TGF β RII + Anti-EGFR1 LC wherein the TGF β RII molecule is connected to the C terminus of the heavy chain separated by a linker and including SEQ ID NOs: 27 and 9.

[0061] Figure 14 shows the amino acid sequences for Anti-EGFR1 HC + Anti-EGFR1 LC -TGF β RII wherein the TGF β RII molecule is connected to the C terminus of the light chain separated by a linker and including SEQ ID NOs: 8 and 28.

[0062] Figure 15 shows the amino acid sequences for TGF β RII-Anti-EGFR1 HC + Anti-EGFR1 LC wherein the TGF β RII molecule is connected to the N terminus of the heavy chain separated by a linker and including SEQ ID NOs: 29 and 9.

[0063] Figure 16 shows the amino acid sequences for Anti-EGFR1 HC + TGF β RII-Anti-EGFR1 LC wherein the TGF β RII molecule is connected to the N terminus of the light chain separated by a linker and including SEQ ID NOs: 8 and 30.

[0064] Figure 17 shows the amino acid sequences for Anti-EGFR1 HC-TGF β RII + Anti-EGFR1 LC -TGF β RII wherein the TGF β RII molecule is connected to the C terminus of the heavy and light chain separated by a linker and including SEQ ID NOs: 31 and 28.

[0065] Figure 18 shows the amino acid sequences for Anti-EGFR1 HC-TGF β RII + TGF β RII-Anti-EGFR1 LC wherein the TGF β RII molecule is connected to the C terminus of the heavy chain and N terminus of the light chain separated by a linker and including SEQ ID NOs: 31 and 30.

[0066] Figure 19 shows the amino acid sequences for TGF β RII- Anti-EGFR1 HC + Anti-EGFR1 LC -TGF β RII wherein the TGF β RII molecule is connected to the N terminus of the heavy chain and C terminus of the light chain separated by a linker and including SEQ ID NOs: 29 and 28.

[0067] Figure 20 shows the amino acid sequences for TGF β RII-Anti-EGFR1 HC + TGF β RII-Anti-EGFR1 LC wherein the TGF β RII molecule is connected to the N terminus of the heavy chain and N terminus of the light chain separated by a linker and including SEQ ID NOs: 29 and 30.

[0068] Figure 21 shows expression constructs developed using the cDNAs as set forth in SEQ ID NOs: 1, 2 and 3.

[0069] Figure 22 shows ProteinA purified samples analyzed on 12% reducing SDS-PAGE.

[0070] Figure 23 shows ProteinA purified samples analyzed on 6% non-reducing SDS-PAGE.

[0071] Figure 24 shows the different possibilities for placement of the PD1 molecule on the anti-CTLA4 antibody.

[0072] Figure 25 shows the amino acid sequences for Anti-CTLA4 HC-PD1 + Anti-CTLA4 LC wherein the PD1 molecule is connected to the C terminus of the heavy chain separated by a linker and including SEQ ID NOs: 32 and 14.

[0073] Figure 26 shows the amino acid sequences for Anti-CTLA4 HC + Anti-CTLA4 LC-PD1 wherein the PD1 molecule is connected to the C terminus of the light chain separated by a linker and including SEQ ID NOs: 13 and 33.

[0074] Figure 27 shows the amino acid sequences for PD1-Anti-CTLA4 HC + Anti-CTLA4 LC wherein the PD1 molecule is connected to the N terminus of the heavy chain separated by a linker and including SEQ ID NOs: 34 and 14.

[0075] Figure 28 shows the amino acid sequences for Anti-CTLA4 HC + PD1-Anti-CTLA4 LC wherein the PD1 molecule is connected to the N terminus of the light chain separated by a linker and including SEQ ID NOs: 13 and 35.

[0076] Figure 29 shows the amino acid sequences for Anti-CTLA4 HC-PD1 + Anti-CTLA4 LC-PD1 wherein the PD1 molecule is connected to the C terminus of the heavy chain and light chain separated by a linker and including SEQ ID NOs: 32 and 33.

[0077] Figure 30 shows the amino acid sequences for Anti-CTLA4 HC-PD1 + PD1-Anti-CTLA4 LC wherein the PD1 molecule is connected to the C terminus of the heavy chain separated by a linker and N terminus of the light chain including SEQ ID NOs: 32 and 35.

[0078] Figure 31 shows the amino acid sequences for PD1-Anti-CTLA4 HC + Anti-CTLA4 LC-PD1 wherein the PD1 molecule is connected to the N terminus of the heavy chain separated by a linker and C terminus of the light chain including SEQ ID NOs: 34 and 33.

[0079] Figure 32 shows the amino acid sequences for PD1-Anti-CTLA4 HC + PD1-Anti-CTLA4 LC wherein the PD1 molecule is connected to the N terminus of the heavy chain separated by a linker and N terminus of the light chain including SEQ ID NOs: 34 and 35.

[0080] Figure 33 shows expression constructs developed using the cDNAs as set forth in SEQ ID NOs: 7, 5 and 6.

[0081] Figure 34 shows EGFR1 target binding ELISA. The Anti-EGFR1 HC-TGF β RII + Anti-EGFR1 LC fusion Mab binds to its immobilized target EGFR1.

[0082] Figure 35 shows TGF β target binding ELISA. The Anti-EGFR1 HC-TGF β RII + Anti-EGFR1 LC fusion Mab binds to its target TGF β .

[0083] Figure 36 shows Bifunctional ELISA. The anti-EGFR1 HC-TGF β RII + Anti-EGFR1 LC fusion Mab binds to both its target EGFR1 and TGF β at the same time.

[0084] Figure 37 shows flow cytometric analysis of the binding of the anti-EGFR1 HC-TGF β RII + Anti-EGFR1 LC fusion Mab to EGFR1-expressing A431 cells.

[0085] Figure 38 shows ADCC against EGFR1-expressing A-431 cells. Anti-EGFR1 HC-TGF β RII + Anti-EGFR1 LC fusion Mab mediates ADCC against EGFR1 -expressing A-431 cells and the effect is dose dependent.

[0086] Figure 39 shows Inhibition of proliferation assay. Anti-EGFR1 HC-TGF β RII + Anti-EGFR1 LC fusion Mab inhibits the proliferation of EGFR1 -expressing A-431 cells.

[0087] Figure 40 shows EGFR1 target binding ELISA. The Anti-EGFR1 HC + Anti-EGFR1 LC- TGF β RII fusion Mab binds to its immobilized target EGFR1.

[0088] Figure 41 shows TGF β target binding ELISA. The anti-EGFR1 HC + Anti-EGFR1 LC -TGF β RII fusion Mab binds to its target TGF β .

[0089] Figure 42 shows Bifunctional ELISA. The anti-EGFR1 HC + Anti-EGFR1 LC -TGF β RII fusion Mab binds to both its target EGFR1 and TGF β at the same time.

[0090] Figure 43 shows Inhibition of proliferation assay. Anti-EGFR1 HC + Anti-EGFR1 LC -TGF β RII fusion Mab inhibits the proliferation of EGFR1 -expressing A-431 cells.

[0091] Figure 44 shows EGFR1 target binding ELISA. The TGF β RII-Anti-EGFR1 HC + Anti-EGFR1 LC fusion Mab binds to its immobilized target EGFR1.

[0092] Figure 45 shows TGF β target binding ELISA. The TGF β RII-anti-EGFR1 HC+ anti-EGFR1 LC fusion Mab binds to its target TGF β .

[0093] Figure 46 shows Bifunctional ELISA. The TGF β RII-Anti-EGFR1 HC + Anti-EGFR1 LC fusion Mab binds to both its target EGFR1 and TGF β at the same time.

[0094] Figure 47 shows Inhibition of proliferation assay. TGF β RII-Anti-EGFR1 HC + Anti-EGFR1 LC fusion Mab inhibits the proliferation of EGFR1-expressing A-431 cells.

[0095] Figure 48 shows EGFR1 target binding ELISA. The Anti-EGFR1 HC + TGF β RII-Anti-EGFR1 LC fusion Mab binds to its immobilized target EGFR1.

[0096] Figure 49 shows TGF β target binding ELISA. The Anti-EGFR1 HC + TGF β RII-Anti-EGFR1 LC fusion Mab binds to its target TGF β .

[0097] Figure 50 shows Bifunctional ELISA. The Anti-EGFR1 HC + TGF β RII-Anti-EGFR1 LC fusion Mab binds to both its target EGFR1 and TGF β at the same time.

[0098] Figure 51 shows flow cytometric analysis of the binding of the Anti-EGFR1 HC + TGF β RII-Anti-EGFR1 LC fusion Mab to EGFR1-expressing A431 cells.

[0099] Figure 52 shows EGFR1 target binding ELISA. The Anti-EGFR1 HC-TGF β RII + TGF β RII-Anti-EGFR1 LC fusion Mab binds to its immobilized target EGFR1.

[00100] Figure 53 shows TGF β target binding ELISA. The Anti-EGFR1 HC-TGF β RII + TGF β RII-Anti-EGFR1 LC fusion Mab binds to its target TGF β .

[00101] Figure 54 shows Bifunctional ELISA. The Anti-EGFR1 HC-TGF β RII + TGF β RII-Anti-EGFR1 LC fusion Mab binds to both its target EGFR1 and TGF β at the same time.

[00102] Figure 55 shows EGFR1 target binding ELISA. The TGF β RII-Anti-EGFR1 HC + TGF β RII-Anti-EGFR1 LC fusion Mab binds to its immobilized target EGFR1.

[00103] Figure 56 shows TGF β target binding ELISA. TGF β RII-Anti-EGFR1 HC + TGF β RII-Anti-EGFR1 LC fusion Mab binds to its target TGF β .

[00104] Figure 57 shows Bifunctional ELISA. The TGF β RII-Anti-EGFR1 HC + TGF β RII-Anti-EGFR1 LC fusion Mab binds to both its target EGFR1 and TGF β at the same time.

[00105] Figure 58 shows Bifunctional ELISA. The Anti-CTLA4 HC-PD1 + Anti-CTLA4 LC fusion Mab binds to both its target CTLA4 and PDL1 at the same time.

[00106] Figure 59 shows Bifunctional ELISA. The Anti-CTLA4 HC + Anti-CTLA4 LC-PD1 fusion Mab binds to both its target CTLA4 and PDL1 at the same time.

[00107] Figure 60 shows Bifunctional ELISA. The PD1-Anti-CTLA4 HC + Anti-CTLA4 LC fusion Mab binds to both its target CTLA4 and PDL1 at the same time.

[00108] Figure 61 shows Bifunctional ELISA. The Anti-CTLA4 HC-PD1 + PD1-Anti-CTLA4 LC-PD1, Anti-CTLA4 HC-PD1 + Anti-CTLA4 LC-PD1 and Anti-CTLA4 HC-PD1 + PD1-Anti-CTLA4 LC fusion Mabs binds to both their target CTLA4 and PDL1 at the same time.

[00109] Figure 62 shows Bifunctional ELISA. The PD1-Anti-CTLA4 HC + Anti-CTLA4 LC-PD1 and PD1-Anti-CTLA4 HC+ PD1-Anti-CTLA4 LC fusion Mabs binds to both their target CTLA4 and PDL1 at the same time.

[00110] Figure 63 shows Bifunctional ELISA. The Anti-EGFR1 HC-PD1 + Anti-EGFR1 LC fusion

[00111] Mab binds to both its target EGFR and PDL1 at the same time.

[00112] Figure 64 shows Bifunctional ELISA. The Anti-EGFR1 HC + Anti-EGFR1 LC-PD1 fusion Mab binds to both its target EGFR and PDL1 at the same time.

DETAILED DESCRIPTION OF THE INVENTION

[00113] In order to facilitate review of the various embodiments of the invention and provide an understanding of the various elements and constituents used in making and using the present invention, the following terms used in the invention description have the following meanings.

[00114] As used herein, the terms "polypeptide," "protein" and "peptide" are used interchangeably to denote a sequence polymer of at least two amino acids covalently linked by an amide bond, regardless of length or post-translational modification (e.g., glycosylation, phosphorylation, lipidation, myristilation, ubiquitination, etc.). D- and L-amino acids, and mixtures of D- and L-amino acids are also included.

[00115] Chimeric polypeptide refers to an amino acid sequence having two or more parts which generally are not found together in an amino acid sequence in nature.

[00116] The term "spacer/linker" as used herein refers to a molecule that connects two monomeric protein units to form a chimeric molecule and still provides for binding of the parts to the desired receptors. Particular examples of spacer/linkers may include an amino acid spacer, wherein the amino acid sequence can essentially be any length, for example, as few as 5 or as many as 200 or more preferably from about 5 to 30 amino acid residues.

[00117] The term "therapeutic," as used herein, means a treatment administered to a subject who exhibits signs of pathology for the purpose of diminishing or eliminating those signs.

[00118] The term "therapeutically effective amount," as used herein means an amount of the chimeric protein that is sufficient to provide a beneficial effect to the subject to which the chimeric protein is administered.

[00119] Another example of a modification is the addition of a heterologous domain that imparts a distinct functionality upon the chimeric polypeptide. A heterologous domain can be any small organic or inorganic molecule or macromolecule, so long as it imparts an

additional function. Particular examples of heterologous domains that impart a distinct function include an amino acid sequence that imparts targeting (e.g., receptor ligand, antibody, etc.), immunopotentiating function (e.g., immunoglobulin, an adjuvant), enable purification, isolation or detection (e.g., myc, T7 tag, polyhistidine, avidin, biotin, lectins, etc.).

[00120] As exemplified herein, polypeptide sequences may include substitutions, variations, or derivitizations of the amino acid sequence of one or both of the polypeptide sequences that comprise the chimeric polypeptide, so long as the modified chimeric polypeptide has substantially the same activity or function as the unmodified chimeric polypeptide.

[00121] As used herein, the term “substantially the same activity or function,” when used in reference to a chimeric polypeptide so modified, means that the polypeptide retains most, all or more of the activity associated with the unmodified polypeptide, as described herein or known in the art.

[00122] Modified chimeric polypeptides that are “active” or “functional” included herein can be identified through a routine functional assay. For example, by using antibody binding assays or co-receptor binding assays one can readily determine whether the modified chimeric polypeptide has activity. As the modified chimeric polypeptides will retain activity or function associated with unmodified chimeric polypeptide, modified chimeric polypeptides will generally have an amino acid sequence “substantially identical” or “substantially homologous” with the amino acid sequence of the unmodified polypeptide.

[00123] As used herein, the term “substantially identical” or “substantially homologous,” when used in reference to a polypeptide sequence, means that a sequence of the polypeptide is at least 50% identical to a reference sequence. Modified polypeptides and substantially identical polypeptides will typically have at least 70%, alternatively 85%, more likely 90%, and most likely 95% homology to a reference polypeptide.

[00124] As set forth herein, substantially identical or homologous polypeptides include additions, truncations, internal deletions or insertions, conservative and non-conservative substitutions, or other modifications located at positions of the amino acid sequence which do

not destroy the function of the chimeric polypeptide (as determined by functional assays, e.g., as described herein). A particular example of a substitution is where one or more amino acids are replaced by another, chemically or biologically similar residue. As used herein, the term “conservative substitution” refers to a substitution of one residue with a chemically or biologically similar residue. Examples of conservative substitutions include the replacement of a hydrophobic residue, such as isoleucine, valine, leucine, or methionine for another, the replacement of a polar residue for another, such as the substitution of arginine for lysine, glutamic for aspartic acids, or glutamine for asparagine, and the like. Those of skill in the art will recognize the numerous amino acids that can be modified or substituted with other chemically similar residues without substantially altering activity.

[00125] Modified polypeptides further include “chemical derivatives,” in which one or more of the amino acids therein have a side chain chemically altered or derivatized. Such derivatized polypeptides include, for example, amino acids in which free amino groups form amine hydrochlorides, p-toluene sulfonyl groups, carbobenzoxy groups; the free carboxy groups form salts, methyl and ethyl esters; free hydroxyl groups that form O-acyl or O-alkyl derivatives, as well as naturally occurring amino acid derivatives, for example, 4-hydroxyproline, for proline, 5-hydroxylysine for lysine, homoserine for serine, ornithine for lysine, and so forth. Also included are D-amino acids and amino acid derivatives that can alter covalent bonding, for example, the disulfide linkage that forms between two cysteine residues that produces a cyclized polypeptide.

[00126] As used herein, the terms “isolated” or “substantially pure,” when used as a modifier of invention chimeric polypeptides, sequence fragments thereof, and polynucleotides, means that they are produced by human intervention and are separated from their native in vivo -cellular environment. Generally, polypeptides and polynucleotides so separated are substantially free of other proteins, nucleic acids, lipids, carbohydrates or other materials with which they are naturally associated.

[00127] Polypeptides of the present invention may be prepared by standard techniques well known to those skilled in the art. Such techniques include, but are not limited to, isolation and purification from tissues known to contain that polypeptide, and expression from cloned DNA that encodes such a polypeptide using transformed cells. Chimeric polypeptides can be obtained by expression of a polynucleotide encoding the polypeptide in a

host cell, such as a bacteria, yeast or mammalian cell, and purifying the expressed chimeric polypeptide by purification using typical biochemical methods (e.g., immunoaffinity purification, gel purification, expression screening etc.). Other well-known methods are described in Deutscher et al., 1990. Alternatively, the chimeric polypeptide can be chemically synthesized. Purity can be measured by any appropriate method, e.g., polyacrylamide gel electrophoresis, and subsequent staining of the gel (e.g., silver stain) or by HPLC analysis.

[00128] The present invention further provides polynucleotide sequences encoding chimeric polypeptides, fragments thereof, and complementary sequences. As used herein, the terms “nucleic acid,” “polynucleotide,” “oligonucleotide,” and “primer” are used interchangeably to refer to deoxyribonucleic acid (DNA) or ribonucleic (RNA), either double- or single-stranded, linear or circular. RNA can be unspliced or spliced mRNA, rRNA, tRNA, or antisense RNAi. DNA can be complementary DNA (cDNA), genomic DNA, or an antisense. Specifically included are nucleotide analogues and derivatives, such as those that are resistant to nuclease degradation, which can function to encode an invention chimeric polypeptide. Nuclease resistant oligonucleotides and polynucleotides are particularly useful for the present nucleic acid vaccines described herein.

[00129] An “isolated” or “substantially pure” polynucleotide means that the nucleic acid is not immediately contiguous with the coding sequences with either the 5' end or the 3' end with which it is immediately contiguous in the naturally occurring genome of the organism from which it is derived. The term therefore includes, for example, a recombinant DNA (e.g., a cDNA or a genomic DNA fragment produced by PCR or restriction endonuclease treatment produced during cloning), as well as a recombinant DNA incorporated into a vector, an autonomously replicating plasmid or virus, or a genomic DNA of a prokaryote or eukaryote.

[00130] The polynucleotides sequences of the present invention can be obtained using standard techniques known in the art (e.g., molecular cloning, chemical synthesis) and the purity can be determined by polyacrylamide or agarose gel electrophoresis, sequencing analysis, and the like. Polynucleotides also can be isolated using hybridization or computer-based techniques that are well known in the art. Such techniques include, but are not limited to: (1) hybridization of genomic DNA or cDNA libraries with probes to detect homologous

nucleotide sequences; (2) antibody screening of polypeptides expressed by DNA sequences (e.g., using an expression library); (3) polymerase chain reaction (PCR) of genomic DNA or cDNA using primers capable of annealing to a nucleic acid sequence of interest; (4) computer searches of sequence databases for related sequences; and (5) differential screening of a subtracted nucleic acid library.

[00131] The invention also includes substantially homologous polynucleotides. As used herein, the term “homologous,” when used in reference to nucleic acid molecule, refers to similarity between two nucleotide sequences. When a nucleotide position in both of the molecules is occupied by identical nucleotides, then they are homologous at that position. “Substantially homologous” nucleic acid sequences are at least 50% homologous, more likely at least 75% homologous, and most likely 90% or more homologous. As with substantially homologous invention chimeric polypeptides, polynucleotides substantially homologous to invention polynucleotides encoding chimeric polypeptides encode polypeptides that retain most or all of the activity or function associated with the sequence to which it is homologous. For polynucleotides, the length of comparison between sequences will generally be at least 30 nucleotides, alternatively at least 50 nucleotides, more likely at least 75 nucleotides, and most likely 110 nucleotides or more. Algorithms for identifying homologous sequences that account for polynucleotide sequence gaps and mismatched oligonucleotides are known in the art, such as BLAST (see Altschul, 1990).

[00132] The polynucleotides of the present invention can, if desired: be naked or be in a carrier suitable for passing through a cell membrane (e.g., polynucleotide-liposome complex or a colloidal dispersion system), contained in a vector (e.g., retrovirus vector, adenoviral vectors, and the like), linked to inert beads or other heterologous domains (e.g., antibodies, ligands, biotin, streptavidin, lectins, and the like), or other appropriate compositions disclosed herein or known in the art. Thus, viral and non-viral means of polynucleotide delivery can be achieved and are contemplated. The polynucleotides of the present invention can also contain additional nucleic acid sequences linked thereto that encode a polypeptide having a distinct functionality, such as the various heterologous domains set forth herein.

[00133] The polynucleotides of the present invention can also be modified, for example, to be resistant to nucleases to enhance their stability in a pharmaceutical

formulation. The described polynucleotides are useful for encoding chimeric polypeptides of the present invention, especially when such polynucleotides are incorporated into expression systems disclosed herein or known in the art. Accordingly, polynucleotides including an expression vector are also included.

[00134] For propagation or expression in cells, polynucleotides described herein can be inserted into a vector. The term “vector” refers to a plasmid, virus, or other vehicle known in the art that can be manipulated by insertion or incorporation of a nucleic acid. Such vectors can be used for genetic manipulation (i.e., “cloning vectors”) or can be used to transcribe or translate the inserted polynucleotide (i.e., “expression vectors”). A vector generally contains at least an origin of replication for propagation in a cell and a promoter. Control elements, including promoters present within an expression vector, are included to facilitate proper transcription and translation (e.g., splicing signal for introns, maintenance of the correct reading frame of the gene to permit in-frame translation of mRNA and stop codons). In vivo or in vitro expression of the polynucleotides described herein can be conferred by a promoter operably linked to the nucleic acid.

[00135] “Promoter” refers to a minimal nucleic acid sequence sufficient to direct transcription of the nucleic acid to which the promoter is operably linked (see Bitter 1987). Promoters can constitutively direct transcription, can be tissue-specific, or can render inducible or repressible transcription; such elements are generally located in the 5’ or 3’ regions of the gene so regulated.

[00136] As used herein, the term “operably linked” means that a selected polynucleotide (e.g., encoding a chimeric polypeptide) and regulatory sequence(s) are connected in such a way as to permit transcription when the appropriate molecules (e.g., transcriptional activator proteins) are bound to the regulatory sequence(s). Typically, a promoter is located at the 5’ end of the polynucleotide and may be in close proximity of the transcription initiation site to allow the promoter to regulate expression of the polynucleotide.

[00137] When cloning in bacterial systems, constitutive promoters, such as T7 and the like, as well as inducible promoters, such as pL of bacteriophage gamma, plac, ptrp, ptac, may be used. When cloning in mammalian cell systems, constitutive promoters, such as SV40, RSV and the like, or inducible promoters derived from the genome of mammalian

cells (e.g., the metallothionein promoter) or from mammalian viruses (e.g., the mouse mammary tumor virus long terminal repeat, the adenovirus late promoter), may be used. Promoters produced by recombinant DNA or synthetic techniques may also be used to provide for transcription of the nucleic acid sequences of the invention.

[00138] Mammalian expression systems that utilize recombinant viruses or viral elements to direct expression may be engineered. For example, when using adenovirus expression vectors, the nucleic acid sequence may be ligated to an adenovirus transcription/translation control complex, e.g., the late promoter and tripartite leader sequence. Alternatively, the vaccinia virus 7.5K promoter may be used (see Mackett 1982; Mackett 1984; Panicali 1982).

[00139] For yeast expression, a number of vectors containing constitutive or inducible promoters may be used (see Ausubel 1988; Grant 1987; Glover 1986; Bitter 1987; and Strathern 1982). The polynucleotides may be inserted into an expression vector for expression in vitro (e.g., using in vitro transcription/translation kits, which are available commercially), or may be inserted into an expression vector that contains a promoter sequence that facilitates expression in either prokaryotes or eukaryotes by transfer of an appropriate nucleic acid into a suitable cell, organ, tissue, or organism in vivo.

[00140] As used herein, a “transgene” is any piece of a polynucleotide inserted by artifice into a host cell, and becomes part of the organism that develops from that cell. A transgene can include one or more promoters and any other DNA, such as introns, necessary for expression of the selected DNA, all operably linked to the selected DNA, and may include an enhancer sequence. A transgene may include a polynucleotide that is partly or entirely heterologous (i.e., foreign) to the transgenic organism, or may represent a gene homologous to an endogenous gene of the organism. Transgenes may integrate into the host cell’s genome or be maintained as a self-replicating plasmid.

[00141] As used herein, a “host cell” is a cell into which a polynucleotide is introduced that can be propagated, transcribed, or encoded polypeptide expressed. The term also includes any progeny of the subject host cell. It is understood that all progeny may not be identical to the parental cell, since there may be mutations that occur during replication. Host cells include but are not limited to bacteria, yeast, insect, and mammalian cells. For example,

bacteria transformed with recombinant bacteriophage polynucleotide, plasmid nucleic acid, or cosmid nucleic acid expression vectors; yeast transformed with recombinant yeast expression vectors; plant cell systems infected with recombinant virus expression vectors (e.g., cauliflower mosaic virus, CaMV; tobacco mosaic virus, TMV), or transformed with recombinant plasmid expression vectors (e.g., Ti plasmid), insect cell systems infected with recombinant virus expression vectors (e.g., baculovirus), or animal cell systems infected with recombinant virus expression vectors (e.g., retroviruses, adenovirus, vaccinia virus), or transformed animal cell systems engineered for stable expression.

[00142] As used herein, the term “transformation” means a genetic change in a cell following incorporation of a polynucleotide (e.g., a transgene) exogenous to the cell. Thus, a “transformed cell” is a cell into which, or a progeny of which, a polynucleotide has been introduced by means of recombinant techniques. Transformation of a host cell may be carried out by conventional techniques known to those skilled in the art. When the host cell is a eukaryote, methods of DNA transformation include, for example, calcium phosphate, microinjection, electroporation, liposomes, and viral vectors. Eukaryotic cells also can be co-transformed with invention polynucleotide sequences or fragments thereof, and a second DNA molecule encoding a selectable marker, as described herein or otherwise known in the art. Another method is to use a eukaryotic viral vector, such as simian virus 40 (SV40) or bovine papilloma virus, to transiently infect or transform eukaryotic cells, and express the protein (see Gluzman 1982). When the host is prokaryotic (e.g., *E. coli*), competent cells that are capable of DNA uptake can be prepared from cells harvested after exponential growth phase and subsequently treated by the CaCl_2 method using procedures well-known in the art. Transformation of prokaryotes also can be performed by protoplast fusion of the host cell.

[00143] Chimeric polypeptides, polynucleotides, and expression vectors containing same of the present invention can be encapsulated within liposomes using standard techniques and introduced into cells or whole organisms. Cationic liposomes are preferred for delivery of polynucleotides. The use of liposomes for introducing various compositions in vitro or in vivo, including proteins and polynucleotides, is known to those of skill in the art.

[00144] Liposomes can be targeted to a cell type or tissue of interest by the addition to the liposome preparation of a ligand, such as a polypeptide, for which a corresponding

cellular receptor has been identified. Monoclonal antibodies can also be used for targeting; many such antibodies specific for a wide variety of cell surface proteins are known to those skilled in the art and are available. The selected ligand is covalently conjugated to a lipid anchor in either preformed liposomes or are incorporated during liposome preparation (see Lee 1994 and Lee 1995).

[00145] As the chimeric polypeptides or polynucleotides of the present invention will be administered to humans, the present invention also provides pharmaceutical formulations comprising the disclosed chimeric polypeptides or polynucleotides. The compositions administered to a subject will therefore be in a “pharmaceutically acceptable” or “physiologically acceptable” formulation.

[00146] As used herein, the terms “pharmaceutically acceptable” and “physiologically acceptable” refer to carriers, diluents, excipients, and the like that can be administered to a subject, preferably without excessive adverse side effects (e.g., nausea, headaches, etc.). Such preparations for administration include sterile aqueous or non-aqueous solutions, suspensions, and emulsions. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oils, such as olive oil, and injectable organic esters, such as ethyl oleate. Aqueous carriers include water, alcoholic/aqueous solutions, emulsions, or suspensions, including saline and buffered media. Vehicles include sodium chloride solution, Ringer’s dextrose, dextrose and sodium chloride, lactated Ringer’s or fixed oils. Intravenous vehicles include fluid and nutrient replenishers, electrolyte replenishers (such as those based on Ringer’s dextrose), and the like. Preservatives and other additives may also be present, such as, for example, antimicrobial, anti-oxidants, chelating agents, and inert gases and the like. Various pharmaceutical formulations appropriate for administration to a subject known in the art are applicable in the methods of the invention (e.g., Remington’s Pharmaceutical Sciences, 18th ed., Mack Publishing Co., Easton, PA (1990); and The Merck Index, 12th ed., Merck Publishing Group, Whitehouse, NJ (1996)).

[00147] Controlling the duration of action or controlled delivery of an administered composition can be achieved by incorporating the composition into particles or a polymeric substance, such as polyesters, polyamine acids, hydrogel, polyvinyl pyrrolidone, ethylene-vinylacetate, methylcellulose, carboxymethylcellulose, protamine sulfate or lactide/glycolide copolymers, polylactide/glycolide copolymers, or ethylenevinylacetate copolymers. The rate

of release of the composition may be controlled by altering the concentration or composition of such macromolecules. Colloidal dispersion systems include macromolecule complexes, nano-capsules, microspheres, beads, and lipid-based systems, including oil-in-water emulsions, micelles, mixed micelles, and liposomes.

[00148] The compositions administered by a method of the present invention can be administered parenterally by injection, by gradual perfusion over time, or by bolus administration or by a microfabricated implantable device. The composition can be administered via inhalation, intravenously, intraperitoneally, intramuscularly, subcutaneously, intracavity (e.g., vaginal or anal), transdermally, topically, or intravascularly. The compositions can be administered in multiple doses. An effective amount can readily be determined by those skilled in the art.

[00149] Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, suitable methods and materials are described below. Other features and advantages of the invention will be apparent from the following detailed description, and from the claims. The invention is further described in the following examples, which do not limit the scope of the invention(s) described in the claims.

[00150] Examples

1. Anti-EGFR1-PD1 fusion protein constructs for cancer targets

[00151] Anti-EGFR (Cetuximab) has been approved for squamous Head and Neck Cancer (locally or regionally advanced in combination with radiotherapy and metastatic after platinum based therapy) and EGFR expressing metastatic colorectal cancer (monotherapy in patients after failure of both oxaliplatin and irinotecan based chemo or in patients intolerant to irinotecan based chemo). Not applicable for colonrectal cancer (CRC) patients having K-RAS mutations.

[00152] Across various studies about 55-60% of mCRC patients respond to cetuximab in first line setting, however, this response too is transient (progression free survival (PFS) advantage of 1.5-2 mths) (EPAR). Significant numbers of patients either do not respond to cetuximab or become resistant to therapy. In the recurrent metastatic head and neck cancer, only 35% patients respond to cetuximab with chemo with only 2-3 month overall survival (OS) and (PFS) advantage.

[00153] Clearly, a significant unmet need exists to improve efficacy of cetuximab therapy in both these indications. Moreover, EGFR is also expressed in gastric cancer, non-small cell lung cancer (NSCLC) and pancreatic cancers. However, cetuximab has failed to prove any significant benefit in these indications over standard of care. Thus, the present invention provides for improvement by combining cetuximab with an immunomodulatory therapy.

[00154] Programmed death-1 (PD-1) is an inhibitory receptor expressed on T cells after activation. It has been shown to down-regulate T-cell activity upon binding its ligand PD-L1 on APCs. Many tumors constitutively express PD-L1 and its' over expression has been associated with impaired tumor immunity, more aggressive disease and decreased survival (see Thompson 2004). Till date PD-L1 expression has been demonstrated to correlate with poor prognosis in patients with renal cell carcinoma (RCC), ovarian cancer and melanoma. Immunohistochemical analysis of freshly isolated tumor samples from patients with ovarian, lung, and breast cancers, renal cell carcinoma, squamous cell carcinoma of the head and neck, esophageal carcinoma, glioblastoma, thymoma, colon carcinoma, pancreatic and melanoma found that the vast majority express B7-H1 (see Flies 2011; Nomi 2007). Several pre-clinical studies have demonstrated increased tumor rejection by blocking PD1-PDL1 interaction. Recently, anti-PD1 and PD-L1 based therapies have demonstrated considerable activity in melanoma and some other solid tumors confirming their application as one of the most promising anti-cancer therapies.

[00155] Cetuximab based therapy may be improved upon by combining it with immunomodulation to remove immunosuppressive environment or delay the development of resistance. Moreover, patients who develop resistance to cetuximab due to mutations in the downstream pathways may still benefit from Anti-EGFR1-PD-1 since the fusion protein of the present invention binds to the EGFR receptor and negates the PD-L1 expressed by the

tumors, allowing T cells to mount an anti-tumor response. Accordingly, the fusion proteins of the present invention can bind to both EGFR and PD-L1 on the surface of the tumor cells.

[00156] The anti-EGFR1-PD1 fusion protein constructs of the present invention may be used in colorectal cancer, squamous head and neck cancer, non-small cell lung cancer, gastric cancer and pancreatic cancer.

[00157] Design and selection of the molecules:

[00158] The antibody fusion molecules of the present invention have dual therapeutic properties. On one hand the molecule retains the complete activity of the Anti-EGFR1 (Cetuximab) and in parallel, it has the PD-L1 receptor binding activity in the tumor environment. The new molecules of the Anti-EGFR1-PD1 fusion proteins that were developed for the cancer therapies herein are devoid of the amino acid lysine 'K' from the C-terminus of heavy chain for the reasons described above. The main objective of the fusion protein design is to keep the anti-EGFR1 molecule intact along with its function unaffected and allows fusion of the PD1 molecule to the various location on the anti-EGFR1 antibody. That being, fusion to the HC C-terminus, LC C-terminus, HC N-terminus, and or LC N-terminus and double fusions on both the chains as shown in Figure 6.

[00159] The following constructs were designed.

Table 1

Constructs.no.	Fusion mAbs name
FMab5 Figure 6	Anti-EGFR1 HC-PD1 + Anti-EGFR1 LC (AA sequences in Figure 7, SEQ ID NO: 15 and 9)
FMab6 Figure 6	Anti-EGFR1 HC + Anti-EGFR1 LC -PD1 (AA sequences in Figure 8, SEQ ID NO: 8 and 16)
FMab7 Figure 6	PD1-Anti-EGFR1 HC + Anti-EGFR1 LC (AA sequences in Figure 9, SEQ ID NO: 17 and 9)
FMab8 Figure 6	Anti-EGFR1 HC + PD1-Anti-EGFR1 LC (AA sequences in Figure 10, SEQ ID NO: 8 and 18)

[00160] Expression of the above fusion constructs in CHO cells:

[00161] The codon-optimized nucleotide sequences of the Anti-EGFR1-PD1 individual domains were optimized for expression in CHO cells. Such optimized sequences (SEQ ID NOs: 1, 2, 7, and 4) were assemble in a mammalian expression vector with help of primers described in Table 2:

Table 2

FMAB7FP1	AGA TAT CGC CAC CAT GAT GTC CTT CGT G	SEQ ID NO: 19
FMAB7FP2	GGC GGC GGA GGC TCT CAG GTG CAG CTG AAG CAG TC	SEQ ID NO: 20
FMAB7RP1	AGT ATA CTC AGC CGG GGG ACA GAG A	SEQ ID NO: 21
FMAB7RP2	TTC AGC TGC ACC TGA GAG CCT CCG CCG CCA CTT C	SEQ ID NO: 22
FMAB7LCRP	ATT AAT TAA TCA ACA CTC GCC CCG GTT GAA GGA CT	SEQ ID NO: 23
FMAB6FP2	CTC TGT CCC CCG GCG GCG GCG GAG GAT CTG GCG GA	SEQ ID NO: 24
FMAB6RP2	GAT CCT CCG CCG CCG CCG GGG GAC AGA GAC AGG GA	SEQ ID NO: 25
FMAB6RP1	AGT ATA CTC ACA CCA GGG TCT GGA AC	SEQ ID NO: 26

[00162] Using the shown above cDNA primers set, constructs were assembled as shown in Figure 11.

[00163] 2. Anti-EGFR1-TGFβRII fusion proteins for treatment of cancer.

[00164] High levels of TGFβ are produced by many types of tumors, including melanomas and cancers of the breast, colon, esophagus, stomach, liver, lung, pancreas, and prostate, as well as hematologic malignancies (see Teicher 2001; Dong 2006). TGFβ is known to be immunosuppressive for T cells and NK cells through blocking of IL-2 and other mechanisms, including generation of T-regs. Several lines of evidence suggest that negating TGFβ activity may enhance anti-tumor effects of T cells (Wrzesinski 2007). Moreover, TGFβ can foster tumor growth through epithelial to mesenchymal transition and promoting angiogenesis. TGFβ expression is also associated with poor prognosis in patients and earlier recurrence. However, considering the pleotropic effects of TGFβ in controlling the immune

response, it has been shown that generalized blocking of TGF β activity may result in widespread auto-inflammatory activity. Hence, localized depletion of TGF β in the tumor vicinity may be an alternative way to modulate immunosuppressive environment. Anti-EGFR1-TGF β R2 fusion protein of the present invention binds to EGFR on the tumor cells and ties up the TGF β around the tumor to enhance immune response against tumor cells.

[00165] Design and selection of the molecules:

[00166] The objective is to design the antibody fusion molecules which have dual therapeutic properties. On one hand the molecule should retain the complete activity of the Anti-EGFR1 (cetuximab) and in parallel; it should have the TGF β binding activity in the tumor environment. The amino acid sequence of the Anti-EGFR1 IgG molecule was retained excepting that the lysine was not expressed at the C-terminus of the heavy chain. Both single and double fusion and expression levels are shown in Table 3, wherein TGF β R2 was fused with Anti-EGFR1.

Table 3

Construct. no.	Fusion Mabs name
FMab1 Figure 12	Anti-EGFR1 HC-TGF β R2 + Anti-EGFR1 LC (AA sequences in Figure 13, SEQ ID NO: 27 and 9)
FMab2 Figure 12	Anti-EGFR1 HC + Anti-EGFR1 LC -TGF β R2 (AA sequences in Figure 14, SEQ ID NO: 8 and 28)
FMab3 Figure 12	TGF β R2-Anti-EGFR1 HC + Anti-EGFR1 LC (AA sequences in Figure 15, SEQ ID NO: 29 and 9)
FMab4 Figure 12	Anti-EGFR1 HC + TGF β R2-Anti-EGFR1 LC (AA sequences in Figure 16, SEQ ID NO: 8 and 30)
FMab9 Figure 12	Anti-EGFR1 HC-TGF β R2 + Anti-EGFR1 LC -TGF β R2) (AA sequences in Figure 17, SEQ ID NO: 31 and 28)
FMab10 Figure 12	Anti-EGFR1 HC-TGF β R2 + TGF β R2-Anti-EGFR1 LC (AA sequences in Figure 18, SEQ ID NO: 31 and 30)

FMab11 Figure 12	TGFβRII-Anti-EGFR1 HC + Anti-EGFR1 LC -TGFβRII (AA sequences in Figure 19, SEQ ID NO: 29 and 28)
FMab12 Figure 12	TGFβRII-Anti-EGFR1 HC + TGFβRII-Anti-EGFR1 LC (AA sequences in Figure 20, SEQ ID NO: 29 and 30)

[00167] Expression of the above fusion constructs in CHO cells:

[00168] The codon-optimized nucleotide sequences of the Anti-EGFR1- TGFβRII individual domains were optimized for expression in CHO cells. Such sequences (SEQ ID NOs: 1, 2, 4, and 3) were assemble in a mammalian expression vector. The expression constructs are set forth in Figure 21.

[00169] Transfection of the above vectors combination to obatin the desired cell line:

[00170] The expression constructs developed above were transfected in the following combination, as set forth in Table 4, into CHO cells to produce the following fusion proteins using the constructs as defined in Figure 21.

Table 4

Sl. No.	Fusion protein Name	Expression constructs combination transfected	Cell line used	Titer g/L
FMab1	Anti-EGFR1 HC- TGFβRII + Anti-EGFR1 LC (HC-C-terminus)	Expression constructs # 2C and 3 C	CHO	0.11
FMab2	Anti-EGFR1 HC + Anti-EGFR1 LC - TGFβRII	Expression constructs # 1 C and 4 C	CHO	0.10
FMab3	TGFβRII -Anti-EGFR1 HC + Anti-EGFR1 LC	Expression constructs # 2 C and 5 C	CHO	0.09
FMab4	Anti-EGFR1 HC + TGFβRII -Anti-EGFR1 LC	Expression constructs # 1 C and 6 C	CHO	0.08
FMab9	Anti-EGFR1 HC- TGFβRII + Anti-EGFR1 LC -TGFβRII	Expression constructs # 3 C and 4 C	CHO	ND/V L

FMab10	Anti-EGFR1 HC- TGFβRII + TGFβRII- Anti-EGFR1 LC	Expression constructs # 3 C and 6 C	CHO	0.06
FMab11	TGFβRII-Anti-EGFR1 HC + Anti-EGFR1 LC - TGFβRII	Expression constructs # 4 C and 5 C	CHO	ND/V L
FMab12	TGFβRII-Anti-EGFR1 HC + TGFβRII-Anti- EGFR1 LC	Expression constructs # 5 C and 6 C	CHO	0.06

[00171] Purification of the Fusion Mabs supernatants using Protein A column:

[00172] The fusion monoclonal antibodies (Mabs) using recombinant protein producing CHO cell culture supernatant.

[00173] Procedure:

[00174] The procedure describes in detail the small scale purification process of IgG using C10/10 or XK26 column and using Mab Select Xtra affinity resin. The samples generated by this protocol can be used for various analysis

[00175] Process flow:

The culture supernatant secreted from recombinant cell line producing monoclonal antibodies or fusion monoclonal antibodies under sterile conditions were tested for titer and endotoxins;

The affinity chromatography using Mab Select Xtra Protein A resin was washed and equilibrated with binding buffer;

The pH of the supernatant was adjusted using 0.5M phosphate to the same pH as the column;

The supernatant was allowed to bind to the column/ pass through the column at the flow rate of 0.5 ml/minute to achieve the maximum binding;

All the fusion Mabs binds through the Fc region and rest of the impurities passed pass through as flow through;

The column was washed with equilibration buffer;

The bound fusion Mabs were eluted using 0.1 M glycine pH 3.0;

The eluted proteins were adjusted back to neutral pH or the stable formulation pH;

The purified proteins were stored at -20°C or at 2-8°C depending on the stability.

[00176] Analysis of Protein A purified Fusion Mabs using SDS PAGE:

[00177] The transfected supernatants obtained were purified using proteinA affinity column. Later these were analyzed on reducing and non-reducing SDS-PAGE to find out the integrity of the molecule, as shown in Figure 22, where in the proteinA purified samples were analyzed on 12% reducing SDS-PAGE. As expected all the fusion partners are giving the expected pattern on SDS-PAGE. The LC fusion and HC are running closely but the bands are separated. This higher mobility may be due to the 8 N-glycosylation sites(TGBRII 3*2=6 + 2 on LC)

[00178] Figure 23 shows the results of the ProteinA purified samples that were analyzed on 6% non-reducing SDS-PAGE and although the amino acid composition is same, there is a difference in mobility. It may be due to the variable levels of glycosylation pattern based on the TGFβRII position and access in the molecule.

[00179] 3. Anti-CTLA4-PD1 fusion protein constructs for cancer targets.

[00180] Immunohistochemical analysis of freshly isolated tumor samples from patients with ovarian, lung, and breast cancers, renal cell carcinoma, squamous cell carcinoma of the head and neck, esophageal carcinoma, glioblastoma, thymoma, colon carcinoma, pancreatic and melanoma found that the vast majority express B7-H1 (see Flies 2011; Nomi 2007). Several pre-clinical studies have demonstrated increased tumor rejection by blocking PD1-PDL1 interaction. Recently, anti-PD1 and PD-L1 based therapies have demonstrated considerable activity in melanoma and some other solid tumors confirming their application as one of the most promising anti-cancer therapies.

[00181] Although, anti-CTLA4 may allow co-stimulation of T cells, they may still be inhibited by PD-L1-PD-1 interaction. This may be one of the reasons for only a minority of patients having response to anti-CTLA4 antibody. Fusion antibody of both anti-CTLA4 and PD1 are more efficacious than either agent alone since anti-CTLA4 allows T cell co-stimulation whereas PD1 binds to PD-L1 on tumor cells to negate the immunosuppression of T cells in tumor microenvironment. This may even be safer than the anti-CTLA4 because the lone use of anti-CTLA4 has led to immune breakthrough adverse events.

[00182] Design and selection of the molecules:

[00183] The objective is to design the antibody fusion molecules which have dual therapeutic properties. On one hand the molecule should retain the complete activity of the anti-CTLA4 (Ipilimumab) and in parallel; it should have the PD L1 receptor binding activity in the tumor environment. The complete amino acid sequence of the anti-CTLA4 IgG molecule was used except the removal of the lysine at the C-terminus of the heavy chain. A 15 amino acid linker was positioned between the PD1 and Anti-CTLA4. The following combinations of constructs, as set forth in Table 5, were designed as shown in Figure 24. The details of the above fusion protein constructs are given below.

Table 5

Constructs.no.	Fusion Mabs name
FMab13 Figure 21	Anti-CTLA4 HC-PD1 + Anti-CTLA4 LC (AA sequences in Figure 25, SEQ ID NO: 32 and 14)
FMab14 Figure 21	Anti-CTLA4 HC + Anti-CTLA4 LC-PD1(AA sequences in Figure 26, SEQ ID NO: 13 and 33)
FMab15 Figure 21	PD1-Anti-CTLA4 HC + Anti-CTLA4 LC (AA sequences in Figure 27, SEQ ID NO: 34 and 14)
FMab16 Figure 21	Anti-CTLA4 HC + PD1-Anti-CTLA4 LC (AA sequences in Figure 28, SEQ ID NO: 13 and 35)
FMab17 Figure 21	Anti-CTLA4 HC-PD1 + Anti-CTLA4 LC-PD1 (AA sequences in Figure 29, SEQ ID NO: 32 and 33)
FMab18 Figure 21	Anti-CTLA4 HC-PD1 + PD1-Anti-CTLA4 LC (AA sequences in Figure 30, SEQ ID NO: 32 and 35)
FMab19 Figure 21	PD1-Anti-CTLA4 HC + Anti-CTLA4 LC-PD1 (AA sequences in Figure 31, SEQ ID NO: 34 and 33)
FMab20 Figure 21	PD1-Anti-CTLA4 HC + PD1-Anti-CTLA4 LC (AA sequences in Figure 32, SEQ ID NO: 34 and 35)

[00184] Expression of the above fusion constructs in CHO cells:

[00185] The complete nucleotide sequence of the Anti-CTLA4-PD1 individual domains were codon optimized for expression in CHO cells (SEQ ID NOs: 7, 4, 5 and 6). The cDNAs were synthesized. The constructs were assembled in mammalian expression vectors. The expression of anti-CTLA4-PD1 fusion proteins using the constructs as set forth in Figure 33.

[00186] The expression constructs developed and shown in Figure 33 were transfected in the following combination into CHO cells (Table 6) to produce the following fusion proteins. The titer obtained for each constructs are mentioned in the last column.

Table 6

Sl. No.	Fusion protein Name	Expression constructs combination transfected	Cell line used	Titer g/L
FMab 13	Anti-CTLA4 HC-PD1 + Anti-CTLA4 LC	Expression constructs # 2 C and 3 C	CHO	0.175
FMab 14	Anti-CTLA4 HC + Anti-CTLA4 LC-PD1	Expression constructs # 1 C and 4 C	CHO	0.221
FMab 15	PD1-Anti-CTLA4 HC + Anti-CTLA4 LC	Expression constructs # 2 C and 5 C	CHO	0.029
FMab 16	Anti-CTLA4 HC + PD1-Anti-CTLA4 LC	Expression constructs # 1 C and 6 C	CHO	0.021
FMab 17	Anti-CTLA4 HC-PD1 + Anti-CTLA4 LC-PD1	Expression constructs # 3 C and 4 C	CHO	0.137
FMab 18	Anti-CTLA4 HC-PD1 + PD1-Anti-CTLA4 LC	Expression constructs # 3 C and 6 C	CHO	0.012
FMab 19	PD1-Anti-CTLA4 HC + Anti-CTLA4 LC-PD1	Expression constructs # 4 C and 5 C	CHO	0.029
FMab 20	PD1-Anti-CTLA4 HC + PD1-Anti-CTLA4 LC	Expression constructs # 5 C and 6 C	CHO	0.014

[00187] Purification of and characterization of Fusion proteins:

[00188] The procedure describes the use of small scale purification process of IgG using C10/1/ or XK26 column and using Mab Select Xtra affinity resin. The samples generated by this protocol can be used for various analysis.

[00189] Process flow:

The culture supernatant secreted from recombinant cell line producing monoclonal antibodies or fusion monoclonal antibodies under sterile conditions were tested for titer and endotoxins;

The affinity chromatography using Mab Select Xtra ProteinA resin was washed and equilibrated with binding buffer;

The pH of the supernatant was adjusted using 0.5M phosphate to the same PH has the column;

The supernatant was allowed to bind to the column/ pass through the column at the flow rate of 0.5 ml/minute to achieve the maximum binding;

All the fusion Mabs binds through the Fc region and rest of the impurities passed through as flow through;

The column was washed with equilibration buffer;

The bound fusion Mabs were eluted using 0.1 M glycine pH 3.0;

The eluted proteins were adjusted back to neutral pH or the stable formulation pH; and

The purified proteins are stored at -20°C or at 2-8°C depending on the stability.

[00190] 4. Anti-EGFR1 HC-TGFβRII + Anti-EGFR1 LC (Fmab 1)

[00191] Binding ELISAs-Procedure:

[00192] The fusion Mab was tested for its ability to bind to its targets in three different ELISAs: 1) EGFR1 target-binding ELISA, 2) TGFβ-target binding ELISA and 3) Bifunctional ELISA.

[00193] For the target binding ELISAs, the targets (rhEGFR-Fc chimera or TGFβ) were coated onto NUNC maxisorb plates overnight at 4°C. The plates were washed and then blocked with superblock at room temperature for 2 hr. Different dilutions of the fusion Mab or the negative control antibody was added to the plate. The plate was incubated at room temperature for 1 hr. Binding of the fusion Mab was detected by the addition of a biotinylated anti-human IgG F(ab)₂ secondary antibody, followed by a 1 hr incubation with peroxidase –conjugated streptavidin at room temperature. TMB substrate solution was added and the reaction stopped with 1N H₂SO₄. The absorbance was measured at 450nm on a BioTek Synergy H4 Hybrid reader.

[00194] For the bifunctional ELISA, rhEGFR-Fc chimera was coated onto NUNC maxisorb plates overnight at 4°C. The plates were washed and then blocked with superblock at room temperature for 2 hr. Different dilutions of the fusion Mab or the negative control antibody was added to the plate. The plate was incubated at room temperature for 1 hr. After washing, TGFβ was added and the plate was incubated at room temperature for 1 hr. The plate was washed and anti-TGFβ-biotin was added and the plate incubated at room temperature for 1 hr. The plate was washed and streptavidin-HRP was added and the plate incubated at room temperature for 1 hr. After washing, TMB substrate solution was added and the reaction stopped with 1N H₂SO₄. The absorbance was measured at 450nm on a BioTek Synergy H4 Hybrid reader.

[00195] Results:

[00196] The binding of the anti-EGFR1 HC-TGFβRII + Anti-EGFR1 LC fusion Mab to both the targets EGFR1 (Figure 34) and TGFβ (Figure 35) was comparable with anti-EGFR1-TGFβRII. The anti-EGFR1 HC-TGFβRII + Anti-EGFR1 LC fusion Mab was also tested in a bifunctional ELISA to determine whether the anti-EGFR1 and TGFβRII domains of the Mab can bind to their respective targets without interfering with each other. As seen in Figure 36, the anti-EGFR1 HC-TGFβRII + Anti-EGFR1 LC fusion Mab binds to both its targets, suggesting that there is no interference in binding to either target due to the construction of the fusion Mab.

[00197] Binding to Cells Expressing EGFR1:

[00198] Procedure:

[00199] A-431 cells were grown in flasks until they reached 70-80% confluency. The cells were trypsinized and harvested. The cells were stained with different dilutions of the anti-EGFR1 HC-TGFβRII + Anti-EGFR1 LC fusion Mab or control Ig at 2 - 8°C for 30 minutes. The cells were washed and incubated with anti-Human IgG - FITC conjugate at 2 - 8°C for 30 minutes. After washing, the cells were analyzed on a flow cytometer. Live cells were gated based on their FSC vs SSC profiles. The total MFI for the gated population were recorded.

[00200] Results:

[00201] The anti-EGFR1 HC-TGF β R2 + Anti-EGFR1 LC fusion binds to EGFR-expressing A-431 cells in a dose dependent manner (Figure 37). The binding of anti-EGFR1 HC-TGF β R2 + Anti-EGFR1 LC is comparable to the binding of anti-EGFR1-TGF β R2.

[00202] Antibody-dependent cytotoxicity Activity

[00203] Procedure:

[00204] A-431 cells were grown in flasks until they reached 70-80% confluency. The cells were trypsinized, harvested and plated into 96-well plates. The cells were labeled with different dilutions of the anti-EGFR1 HC-TGF β R2 + Anti-EGFR1 LC fusion Mab or control Ig at 2 - 8°C for 30 minutes. The labeled cells were co-incubated with freshly isolated human PBMC at 37°C, 5% CO₂ for 24 hours. Cytotoxicity was measured using the Cyto-Tox-Glo cytotoxicity assay kits.

[00205] Results:

[00206] The anti-EGFR1 HC-TGF β R2 + Anti-EGFR1 LC fusion mediates ADCC of EGFR-expressing A-431 cells by human PBMC effector cells. The ADCC is dose dependent (Figure 38). These results suggest that the Fc portion of the fusion Mab is intact and functional.

[00207] Inhibition of Proliferation

[00208] Procedure:

[00209] A-431 cells were grown in flasks until they reached 70-80% confluency. The cells were trypsinized, harvested and plated into 96-well plates. Different dilutions of the anti-EGFR1 HC-TGF β R2 + Anti-EGFR1 LC fusion Mab or control Ig were added to the cells. The plates were incubated at 37°C, 5% CO₂ for three days. On the third day, cell proliferation was measured by the AlamarBlue method.

[00210] Results:

[00211] Anti-EGFR antibodies such as Cetuximab are known to inhibit the proliferation of EGFR1-expressing cells. As seen in Figure 39, the anti-EGFR portion of the anti-EGFR1 HC-TGF β R2 + Anti-EGFR1 LC fusion Mab is intact and has anti-proliferative activity.

[00212] 5. Anti-EGFR1 HC + Anti-EGFR1 LC -TGF β R2 (Fmab2)

[00213] Binding ELISAs: Procedure:

[00214] The anti-EGFR1 HC + Anti-EGFR1 LC -TGF β R2 fusion Mab was tested for its ability to bind to its targets in three different ELISAs : 1) EGFR1 target-binding ELISA, 2) TGF β -target binding ELISA and 3) Bifunctional ELISA.

[00215] For the target binding ELISAs, the targets (rhEGFR-Fc chimera or TGF β) were coated onto NUNC maxisorb plates overnight at 4°C. The plates were washed and then blocked with superbloc at room temperature for 2 hr. Different dilutions of the fusion Mab or the negative control antibody was added to the plate. The plate was incubated at room temperature for 1 hr. Binding of the fusion Mab was detected by the addition of a biotinylated anti-human IgG F(ab)₂ secondary antibody, followed by a 1 hr incubation with peroxidase –conjugated streptavidin at room temperature. TMB substrate solution was added and the reaction stopped with 1N H₂SO₄. The absorbance was measured at 450nm on a BioTek Synergy H4 Hybrid reader.

[00216] For the bifunctional ELISA, rhEGFR-Fc chimera was coated onto NUNC maxisorb plates overnight at 4°C. The plates were washed and then blocked with superbloc at room temperature for 2 hr. Different dilutions of the fusion Mab or the negative control antibody was added to the plate. The plate was incubated at room temperature for 1 hr. After washing, TGF β was added and the plate is incubated at room temperature for 1 hr. The plate was washed and anti-TGF β -biotin was added and the plate incubated at room temperature for 1 hr. The plate was washed and streptavidin-HRP was added and the plate incubated at room temperature for 1 hr. After washing, TMB substrate solution was added and the reaction

stopped with 1N H₂SO₄. The absorbance was measured at 450nm on a BioTek Synergy H4 Hybrid reader.

[00217] Results:

[00218] The binding of anti-EGFR1 HC + Anti-EGFR1 LC -TGFβRII fusion Mab to both the targets EGFR1 (Figure 40) and TGFβ (Figure 41) was comparable to anti-EGFR1-TGFβRII. The anti-EGFR1 HC + Anti-EGFR1 LC -TGFβRII fusion Mab was also tested in a bifunctional ELISA to determine whether the anti-EGFR1 and TGFβRII domains of the Mab can bind to their respective targets without interfering with each other. As seen in Figure 42, the anti-EGFR1 HC + Anti-EGFR1 LC -TGFβRII fusion Mab binds to both its targets, suggesting that there is no interference in binding to either target due to the construction of the fusion Mab.

[00219] Inhibition of Proliferation

[00220] Procedure:

[00221] A-431 cells were grown in flasks until they reached 70-80% confluency. The cells were trypsinized, harvested and plated into 96-well plates. Different dilutions of the anti-EGFR1 HC + Anti-EGFR1 LC -TGFβRII fusion Mab or control Ig were added to the cells. The plates were incubated at 37°C, 5% CO₂ for three days. On the third day, cell proliferation was measured by the AlamarBlue method.

[00222] Results:

[00223] Anti-EGFR antibodies such as Cetuximab are known to inhibit the proliferation of EGFR1-expressing cells. As seen in Figure 43, the anti-EGFR portion of the anti-EGFR1 HC + Anti-EGFR1 LC -TGFβRII fusion Mab is intact and has anti-proliferative activity.

[00224] 6. TGFβRII-Anti-EGFR1 HC + Anti-EGFR1 LC (Fmab 3)

[00225] Binding ELISAs: Procedure:

[00226] The fusion Mab was tested for its ability to bind to its targets in three different ELISAs : 1) EGFR1 target-binding ELISA, 2) TGF β -target binding ELISA and 3) Bifunctional ELISA.

[00227] For the target binding ELISAs, the targets (rhEGFR-Fc chimera or TGF β) were coated onto NUNC maxisorb plates overnight at 4°C. The plates were washed and then blocked with superblock at room temperature for 2 hr. Different dilutions of the fusion Mab or the negative control antibody was added to the plate. The plate was incubated at room temperature for 1 hr. Binding of the fusion Mab was detected by the addition of a biotinylated anti-human IgG F(ab)₂ secondary antibody, followed by a 1 hr incubation with peroxidase –conjugated streptavidin at room temperature. TMB substrate solution was added and the reaction stopped with 1N H₂SO₄. The absorbance was measured at 450nm on a BioTek Synergy H4 Hybrid reader.

[00228] For the bifunctional ELISA, rhEGFR-Fc chimera was coated onto NUNC maxisorb plates overnight at 4°C. The plates were washed and then blocked with superblock at room temperature for 2 hr. Different dilutions of the fusion Mab or the negative control antibody was added to the plate. The plate was incubated at room temperature for 1 hr. After washing, TGF β was added and the plate was incubated at room temperature for 1 hr. The plate was washed and anti-TGF β -biotin was added and the plate incubated at room temperature for 1 hr. The plate was washed and streptavidin-HRP was added and the plate incubated at room temperature for 1 hr. After washing, TMB substrate solution was added and the reaction stopped with 1N H₂SO₄. The absorbance was measured at 450nm on a BioTek Synergy H4 hybrid reader.

[00229] Results:

[00230] The binding of the TGF β RII-Anti-EGFR1 HC + Anti-EGFR1 LC fusion Mab to both the targets EGFR1 (Figure 44) and TGF β (Figure 45) was comparable to anti-EGFR1-TGF β RII. The TGF β RII-Anti-EGFR1 HC + Anti-EGFR1 LC fusion Mab was also tested in a bifunctional ELISA to determine whether the anti-EGFR1 and TGF β RII domains of the Mab can bind to their respective targets without interfering with each other. As seen in Figure 46, the binding of TGF β RII-Anti-EGFR1 HC + Anti-EGFR1 LC fusion Mab is

reduced as compared to anti-EGFR1-TGF β RII, suggesting that there is some interference in binding to either target due to the construction of the fusion Mab.

[00231] Binding to Cells Expressing EGFR1:

[00232] Procedure:

[00233] A-431 cells were grown in flasks until they reached 70-80% confluency. The cells were trypsinized and harvested. The cells were stained with different dilutions of the TGF β RII-Anti-EGFR1 HC + Anti-EGFR1 LC fusion Mab or control Ig at 2 - 8⁰C for 30 minutes. The cells were washed and incubated with anti-Human IgG - FITC conjugate at 2 - 8⁰C for 30 minutes. After washing, the cells were analyzed on a flow cytometer. Live cells were gated based on their FSC vs SSC profiles. The total MFI for the gated population were recorded.

[00234] Results:

[00235] The TGF β RII-Anti-EGFR1 HC + Anti-EGFR1 LC fusion binds to EGFR-expressing A-431 cells in a dose dependent manner. The binding of TGF β RII-Anti-EGFR1 HC + Anti-EGFR1 LC is comparable to the binding of anti-EGFR1-TGF β RII.

[00236] Inhibition of Proliferation

[00237] Procedure:

[00238] A-431 cells were grown in flasks until they reached 70-80% confluency. The cells were trypsinized, harvested and plated into 96-well plates. Different dilutions of the TGF β RII-Anti-EGFR1 HC + Anti-EGFR1 LC fusion Mab or control Ig were added to the cells. The plates were incubated at 37⁰C, 5% CO₂ for three days. On the third day, cell proliferation was measured by the AlamarBlue method.

[00239] Results:

[00240] Anti-EGFR antibodies such as Cetuximab are known to inhibit the proliferation of EGFR1-expressing cells. As seen in Figure 47, the anti-EGFR portion of the TGF β RII-Anti-EGFR1 HC + Anti-EGFR1 LC fusion Mab is intact and has anti-proliferative activity.

[00241] 7. Anti-EGFR1 HC + TGF β RII-Anti-EGFR1 LC (Fmab 4)

[00242] Binding ELISAs: Procedure:

[00243] The fusion Mab was tested for its ability to bind to its targets in three different ELISAs : 1) EGFR1 target-binding ELISA, 2) TGF β -target binding ELISA and 3) Bifunctional ELISA.

[00244] For the target binding ELISAs, the targets (rhEGFR-Fc chimera or TGF β) were coated onto NUNC maxisorb plates overnight at 4°C. The plates were washed and then blocked with superblock at room temperature for 2 hr. Different dilutions of the fusion Mab or the negative control antibody was added to the plate. The plate was incubated at room temperature for 1 hr. Binding of the fusion Mab was detected by the addition of a biotinylated anti-human IgG F(ab)₂ secondary antibody, followed by a 1 hr incubation with peroxidase –conjugated streptavidin at room temperature. TMB substrate solution was added and the reaction stopped with 1N H₂SO₄. The absorbance was measured at 450nm on a BioTek Synergy H4 Hybrid reader.

[00245] For the bifunctional ELISA, rhEGFR-Fc chimera was coated onto NUNC maxisorb plates overnight at 4°C. The plates were washed and then blocked with superblock at room temperature for 2 hr. Different dilutions of the fusion Mab or the negative control antibody was added to the plate. The plate was incubated at room temperature for 1 hr. After washing, TGF β was added and the plate was incubated at room temperature for 1 hr. The plate was washed and anti-TGF β -biotin was added and the plate incubated at room temperature for 1 hr. The plate was washed and streptavidin-HRP was added and the plate incubated at room temperature for 1 hr. After washing, TMB substrate solution was added and the reaction stopped with 1N H₂SO₄. The absorbance was measured at 450nm on a BioTek Synergy H4 hybrid reader.

[00246] Results:

[00247] The binding of the Anti-EGFR1 HC + TGF β RII-Anti-EGFR1 LC fusion Mab to both the targets EGFR1 (Figure 48) and TGF β (Figure 49) was comparable with anti-EGFR1-TGF β RII. The Anti-EGFR1 HC + TGF β RII-Anti-EGFR1 LC fusion Mab was also tested in a bifunctional ELISA to determine whether the anti-EGFR1 and TGF β RII domains of the Mab can bind to their respective targets without interfering with each other. As seen in Figure 50, the binding of Anti-EGFR1 HC + TGF β RII-Anti-EGFR1 LC fusion Mab is reduced as compared to anti-EGFR1-TGF β RII, suggesting that there is some interference in binding to either target due to the construction of the fusion Mab.

[00248] Binding to Cells Expressing EGFR1:

[00249] Procedure:

[00250] A-431 cells were grown in flasks until they reached 70-80% confluency. The cells were trypsinized and harvested. The cells were stained with different dilutions of Anti-EGFR1 HC + TGF β RII-Anti-EGFR1 LC fusion Mab or control Ig at 2 - 8 $^{\circ}$ C for 30 minutes. The cells were washed and incubated with anti-Human IgG - FITC conjugate at 2 - 8 $^{\circ}$ C for 30 minutes. After washing, the cells were analyzed on a flow cytometer. Live cells were gated based on their FSC vs SSC profiles. The total MFI for the gated population were recorded.

[00251] Results:

[00252] The Anti-EGFR1 HC + TGF β RII-Anti-EGFR1 LC fusion binds to EGFR-expressing A-431 cells in a dose dependent manner (Figure 51). The binding of Anti-EGFR1 HC + TGF β RII-Anti-EGFR1 LC is reduced compared to the binding of anti-EGFR1-TGF β RII.

[00253] 8. Anti-EGFR1 HC-TGF β RII + TGF β RII-Anti-EGFR1 LC (Fmab 10)

[00254] Binding ELISAs: Procedure:

[00255] The fusion Mab was tested for its ability to bind to its targets in three different ELISAs : 1) EGFR1 target-binding ELISA, 2) TGF β -target binding ELISA and 3) Bifunctional ELISA.

[00256] For the target binding ELISAs, the targets (rhEGFR-Fc chimera or TGF β) were coated onto NUNC maxisorb plates overnight at 4°C. The plates were washed and then blocked with superbloc at room temperature for 2 hr. Different dilutions of the fusion Mab or the negative control antibody was added to the plate. The plate was incubated at room temperature for 1 hr. Binding of the fusion Mab was detected by the addition of a biotinylated anti-human IgG F(ab)₂ secondary antibody, followed by a 1 hr incubation with peroxidase –conjugated streptavidin at room temperature. TMB substrate solution was added and the reaction stopped with 1N H₂SO₄. The absorbance was measured at 450nm on a BioTek Synergy H4 Hybrid reader.

[00257] For the bifunctional ELISA, rhEGFR-Fc chimera was coated onto NUNC maxisorb plates overnight at 4°C. The plates were washed and then blocked with superbloc at room temperature for 2 hr. Different dilutions of the fusion Mab or the negative control antibody was added to the plate. The plate was incubated at room temperature for 1 hr. After washing, TGF β was added and the plate was incubated at room temperature for 1 hr. The plate was washed and anti-TGF β -biotin was added and the plate incubated at room temperature for 1 hr. The plate was washed and streptavidin-HRP was added and the plate incubated at room temperature for 1 hr. After washing, TMB substrate solution was added and the reaction stopped with 1N H₂SO₄. The absorbance was measured at 450nm on a BioTek Synergy H4 hybrid reader.

[00258] Results:

[00259] The binding of Anti-EGFR1 HC-TGF β R2 + TGF β R2-Anti-EGFR1 LC fusion Mab to the target EGFR1 was slightly reduced (Figure 52) but was higher for TGF β (Figure 53) when compared to the binding of anti-EGFR1-TGF β R2. The Anti-EGFR1-TGF β R2 + TGF β R2-Anti-EGFR1 LC fusion Mab was also tested in a bifunctional ELISA to determine whether the anti-EGFR1 and TGF β R2 domains of the Mab can bind to their respective targets without interfering with each other. As seen in Figure 54, the binding of Anti-EGFR1 HC-TGF β R2 + TGF β R2-Anti-EGFR1 LC fusion Mab is comparable to anti-

EGFR1-TGF β RII, suggesting that there is no interference in binding to either target due to the construction of the fusion Mab.

[00260] 9. TGF β RII-Anti-EGFR1 HC + TGF β RII-Anti-EGFR1 LC (Fmab 12)

[00261] Binding ELISAs: Procedure:

[00262] The fusion Mab was tested for its ability to bind to its targets in three different ELISAs : 1) EGFR1 target-binding ELISA, 2) TGF β -target binding ELISA and 3) Bifunctional ELISA.

[00263] For the target binding ELISAs, the targets (rhEGFR-Fc chimera or TGF β) were coated onto NUNC maxisorb plates overnight at 4°C. The plates were washed and then blocked with superblock at room temperature for 2 hr. Different dilutions of the fusion Mab or the negative control antibody was added to the plate. The plate was incubated at room temperature for 1 hr. Binding of the fusion Mab was detected by the addition of a biotinylated anti-human IgG F(ab)₂ secondary antibody, followed by a 1 hr incubation with peroxidase –conjugated streptavidin at room temperature. TMB substrate solution was added and the reaction stopped with 1N H₂SO₄. The absorbance was measured at 450nm on a BioTek Synergy H4 Hybrid reader.

[00264] For the bifunctional ELISA, rhEGFR-Fc chimera was coated onto NUNC maxisorb plates overnight at 4°C. The plates were washed and then blocked with superblock at room temperature for 2 hr. Different dilutions of the fusion Mab or the negative control antibody was added to the plate. The plate was incubated at room temperature for 1 hr. After washing, TGF β was added and the plate was incubated at room temperature for 1 hr. The plate was washed and anti-TGF β -biotin was added and the plate incubated at room temperature for 1 hr. The plate was washed and streptavidin-HRP was added and the plate incubated at room temperature for 1 hr. After washing, TMB substrate solution was added and the reaction stopped with 1N H₂SO₄. The absorbance was measured at 450nm on a BioTek Synergy H4 hybrid reader.

[00265] Results:

[00266] The binding of TGF β RII-Anti-EGFR1 HC + TGF β RII-Anti-EGFR1 LC fusion Mab to the target EGFR1 was slightly reduced (Figure 53) but was higher for TGF β (Figure 56) when compared to the binding of anti-EGFR1-TGF β RII. The TGF β RII-Anti-EGFR1 HC + TGF β RII-Anti-EGFR1 LC fusion Mab was also tested in a bifunctional ELISA to determine whether the anti-EGFR1 and TGF β RII domains of the Mab can bind to their respective targets without interfering with each other. As seen in Figure 57, the binding of TGF β RII-Anti-EGFR1 HC + TGF β RII-Anti-EGFR1 LC fusion Mab is reduced compared to anti-EGFR1-TGF β RII, suggesting that there is interference in binding to either target due to the construction of the fusion Mab.

[00267] References

[00268]

[00269] Altschul, et al., 1990, Basic local alignment search tool, *J. Mol. Biol.* 15:403-10.

[00270] Ausubel, et al., (ed), 1998, Current Protocols in Molecular Biology, Vol. 2, Ch. 13, Greene Publish. Assoc. & Wiley Interscience.

[00271] Bitter, et al., 1987, Expression and Secretion Vectors for Yeast; *Methods in Enzymology*, 153:5 16-544.

[00272] Bitter, 1987, Heterologous Gene Expression in Yeast, *Methods in Enzymology*, Vol. 152, pp. 673-684.

[00273] Dong M., et al., 2006, Role of transforming growth factor- β in hematologic malignancies. *Blood*. 107:4589-4596.

[00274] Deutscher, M., et al., Guide to Protein Purification: Methods in Enzymology, Vol. 182, Academic Press (1990).

[00275] Flies, et al., 2011, Blockade of the B7-H1/PD-1 Pathway for Cancer Immunotherapy, *J Biol Med.*, 84(4): 409-421.

[00276] Glover, 1986, DNA Cloning, Vol. II, Ch. 3, IRL Press, Wash., D.C.

[00277] Gluzman (ed.), 1982, Eukaryotic Viral Vectors, Cold Spring Harbor, N.Y.: Cold Spring Harbor Laboratory.

[00278] Grant, et al., 1987, Expression and Secretion Vectors for Yeast, *Methods in Enzymology*, Vol. 153, 516-544.

- [00279] Lee, et al., 1994, Delivery of liposomes into cultured KB cells via folate receptor-mediated endocytosis. *J Biol. Chem.*, 269:3 198.
- [00280] Lee, et al., 1995, Folate-mediated tumor cell targeting of liposome-entrapped doxorubicin in vitro. *Biochem. Biophys. Acta*, 1233: 134-144.
- [00281] Mackett, et al., 1982, Vaccinia virus: a selectable eukaryotic cloning and expression vector, *Proc. Natl. Acad. Sci. USA*, 79:7415-7419.
- [00282] Mackett, et al., 1984, General method for production and selection of infectious vaccinia virus, *J. Virol.*, 49:857-864.
- [00283] Nomi, et al., 2007, Clinical significance and therapeutic potential of the programmed death-1 ligand/programmed death-1 pathway in human pancreatic cancer. *Clin Cancer Res.*, 13:2151-7.
- [00284] Okazaki, et al., 2007, PD-1 and PD-1 ligands: from discovery to clinical Application. *International Immunology*, Vol. 19, No. 7, pp. 813-824.
- [00285] Panicali, et al., 1982, Construction of poxviruses as cloning vectors: insertion of the thymidine, *Proc. Natl. Acad. Sci. USA*, 79:4927-4931.
- [00286] Pardoll, DM., 2012, The blockage of immune checkpoints in cancer immunotherapy. *Nat. Rev. Cancer*, 12(4): 252-64.
- [00287] Strathern, et al., 1982, The Molecular Biology of the Yeast *Saccharomyces*, Cold Spring Harbor Press, Vols. I and II.
- [00288] Thompson RH, et al., 2004, Costimulatory B7-H1 in renal cell carcinoma patients: Indicator of tumor aggressiveness and potential therapeutic target. *Proc Natl Acad Sci U S A.*, 101(49):17174-9.

[00289] Teicher BA., 2001, Malignant cells, directors of the malignant process: role of transforming growth factor beta. *Cancer Metastasis Rev*; 20:133–143.

[00290] Wrzesinski, et al., 2007, Transforming Growth Factor- β and the Immune Response: Implications for Anticancer Therapy, *Clin Cancer Res*, 13; 5262.

Claims

1. A method for preparing a therapeutically active fusion protein having bispecific binding activity, wherein the fusion protein comprises a tumor targeting moiety and at least one immunomodulatory moiety that counteracts immune tolerance of a cancer cell, wherein the tumor targeting moiety is an antibody that binds to EGFR1 and wherein the fusion protein is prepared by the following steps:

preparing a codon optimized nucleotide sequence encoding the fusion protein for expression in host CHO cells, wherein the codon optimized nucleotide sequence comprises SEQ ID NO: 1 and SEQ ID NO: 2 for encoding the antibody that binds to EGFR1, wherein the codon optimized nucleotide sequence comprises SEQ ID NO: 3 or 7 for encoding the at least one immunomodulatory moiety, wherein the at least one immunomodulatory moiety is selected from the group consisting of TGF β R2 and PD1 respectively, wherein the immunomodulatory moiety is linked to the antibody by the entire amino acid sequence expressed by codon optimized nucleotide sequence SEQ ID NO: 4, and wherein the codon optimized nucleotide sequence SEQ ID NO: 1 for encoding the heavy chain of the antibody that binds to EGFR1 is lacking nucleotides for expression of a lysine at the C-terminal end of the heavy chain of the antibody;

cloning the codon optimized nucleotide sequence of said fusion protein in host CHO cells capable of transient or stable expression;

growing the host CHO cells in a media under suitable conditions for growing and allowing the host cell to express the fusion protein; and

collecting secreted fusion proteins.

2. The method of claim 1, wherein the linker expressed by the codon optimized nucleotide sequence SEQ ID NO: 4 is of sufficient length to allow bi-specific binding of the fusion protein.

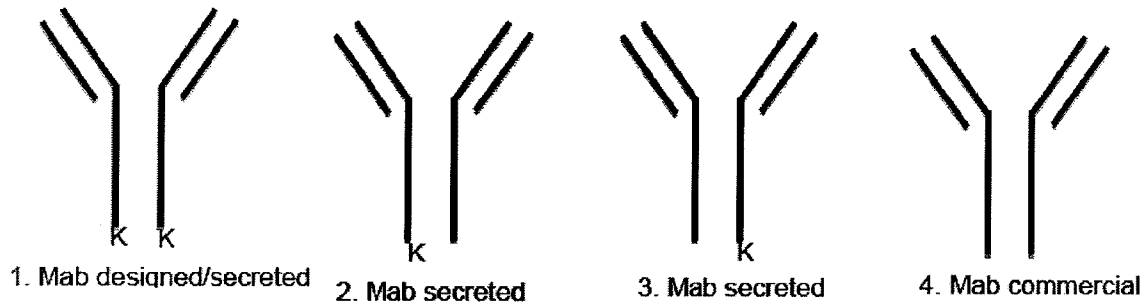
3. The method of claim 1, wherein the linker expressed by the codon optimized nucleotide sequence SEQ ID NO: 4 binds to the N or C terminus of the heavy chain of the antibody, to the N or C terminus of the light chain of the antibody, or to both N and C termini of both chains.

4. The method of claim 1, wherein the codon optimized nucleotide sequences comprise SEQ ID NOs: 1, 2, 4 and 7.
5. The method of claim 1, wherein the codon optimized nucleotide sequences comprise SEQ ID NOs: 1, 2, 3 and 4.
6. A preparation comprising homogeneous therapeutically active fusion proteins, wherein the fusion proteins comprise a tumor targeting moiety and at least one immunomodulatory moiety selected from the group consisting of TGF β RII and PD1, wherein the tumor targeting moiety is an antibody that binds to EGFR1 wherein the fusion proteins are prepared according to the method of claim 1, wherein the codon optimized nucleotide sequence encodes the fusion protein for expression in host CHO cells, wherein the codon optimized nucleotide sequence comprises SEQ ID NO: 1 and SEQ ID NO: 2 for encoding the antibody that binds to EGFR1, and the codon optimized nucleotide sequence comprises SEQ ID NO: 3 or 7 that encode for immunomodulatory molecule TGF β RII and PD1 respectively, wherein the immunomodulatory moiety is linked to the antibody by the entire amino acid sequence expressed by codon optimized nucleotide sequence SEQ ID NO: 4, and wherein the codon optimized nucleotide sequence SEQ ID NO: 1 for encoding the heavy chain of the antibody that binds to EGFR1 is lacking nucleotides for expression of a lysine at the C-terminal end of the heavy chain of the antibody.
7. A vector comprising the codon optimized nucleotide sequence defined by SEQ ID NOs: 1, 2, 4 and 7 or SEQ ID NOs: 1, 2, 3 and 4 encoding the fusion protein as defined in claim 1.
8. The method of claim 1, wherein the fusion protein comprises the amino acid sequences defined by SEQ ID NO: 9 and one of SEQ ID NO: 17, 27, or 29.
9. The method of claim 1, wherein the fusion protein comprises the amino acid sequences defined by SEQ ID NO: 8 and one of SEQ ID NO: 16, 18, 28 or 30.
10. The method of claim 1, wherein the fusion protein comprises the amino acid sequences defined by SEQ ID NO: 31 and one of SEQ ID NO: 28 or 30.

11. The method of claim 1, wherein the fusion protein comprises the amino acid sequences defined by SEQ ID NO: 29 and one of SEQ ID NO: 28 or 30.

12. Use of a preparation comprising homogeneous therapeutically active fusion proteins to treat cancer, wherein the cancer expresses EGFR1, wherein the fusion proteins comprise a tumor targeting moiety and at least one immunomodulatory moiety selected from the group consisting of TGF β RII and PD1, wherein the tumor targeting moiety is an antibody that binds to EGFR1, wherein the fusion proteins are prepared according to the method of claim 1, wherein the codon optimized nucleotide sequence encodes the fusion protein for expression in host CHO cells, wherein the codon optimized nucleotide sequence comprises SEQ ID NO: 1 and SEQ ID NO: 2 for encoding the antibody that binds to EGFR1, and comprises codon optimized nucleotide sequence SEQ ID NO: 3 or 7 that encode for immunomodulatory molecule TGF β RII and PD1 respectively, wherein the immunomodulatory moiety is linked to the antibody by the entire amino acid sequence expressed by codon optimized nucleotide sequence SEQ ID NO: 4, and wherein the codon optimized nucleotide sequence SEQ ID NO: 1 for encoding the heavy chain of the antibody that binds to EGFR1 is lacking nucleotides for expression of a lysine at the C-terminal end of the heavy chain of the antibody.

13. The method according to claim 1, wherein collected secreted fusion proteins are further purified.

**Figure 1**

SEQ ID NO: 1

Codon optimized Anti-EGFR1 Heavy chain sequence:

```

1 CAGGTGCAGC TGAAGCAGTC TGGCCCTGGC CTGGTGCAGC CCTCCCAGTC CCTGTCCATC
61 ACCTGTACCG TGTCCGGCTT CTCCCTGACC AACTACGGCG TGCACCTGGGT GCGACAGTCC
121 CCCGCCAAGG GCCTGGAATG GCTGGGAGTG ATTTGGAGCG GCGGCAACAC CGACTACAAC
181 ACCCCCTTCA CCTCCCGGCT GTCCATCAAC AAGGACAACCT CCAAGTCCCA GGTGTTCTTC
241 AAGATGAACT CCCTGCAGTC CAACGACACC GCCATCTACT ACTGCGCCAG AGCCCTGACC
301 TACTATGACT ACGAGTTCGC CTACTGGGGC CAGGGCACCC TGGTGACAGT GTCCGCCGCT
361 TCCACCAAGG GCCCCCTCCGT GTTCCCTCTG GCCCCCTCCA GCAAGTCCAC CTCTGGCGGC
421 ACCGCTGCCC TGGGCTGCCT GGTGAAAGAC TACTTCCCCG AGCCCGTGAC CGTGTCTCTG
481 AACTCTGGCG CCCTGACCTC GGGCGTGAC ACCTTCCCTG CCGTGCTGAA GTCTCTCCGC
541 CTGTACTCCC TGTCCTCCGT GGTGACCGTG CCCTCCAGCT CTCTGGGCAC CCAGACCTAC
601 ATCTGCAACG TGAACCACAA GCCCTCCAAC ACCAAGGTGG ACAAGCGGGT GGAACCCAAG
661 TCCTGCGACA AGACCCACAC CTGTCCCCC TGCCCTGCCC CTGAACTGCT GGGCGGACCT
721 TCCGTGTTC TGTTCCTCCC AAAGCCCAAG GACACCCCTGA TGATCTCCCG GACCCCGGAA
781 GTGACCTGCG TGGTGGTGGA CGTGTCCAC GAGGACCCCTG AAGTGAAGTT CAATTGGTAC
841 GTGGACGGCG TGGAAAGTCA CAACGCCAAG ACCAAGCCCA GAGAGGAACA GTACAACTCC
901 ACCTACCGGG TGGTGTCTGT GCTGACCGTG CTGCACCAGG ACTGGCTGAA CGGCAAAGAG
961 TACAAGTGCA AGGTGTCCAA CAAGGCCCTG CCTGCCCCCA TCGAAAAGAC CATCTCCAAG
1021 GCCAAGGGCC AGCCCCGCGA GCCCCAGGTG TACACCCTGC CCCCTAGCCG GGACGAGCTG
1081 ACCAAGAACC AGGTGTCCCT GACCTGTCTG GTGAAAGGCT TCTACCCCTC CGATATCGCC
1141 GTGGAATGGG AGTCCAACGG CCAGCCCGAG AACAACACTA AGACCACCCC CCCTGTGCTG
1201 GACTCCGACG GCTCATCTT CCTGTACTCC AAGCTGACCG TGGACAAGTC CCGGTGGCAG
1261 CAGGGCAACG TGTCTCCTG CTCCGTGATG CAGAGGCCCC TGCACAACCA CTACACCCAG
1321 AAGTCCCTGT CTCTGTCCCC CGGC

```

SEQ ID NO: 2

Codon optimized Anti-EGFR1 light chain nucleotide sequence:

```

1 GACATCCTGC TGACCCAGTC CCCCCTGATC CTGTCCGTGT CTCCTGGCGA GCGGGTGTCC
61 TTCTCCTGCC GGGCCTCTCA GTCCATCGGC ACCAACATCC ACTGGTATCA GCAGCGGACC
121 AACGGCTCCC CTCGGCTGCT GATTAAGTAC GCCTCCGAGT CCATCTCCGG CATCCCTTCC
181 CGGTTCTCCG GCTCCGGCTC TGGCACCGAC TTCACCCCTGT CCATCAACTC CGTGGAAATCC
241 GAGGACATTG CCGACTACTA CTGCCAGCAG AACACAACCT GGCCCACCAC CTTCCGGCGCT
301 GGCACCAAGC TGGAAGTGAA GCGGACCGTG GCCGCTCCCT CCGTGTTCAT CTTCCACCCC
361 TCCGACGAGC AGCTGAAGTC CGGCACCGCC TCCGTGGTGT GCCTGCTGAA CAACTTCTAC
421 CCCC GCGAGG CCAAGGTGCA GTGGAAGGTG GACAACGCCC TGCAGTCCGG CAACTCCCAG
481 GAATCCGTGA CCGAGCAGGA CTCCAAGGAC AGCACCTACT CCCTGTCTC CACCCTGACC
541 CTGTCCAAGG CCGACTACGA GAAGCACAAG GTGTACGCCT GCGAAGTGAC CCACCAGGGC
601 CTGTCCAGCC CCGTGACCAA GTCTTCAAC CGGGGCGAGT GT

```

Figure 2

SEQ ID NO: 3

Codon optimized nucleotide sequence of TGF β RII:

```

1 ACAATCCCTC CACACGTGCA GAAATCCGTG AACAAACGACA TGATCGTGAC CGACAACAAT
61 GGCGCCGTGA AGTTCCCCCA GCTGTGCAAG TTCTGCGACG TGCGGTTCTC TACCTGCGAC
121 AACCAGAAAT CCTGCATGTC CAACTGCTCC ATCACCTCCA TCTGCGAGAA GCCCCAGGAA
181 GTGTGCGTGG CCGTGTGGCG GAAGAACGAC GAGAACATCA CCCTGGAAAC CGTGTGCCAC
241 GACCCCAAGC TGCCCTACCA CGACTTCATC CTGGAAGATG CCGCCTCCCC CAAGTGCATC
301 ATGAAGGAAA AGAAGAAGCC CGGCGAGACT TTCTTCATGT GCAGCTGCTC CTCCGACGA G
361 TGCAACGACA ACATCATCTT CTCCGAAGAG TACAACACCT CCAACCCCGA C

```

SEQ ID NO: 4

Codon optimized nucleotide sequence of the Linker:

```

1 GGAGGCGGAG GATCTGGCGG AGGTGGAAGT GGCGGCGGAG GCTCT

```

SEQ ID NO: 5

Codon optimized nucleotide sequence of AntiCTLA4 heavy chain:

```

1 CAGGTGCAGC TGGTGAATC TGGTGGCGGA GTGGTGCAGC CTGGCAGATC CCTGAGACTG
61 TCTTGTGCCG CCTCCGGCTT CACCTTCTCC TGTACACCA TGCCTGGGT GCGACAGGCC
121 CCTGGCAAGG GACTGGAATG GGTCACCTTC ATCTCTTACG ACGGCAACAA CAAGTACTAC
181 GCGGACTCCG TGAAGGGCCG GTTCACCATC TCCCGGACA ACTCCAAGAA CACCTGTAC
241 CTGCAGATGA ACTCCCTGCG GGCCGAGGAC ACCGCCATCT ACTACTGTGC TAGAACCGGC
301 TGGCTGGGCC CCTTCGATTA TTGGGGCCAG GGCACCCTCG TGACCGTCTC GAGCGCTAGC
361 ACAAAGGGCC CTAGTGTGTT TCCTCTGGCT CCTCTTCCA AATCCACTTC TGGTGGCACT
421 GCTGCTCTGG GATGCCTGGT GAAGGATTAC TTCTCTGAAC CTGTGACTGT CTCATGGAAC
481 TCTGGTGCTC TGACTTCTGG TGTCCACACT TTCCCTGCTG TGCTGCAGTC TAGTGGACTG
541 TACTCTCTGT CATCTGTGGT CACTGTGCCC TCTTCATCTC TGGGAACCCA GACCTACATT
601 TGTAATGTGA ACCACAAACC ATCCAACACT AAAGTGGACA AACGGGTGGA ACCCAAATCC
661 TGTGACAAAA CCCACACCTG CCCACCTTGT CCTGCCCTG AACTGCTGGG AGGACCTTCT
721 GTGTTTCTGT TCCCCCCAA ACCAAAGGAT ACCCTGATGA TCTCTAGAAC CCCTGAGGTG
781 ACATGTGTGG TGGTGGATGT GTCTCATGAG GACCCTGAGG TCAAATTCAA CTGGTACGTG
841 GATGGAGTGG AAGTCCACAA TGCCAAAACC AAGCCTAGAG AGGAACAGTA CAATTCAACC
901 TACAGAGTGG TCAGTGTGCT GACTGTGCTG CATCAGGATT GGCTGAATGG CAAGGAATAC
961 AAGTGTAAG TCTCAAACAA GGCCCTGCCT GCTCCAATTG AGAAAACAAT CTCAAAGGCC
1021 AAGGGACAGC CTAGGGAACC CCAGGTCTAC ACCCTGCCAC CTTCAAGAGA TGAAGTACC
1081 AAAAACCAGG TGTCCCTGAC ATGCCTGGTC AAAGGCTTCT ACCCTTCTGA CATTGCTGTG
1141 GAGTGGGAGT CAAATGGACA GCCTGAGAAC AACTACAAAA CAACCCCCC TGTGCTGGAT
1201 TCTGATGGCT CTTTCTTTCT GTACTCCAAA CTGACTGTGG ACAAGTCTAG ATGGCAGCAG
1261 GGGAATGTCT TTTCTTGCTC TGTCATGCAT GAGGCTCTGC ATAACCACTA CACTCAGAAA
1321 TCCCTGTCTC TGTCTCCCGG G

```

Figure 2 Cont.

SEQ ID NO: 6

Codon optimized nucleotide sequence of Anti-CTLA4 light chain:

```

1 GAGATCGTGC TGACCCAGTC TCCTGGCACC CTGTCTCTGA GCCCTGGCGA GAGAGCTACC
61 CTGTCTTGCA GAGCCTCTCA GTCCGTGGGC TCCTCTTACC TGGCCTGGTA TCAACAAAAA
121 CCCGGCCAAG CTCCCCGGCT GCTGATCTAC GGTGCCTTTT CTCGCGCCAC CGGCATCCCC
181 GACCGGTTCT CCGGATCTGG CTCTGGCACC GACTTCACCC TGACCATCTC CCGGCTGGAA
241 CCCGAGGACT TCGCCGTGTA CTACTGCCAG CAGTACGGCT CCTCCCCCTG GACCTTTGGC
301 CAGGGCACCA AGGTGGAAAT CAAACGTACG GTCGCGGCGC CTTCCTGTT CATCTTCCCA
361 CCCTCCGACG AGCAGCTGAA GTCCGGCACC GCCTCCGTGG TGTGCCTGCT GAACAACCTC
421 TACCCCCGCG AGGCCAAGGT GCAGTGGAAG GTGGACAACG CCCTGCAGTC CGGCAACTCC
481 CAGGAATCCG TGACCGAGCA GGACTCCAAG GACAGCACCT ACTCCCTGTC CTCCACCCTG
541 ACCCTGTCCA AGGCCGACTA CGAGAAGCAC AAGGTGTACG CCTGCGAAGT GACCCACCAG
601 GGCCTGTCCA GCCCCGTGAC CAAGTCCTTC AACCAGGGCG AGTGT

```

SEQ ID NO: 7

Codon optimized nucleotide sequence of PD1

```

1 CCTGGCTGGT TTCTGGACTC CCCTGACCGG CCCTGGAACC CCCCACCTT CTCTCCTGCC
61 CTGCTGGTGG TGACAGAGGG CGACAACGCC ACCTTCACCT GTTCCTTCAG CAACACCTCC
121 GAGTCCTTCG TGCTGAACTG GTACAGAATG TCCCCAGCA ACCAGACCGA CAAGCTGGCC
181 GCCTTCCCCG AGGACAGATC CCAGCCTGGC CAGGACTGCC GGTTCAGAGT GACCCAGCTG
241 CCCAACGGCC GGGACTTCCA CATGTCCGTG GTGCGAGCCA GACGGAACGA CTCCGGCACC
301 TACCTGTGCG GCGCCATCTC TCTGGCCCCC AAGGCCAGCA TCAAAGAGTC CCTGCGGGCC
361 GAGCTGAGAG TGACCGAGAG AAGGGCCGAG GTGCCCACCG CCCACCCTAG CCCATCTCCA
421 AGACCTGCCG GCCAGTTCCA GACCCTGGTG

```

Figure 2 Cont.

5/66

SEQ ID NO: 8

Amino acid sequence of Anti-EGFR1 Heavy Chain constant

QVQLKQSGPGLVQPSQSLSTCTVSGFSLTNYGVHWVRQSPGKGLEWL
GVIWSSGNTDYNTPFTSRLSINKDNSKSQVFFKMNSLQSNDAIYYCAR
ALTYDYEFAYWGQGLVTVSAASTKGPSVFPLAPSSKSTSGGTAALG
CLVKDYFPEPTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVTVPSSSL
GTQTYICNVNHKPSNTKVDKRVEPKSCDKTHTCPPCPAPELLGGPSVFL
FPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTK
PREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISK
AKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQP
ENNYKTTTPVLDSGDSFFLYSKLTVDKSRWQQGNVVFSCSVMHEALHNH
YTQKSLSLSPG

SEQ ID NO: 9

Amino acid sequence of Anti-EGFR1 Light chain

DILLTQSPVILSVSPGERVSFSCRASQSIGTNIHWYQQR TNGSPRLLIKYA
SEISGIPSRFSGSGGTDFTLINSVESEDIADYYCQQNNNWPTTFGAGT
KLELKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVD
NALQSGNSQESVTEQDSKDYSLSTLTLSKADYEEKHKVYACEVTHQ
GLSSPVTKSFNRGEC

SEQ ID NO: 10

Amino acid sequence of linker

GGGGSGGGGSGGGGS

SEQ ID NO: 11

Amino acid sequence of PD1

PGWFLDSPDRPWNPPTFSPALLVVTEGDNATFTCSFSNTSESVLNWYR
MSPSNQTDKLAAPEDRSQPGQDCRFRVTQLPNGRDFHMSVVRARRND
SGTYLCGAISLAPKAQIKESLRAELRV TERRAEVPTAHPSPSPRPAGQFQ
TLV

Figure 3

6/66

SEQ ID NO: 8**Amino acid sequence of Anti-EGFR1 Heavy Chain constant**

QVQLKQSGPGLVQPSQSLSITCTVSGFSLTNYGVHWVRQSPGKGLE
 WLGVIWSGGNTDYNTPTFTSRLSINKDNSKSQVFFKMNSLQSNDAIY
 YCARALTYDYEFAYWGQGLVTVSAASTKGPSVFPLAPSSKSTSG
 GTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSS
 VVTVPSSSLGTQTYICNVNHKPSNTKVDKRVEPKSCDKTHTCPPCPA
 PELLGGPSVFLFPPKPKDTLMISRTPEVTCVVDVSHEDPEVKFNWY
 VDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCK
 VSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLV
 KGFYPSDIAVEWESNGQPENNYKTTTPVLDSDGSFFLYSKLTVDKSR
 WQQGNVFSCSVMHEALHNHYTQKSLSLSPG

SEQ ID NO: 9**Amino acid sequence of Anti-EGFR1 Light chain**

DILLTQSPVILSVSPGERVSFSCRASQSIGTNIHWYQQRTNGSPRLLIK
 YASESISGIPSRFSGSGSGTDFTLSINSVESEDIADYYCQQNNNWPTTF
 GAGTKLELKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAK
 VQWKVDNALQSGNSQESVTEQDSKDSTYLSSTLTLSKADYEKHKV
 YACEVTHQGLSSPVTKSFNRGEC

SEQ ID NO: 10**Amino acid sequence of linker**

GGGGSGGGGSGGGGS

SEQ ID NO: 12**Amino acid sequence of TGFRII**

TIPPHVQKSVNNDMIVTDNNGAVKFPQLCKFCDVRFSTCDNQKSCMSNCSITS
 ICEKPQEVCVAVWRKNDENITLETVCHDPKLPYHDFILEDAAASPKCIMKEKKK
 PGETFFMCSSSDECNDNIIFSEEYNTSNPD

Figure 4

SEQ ID NO: 13**Amino acid sequence of Anti-CTLA-4 Heavy Chain constant**

QVQLVESGGGVVQPGRSLRLSCAASGFTFSSYTMHWVRQAPGKGLEWVTFISY
DGNNKYYADSVKGRFTISRDN SKNTLYLQMNSLRAEDTAIYYCARTGWLGPFD
YWGQGTLLTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSW
NSGALTSGVHTFPAVLQSSGLYSLSSVTVPSSSLGTQTYICNVNHKPSNTKV
DKRVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVTV
DVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGK
EYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVK
GFYPSDIAVEWESNGQPENNYKTTTPVLDSDGSFFLYSKLTVDKSRWQQGNVF
SCSVMHEALHNHYTQKSLSLSPG

SEQ ID NO: 14**Amino acid sequence of Anti-CTLA-4 Light chain**

EIVLTQSPGTLSSLSPGERATLSCRASQSVGSSYLAWEYQQKPGQAPRLLIYGAF
SRATGIPDRFSGSGSGTDFTLTISRLEPEDFAVYYCQQYGSSPWTFTGQGTKVE
IKRTVAAPS FFI FPPSDEQLKSGTASVVCLLNFFYPREAKVQWKVDNALQSGN
SQESVTEQDSKDYSLSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNR
GEC

SEQ ID NO: 10**Amino acid sequence of linker**

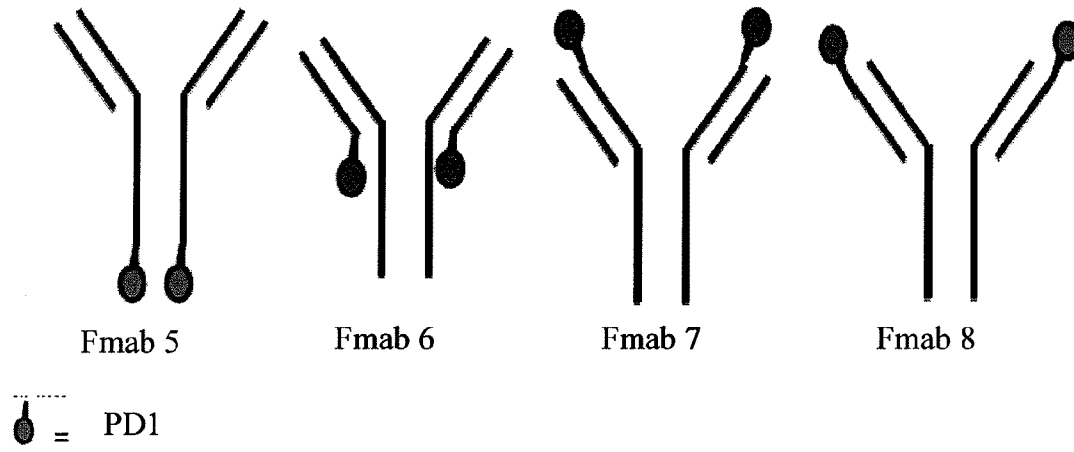
GGGGSGGGGSGGGGS

SEQ ID NO: 11**Amino acid sequence of PD1**

PGWFLDSPDRPWNPPTFSPALLVTEGDNATFTCSFSNTSESFVLNW
YRMSPSNQTDKLAAPFEDRSQPGQDCRFRVTQLPNGRDFHMSVVR
ARRNSGTYLCGAISLAPKAQIKESLRAELRVTERRAEVPTAHPSPS
PRPAGQFQTLV

Figure 5

8/66

**Figure 6**

Anti-EGFR1-PD1 (HC-C-terminus):

Amino acid sequence of Anti-EGFR1 HC constant- PD1 fusion protein:

SEQ ID NO: 15

QVQLKQSGPGLVQPSQSLSTCTVSGFSLTNYGVHWVRQSPGKGLE
 WLGVIWSSGGNTDYNTPFTSRLSINKDNSKSQVFFKMNSLQSNDAIY
 YCARALTYDYEFAYWGQGTLLVTVSAASTKGPSVFPLAPSSKSTSG
 GTAALGCLVKDYFPEPTVSWNSGALTSGVHTFPAVLQSSGLYSLSS
 VVTVPSSSLGTQTYICNVNHKPSNTKVDKRVEPKSCDKTHTCPPCPA
 PELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWY
 VDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCK
 VSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLV
 KGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSR
 WQQGNVFSCSVMHEALHNHYTQKSLSLSPGSGGGGSGGGGSGGGGS
 PGWFLDSPDRPWNPPTFSPALLVVTEDGNATFTCSFSTNTSEFVLNW
 YRMSPSNQTDKLAAPEDRSQPGQDCRFRVTQLPNGRDFHMSVVR
 ARRNDSGTYLCAISLAPKAQIKESLRAELRVTERRAEVPTAHPSPS
 PRPAGQFQTLV

Amino acid sequence of Anti-EGFR1 light chain: SEQ ID NO: 9

DILLTQSPVILSVSPGERVSFSCRASQSIGTNIHWYQQRNNGSPRLLIK
 YASESISGIPSRFSGSGSGTDFTLSINSVESEDIADYYCQQNNNWPTTF
 GAGTKLELKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAK
 VQWKVDNALQSGNSQESVTEQDSKDSSTYSLSSTLTLSKADYEKHKV
 YACEVTHQGLSSPVTKSFNRGEC

Figure 7

Anti-EGFR1-PD1 (LC-C-terminus):

Amino acid sequence of Anti-EGFR1 heavy chain: SEQ ID NO: 8

QVQLKQSGPGLVQPSQSLSITCTVSGFSLTNYGVHWVRQSPGKGLEWL
 GVIWSGGNTDYNTPFTSRLSINKDNSKSQVFFKMNSLQSNDAIYYCAR
 ALTYDYEFAYWGQGLVTVSAASTKGPSVFPLAPSSKSTSGGTAALG
 CLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSL
 GTQTYICNVNHKPSNTKVDKRVEPKSCDKTHTCPPCPAPELLGGPSVFL
 FPPKPKDITLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTK
 PREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISK
 AKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQP
 ENNYKTTTPVLDSGDSFFLYSKLTVDKSRWQQGNVDFCSVMHEALHNH
 YTQKSLSLSPG

Amino acid sequence of Anti-EGFR1 LC constant-PD1 fusion protein: SEQ ID
 NO: 16

DILLTQSPVILSVSPGERVSFSCRASQSIGTNIHWYQQRNGSPRLLIKYA
 SESISGIPSRFSGSGSGTDFTLSINSVESEDIADYYCQQNNNWPTTFGAGT
 KLELKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVD
 NALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQ
 GLSSPVTKSFNRGECGGGGSGGGGSGGGGSPGWFLDSPDRPWNPPFTFSP
 ALLVVTEGDNATFTCSFSNTSESVFLNWYRMSPSNQTDKLAAPFEDRSQ
 PGQDCRFRVTQLPNGRDFHMSVVRARRNDSGTYLCGAISLAPKAQIKES
 LRAELRVTERRAEVPTAHPSPSPRPAGQFQTLV

Figure 8

Anti-EGFR1-PD1 (HC-N-terminus):

Amino acid sequence of PD1-Anti-EGFR1 HC variable fusion protein:

SEQ ID NO: 17

PGWFLDSPDRPWNPTTFSPALLVVTEGDNATFTCSFSNTSESFVLNW
YRMSPSNQTDKLAAPEDRSQPGQDCRFRVTQLPNGRDFHMSVVR
ARRNDSTYLCAISLAPKAQIKESLRAELRVTERRAEVPTAHPSPS
PRPAGQFQTLVGGGSGGGGSGGGGSQVQLKQSGPGLVQPSQSLST
CTVSGFSLTNYGVHWVRQSPGKGLEWLGVWSSGGNTDYNTPTTSR
LSINKDNSKSQVFFKMNSLQSNDAIYYCARALTYDYEFAYWGQG
TLVTVSAASTKGPSVFLAPSSKSTSGGTAALGCLVKDYFPEPTVS
WNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNH
KPSNTKVDKRVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTL
MISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQY
NSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQ
PREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPEN
NYKTTTPVLDSGDSFFLYSKLTVDKSRWQQGNVVFSCSVMHEALHN
HYTQKSLSLSPG

Amino acid sequence of Anti-EGFR1 light chain: SEQ ID NO: 9

DILLTQSPVILSVSPGERVSFSCRASQSIGTNIHWYQQRTNGSPRLLIK
YASESISGIPSRFSGSGSGTDFTLSINSVESEDIADYYCQQNNNWPTTF
GAGTKLELKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAK
VQWKVDNALQSGNSQESVTEQDSKDSSTYSLSSTLTLSKADYEKHKV
YACEVTHQGLSPVTKSFNRGEC

Figure 9

Anti-EGFR1-PD1 (LC-N-terminus):

Amino acid sequence of Anti-EGFR1 heavy chain: SEQ ID NO: 8

QVQLKQSGPGLVQPSQSLSTCTVSGFSLTNYGVHWVRQSPGKGLE
 WLGVWISGGNTDYNTPTFTSRLSINKDNSKSQVFFKMNSLQSNDAIY
 YCARALTYDYEFAYWGQGLTVTVSAASTKGPSVFPLAPSSKSTSG
 GTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSS
 VVTVPSSSLGTQTYICNVNHKPSNTKVDKRVEPKSCDKTHTCPPCPA
 PELLGGPSVFLFPPKPKDTLMISRTPEVTCVVDVSHEDPEVKFNWY
 VDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCK
 VSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLV
 KGFYPSDIAVEWESNGQPENNYKTTTPVLDSDGSFFLYSKLTVDKSR
 WQQGNVFSCSVMHEALHNHYTQKSLSLSPG

Amino acid sequence of PD1-Anti-EGFR1 LC variable fusion protein: SEQ
 ID NO: 18

PGWFLDSPDRPWNPPTFSPALLVVTEGDNATFTCSFSNTSESFVLNW
 YRMSPSNQTDKLAAPEDRSQPGQDCRFRVTQLPNGRDFHMSVVR
 ARRNDSGTYLCGAISLAPKAQIKESLRAELRVTERRAEVPTAHPSPS
 PRPAGQFQTLVGGGGSGGGGSGGGGSDILLTQSPVILSVSPGERVSFS
 CRASQSIGTNIHWYQQRNGSPRLLIKYASESISGIPSRFSGSGSGTDF
 TLSINSVESEDIADYYCQQNNNWPTTFGAGTKLELKRTVAAPSVFIF
 PPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVT
 EQDSKDSYSLSSLTTLTKADYEKHKVYACEVTHQGLSSPVTKSFNR
 GEC

Figure 10

Expression constructs developed using the cDNAs:

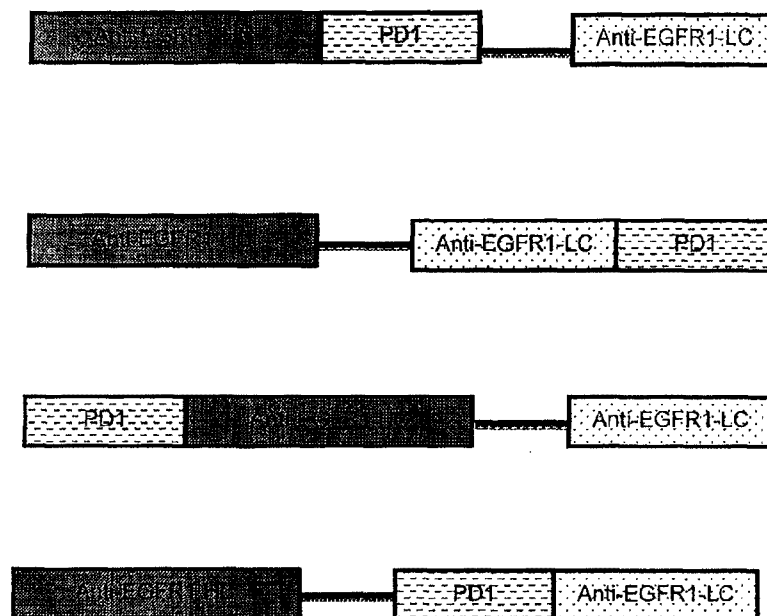
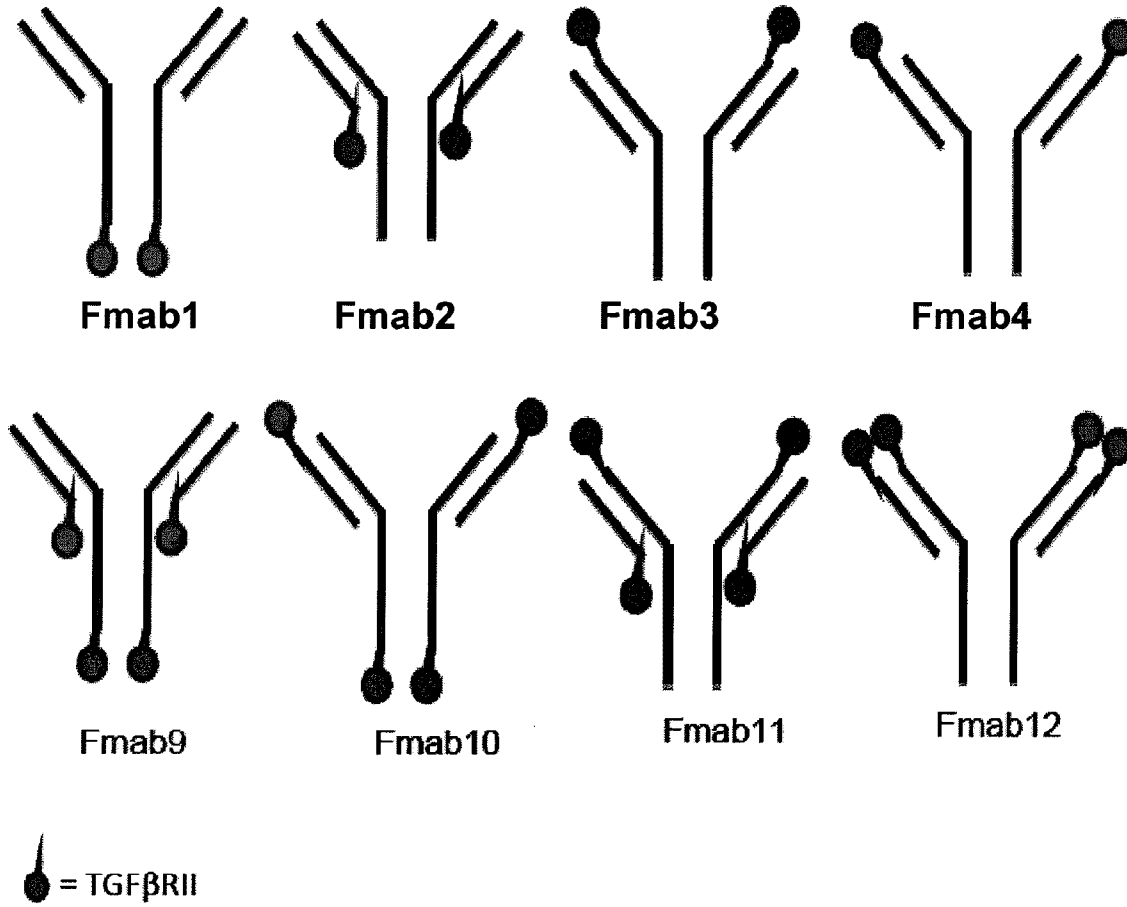


Figure 11

**Figure 12**

Anti-EGFR1-TGFR1I (HC-C-terminus):

Amino acid sequence of Anti-EGFR1 HC constant-TGFR1I fusion protein: SEQ ID NO: 27

QVQLKQSGPGLVQPSQSLITCTVSGFSLTNYGVHWVRQSPGKGLEWLGVIWSGGNTDYNT
 FTSRLSINKDNSKSQVFFKMNSLQSDNTAIYYCARALTYDYEFAYWGQGLVTVSAASTKG
 PSVPPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSS
 VVTVPSSSLGTQTYICNVNHKPSNTKVDKRVKPKSCDKTHTCPPCPAPELLGGPSVFLFPPK
 PKDTLMISRTPEVTCVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTV
 LHQDNLNGKEYCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVK
 GFYPSDIAVEWESNGQPENNYKTTTPVLDSDGSFFLYSKLTVDKSRWQQGNVFSQVMHEAL
 HNHYTQKSLSLSPGGGGSGGGSGGGGSTIPPHVQKSVNNDMIVTDNNGAVKFPQLCKFCD
 VRFSTCDNQKSCMSNCSITSICEKPQEVCAVWRKNDENITLETVCHDPKLPYHDFILEDAA
 SPKCIMKEKKKPGETFFMCSCSSDECNDNIIPSEYNTSNPD

Amino acid sequence of Anti-EGFR1 light chain: SEQ ID NO: 9

DILLTQSPVILSVSPGERVSFSCRASQSIGTNIHWYQQRNGSPRLLIKYASESISGIPSRF
 SGSGSGTDFTLINSVESEDIADYYCQQNMNWPITFGAGTKLELKRTVAAPSVFIFPPSDEQ
 LKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSSTYLSSTLTLSKADY
 EKHKVYACEVTHQGLSSPVTKSFNRGEC

Figure 13

Anti-EGFR1-TGF β R11 (LC-C-terminus):

Amino acid sequence of Anti-EGFR1 heavy chain: SEQ ID NO: 8
 QVQLKQSGPGLVQPSSLSITCTVSGFSLTNYGVHWVRQSPGKGLEWLGVIWSSGNTDYNTP
 FTSRLSINKDNSKSQVFFKMNSLQSNDAIYYCARALTYDYEFAYWGQGLTVTVSAASTKG
 PSVFPPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSS
 VVTVPSSSLGTQTYICNVNHKPSNTKVDKRVPEPKSCDKTHTCPPCPAPELLGGPSVFLFPPK
 PKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTV
 LHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVK
 GFYPSDIAVEWESNGQPENNYKTTTPVLDSDGSFFLYSKLTVDKSRWQQGNVFCSSVMHEAL
 HNHYTQKSLSLSPG

Amino acid sequence of Anti-EGFR1 LC constant-TGF β R11 fusion
 protein: SEQ ID NO: 28

DILLTQSPVILSVSPGERVSFSCRASQSIGTNIHWYQQRNGSPRLLIKYASESISGIPSRF
 SSGSGSGTDFTLSINSVESEDIADYYCQQNNNWPTTFGAGTKLELKRVAAPSVFIFPPSDEQ
 LKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKSTYLSSTLTLSKADY
 EKHKVYACEVTHQGLSSPVTKSFNRGECGGGGSGGGSGGGGSTIPPHVQKSVNNDMIVTDN
 NGAVKFPQLCKFCDVRFSTCDNQKSCMSNCSITSICEKPQEVCAVWRKNDENITLETVCHD
 PKLPYHDFILEDAAAPKCMKEKKKPGETFFMCSSSDECNDNIIFSEYNTSNPD

Figure 14

Anti-EGFR1-TGF β RII (HC-N-terminus):

Amino acid sequence of TGF β RII-Anti-EGFR1 HC variable fusion protein: SEQ ID NO: 29

TIPPHVQKSVNNDMIIVTDNNGAVKFPQLCKFCDFRSTCDNQKSCMSNCSITSICEKPQEV
 VAVWRKNDENITLETVCHDPKLPYHDFILEDAAAPKCMKEKKKPGETFFMCSCSSDECNDN
 IIFSEYNTSNPDGGGSGGGSGGGSGVQLKQSGPGLVQPSQSLSTCTVSGFSLTNYGV
 HWVRQSPGKLEWLGVWSGGNTDYNTPTTSRLSINKDNSKSQVFFKMNSLQSNDAIYYCA
 RALTYDYEFAYWGGTLVTVSAASTKGPSVFFLAPSSKSTSGGTAALGCLVKDYFPEPVT
 SWNSGALTSGVHTFPAVLQSSGLYSLSSVTVPSSSLGTQTYICNVNHKPSNTKVDKRV
 EPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVD
 GVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQ
 PREPQVYITLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTPPVLDSDGSF
 FLYSKLTVDKSRWQQGNVFSQSVMEALHNHYTQKSLSLSPG

Amino acid sequence of Anti-EGFR1 light chain: SEQ ID NO: 9

DILLTQSPVILSVSPGERVSFSCRASQSIGTNIHWYQQRTNGSPRLLIKYASESISGIPSRF
 SGSGSGTDFTLINSVESEDIADYYCQQNNNWPTTFGAGTKLELKRTVAAPSVFIFPPSDEQ
 LKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYLSSTLTLSKADY
 EKHKVYACEVTHQGLSSPVTKSFNRGEC

Figure 15

Anti-EGFR1-TGF β R11 (LC-N-terminus):

Amino acid sequence of Anti-EGFR1 heavy chain: SEQ ID NO: 8
 QVQLKQSGPGLVQPSSLSITCTVSGFSLTNYGVHWVRQSPGKGLEWLGVIWSSGNTDYNTF
 FTSRLSINKDNSKSQVFFKMNSLQSNDAIYYCARALTYDYEFAYWGQGLTVTVSAASTKG
 PSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSS
 VVTVPSSSLGTQTYICNVNHKPSNTKVDKRVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPK
 PKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTV
 LHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVK
 GFYPSDIAVEWESNGQPENNYKTTTPVLDSDGSFFLYSKLTVDKSRWQQGNVFSQSVMEAL
 HNHYTQKSLSLSPG

Amino acid sequence of TGF β R11-Anti-EGFR1 LC variable fusion
 protein: SEQ ID NO: 30

TIPPHVQKSVNNDMIVTDNNGAVKFPQLCKFCDVRFSTCDNQKSCMSNCSITSICEKPQEV
 VAVWRKNENITLETVCHDPKLPYHDFILEDAAAPKCMKEKKKPGETFFMCSSSDECNDN
 IIFSEEYNTSNPDGGGSGGGSGGGSDILLTQSPVILSVSPGERVSFSCRASQSIGTNIH
 WYQRTNGSPRLLIKYASESISGIPSRFSGSGSCTDFTLSINSVESEDIADYYCQNNNWPT
 TFGAGTKLELKRVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGN
 SQESVTEQDSKDYSLSSSTLTLSKADYEEKHKVYACEVTHQGLSSPVTKSFNRGEC

Figure 16

Anti-EGFR1-TGFβRII (HC-C-terminus & LC- C-terminus):

Amino acid sequence of Anti-EGFR1 HC constant-TGFβRII fusion
protein: SEQ ID NO: 31

QVQLKQSGPGLVQPSSQLSITCTVSGFSLTNYGVHWVRQSPGKGLEWLGVIWSSGNTDYNT
FTSRLSINKDNSKSKQVFFKMNSLQSDTAIYYCARALTYDYEFAYWGQGLVTVSAASTKG
PSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSS
VVTVPSSSLGTQTYICNVNHKPSNTKVDKRVEPKSCDKTHCTPPCPAPELLGGPSVFLFPPK
PKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTV
LHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVK
GFYPSDIAVEWESNGQPENNYKTTPVLDSDGSFFLYSKLTVDKSRWQQGNVSCSVMHEAL
HNHYTQKSLSLSPGGGGGSGGGGSGGGGSTIPPHVQKSVNNDMIVTDNNGAVKFPQLCKFCD
VRFSTCDNQKSCMSNCSITSICEKPQEVCAVWRKNDENITLETVCHDPKLPYHDFILEDAA
SPKCMKEKKKPGETFFMCSSSDECNDNIIFSEEYNTSNPD

Amino acid sequence of Anti-EGFR1 LC constant- TGFβRII fusion:
SEQ ID NO: 28

DILLTQSPVILSVSPGERVSFSCRASQSIGTNIHWYQORTNGSPRLLIKYASESISGIPSRF
SGSGSGTDFTLSINSVESEDIADYYCQONNNWPTTFGAGTKLELKRTVAAPSVFIFPPSDEQ
LKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDYSLSSLTLSKADY
EKHKVYACEVTHQGLSSPVTKSFNRGECGGGGSGGGGSGGGGSTIPPHVQKSVNNDMIVTDN
NGAVKFPQLCKFCDVRFSTCDNQKSCMSNCSITSICEKPQEVCAVWRKNDENITLETVCHD
PKLPYHDFILEDAA SPKCMKEKKKPGETFFMCSSSDECNDNIIFSEEYNTSNPD

Figure 17

Anti-EGFR1-TGF β R11 (HC-C-terminus & LC- N-terminus):

Amino acid sequence of Anti-EGFR1 HC constant-TGF β R11 fusion protein: SEQ ID NO: 31

QVQLKQSGPGLVQPSSLSITCTVSGFSLTNYGVHWVRQSPGKGLEWLGVIWSSGNTDYNTP
 FTSRLSINKDNSKSQVFFKMNSLQSNDAIYYCARALTYDYEFAYWGQGLVTVSAASTKG
 PSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSS
 VVTVPSSSLGTQTYICNVNHKPSNTKVDKRVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPK
 PKDTLMISRTPEVTCVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTV
 LHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVK
 GFYPSDIAVEWESNGQPENNYKTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFCSCVMHEAL
 HNHYTQKSLSLSPGGGGSGGGSGGGGSTIPPHVQKSVNNDMIVTDNNGAVKFPQLCKFCD
 VRFSTCDNQKSCMSNCSITSICEKPQEVCAVWRKNDENITLETVCHDPKLPYHDFILEDAA
 SPKCIMKEKKKPGETFFMCSCSSDECNDNIIFSEYNTSNPD

Amino acid sequence of TGF β R11-Anti-EGFR1 LC variable fusion protein: SEQ ID NO: 30

TIPPHVQKSVNNDMIVTDNNGAVKFPQLCKFCDVRFSTCDNQKSCMSNCSITSICEKPQEVCA
 VAVWRKNDENITLETVCHDPKLPYHDFILEDAAAPKCIMKEKKKPGETFFMCSCSSDECNDN
 IIFSEYNTSNPDGGGGSGGGSGGGSDILLTQSPVILSVSPGERVSFSCRASQSIGTNIH
 WYQQRTNGSPRLIKYASESISGIPSRFSGSGTDFTLINSVESEDIADYYCQQNNNWPT
 TFGAGTKLELKRTVAAPSVFIFPPSDEQLKSGTASVVCCLNNFYPREAKVQWKVDNALQSGN
 SQESVTEQDSKDYSLSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC

Figure 18

Anti-EGFR1-TGF β R11 (HC-N-terminus & LC- C-terminus):

Amino acid sequence of TGF β R11-Anti-EGFR1 HC variable fusion protein: SEQ ID NO: 29

TIPPHVQKSVNNDMIVTDNNGAVKFPQLCKFCDVRFSTCDNQKSCMSNCSITSICEKPQEV
 VAVWRKNDENITLETVCHDPKLPYHDFILEDAAAPKCMKEKKKPGETFFMCS CSSDECNDN
 IIFSEYNTSNPDGGGSGGGSGGGSGVQLKQSGPGLVQPSQSLITCTVSGFSLTNYGV
 HWVRQSPGKGLEWLGVWSGGNTDYNTPTTSRLSINKDNSKSQVFFKMNSLQSNDAIYYCA
 RALTYDYEFAYWGQGLVTVSAASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVT
 SWNSGALTSGVHTFPAVLQSSGLYSLSSVTVPSSSLGTQTYICNVNHKPSNTKVDKRV
 EPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVD
 GVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQ
 PREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTPPVLDSDGSF
 FLYSKLTVDKSRWQQGNVFCFSVMHEALHNHYTQKSLSLSPG

Amino acid sequence of Anti-EGFR1 LC constant-TGF β R11 fusion protein: SEQ ID NO: 28

DILLTQSPVILSVSPGERVVSFSCRASQSIGTNIHWYQRTNGSPRLLIKYASESISGIPSRF
 SSGSGTDFTLINSVESEDIADYYCQNNNWPTTFGAGTKLELKRTVAAPSVFIFPPSDEQ
 LKSGTASVVCLLNFPYPREAKVQWKVDNALQSGNSQESVTEQDSKDSSTYSLSSTLTLSKADY
 EKHKVYACEVTHQGLSPVTKSFNRGECGGGSGGGSGGGSGTIPPHVQKSVNNDMIVTDN
 NGAVKFPQLCKFCDVRFSTCDNQKSCMSNCSITSICEKPQEVCAVAVWRKNDENITLETVCHD
 PKLPYHDFILEDAAAPKCMKEKKKPGETFFMCS CSSDECNDNIIIFSEYNTSNPD

Figure 19

Anti-EGFR1-TGF β RII (HC-N-terminus & LC- N-terminus):

Amino acid sequence of TGF β RII-Anti-EGFR1 Hc variable fusion protein: SEQ ID NO: 29

TIPPHVQKSVNNDMIVTDNNGAVKFPQLCKFCDVRFSTCDNQKSCMSNCSITSICEKPQEV
 VAVWRKNDENITLETVCHDPKLPYHDFILEDAAAPKCIMKEKKKPGETFFMCSCSSDECNDN
 IIFSEYNTSNPDGGGGSGGGSGGGSGVQLKQSGPGLVQPSQSLSTCTVSGFSLTNYGV
 HWVRQSPGKGLEWLGVIWSSGNTDYNTPFTRSRLSINKDNSKSQVFFKMNSLQSNDAIYYCA
 RALTYDYEFAYWQGTLVTVSAASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVT
 SWNSGALTSGVHTFPAVLQSSGLYSLSSVTVPSSSLGTQTYICNVNHKPSNTKVDKRV
 EPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVD
 GVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQ
 PREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTTPVLDSDGSF
 FLYSKLTVDKSRWQQGNVFSQSVMHENHNYTQKSLSLSPG

Amino acid sequence of TGF β RII-Anti-EGFR1 LC variable fusion protein: SEQ ID NO: 30

TIPPHVQKSVNNDMIVTDNNGAVKFPQLCKFCDVRFSTCDNQKSCMSNCSITSICEKPQEV
 VAVWRKNDENITLETVCHDPKLPYHDFILEDAAAPKCIMKEKKKPGETFFMCSCSSDECNDN
 IIFSEYNTSNPDGGGGSGGGSGGGSDILLTQSPVILSVSPGERVVSFSCRASQSIGTNIH
 WYQORTNGSPRLLIKYASESISGIPSRFSGSGSGTDFTLINSVESEDIADYYCQNNNWPT
 TFGAGTKLELKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGN
 SQESVTEQDSKDSSTLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC

Figure 20

Expression constructs developed using the cDNAs:

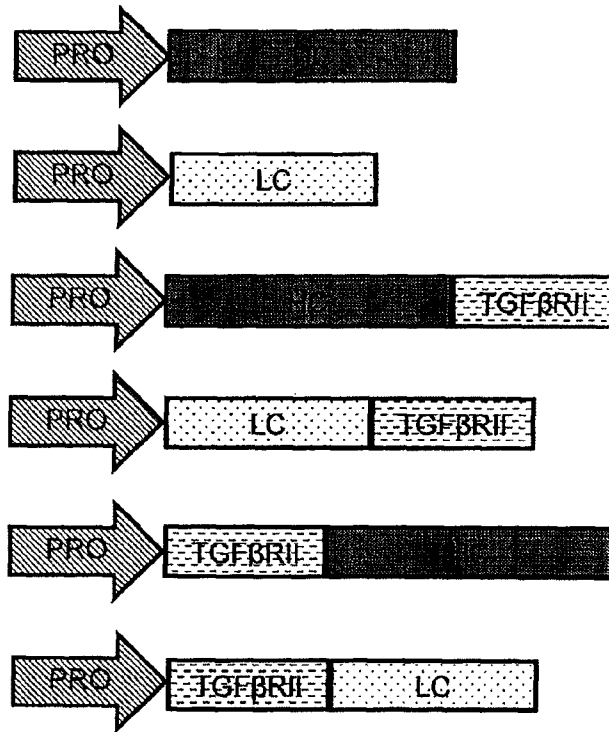
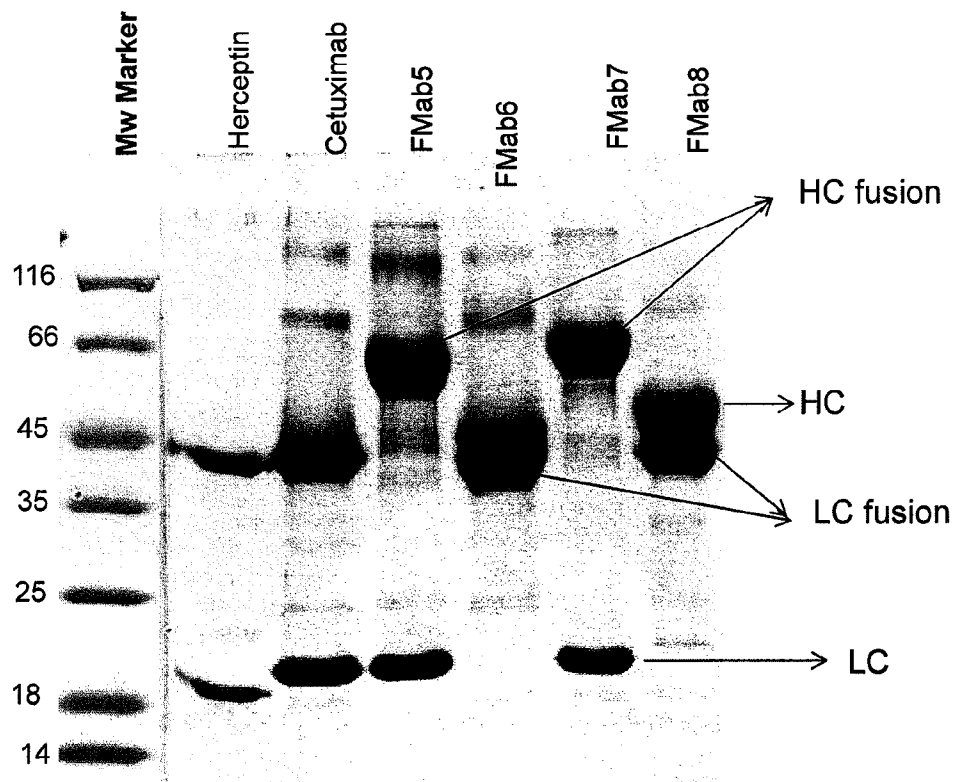
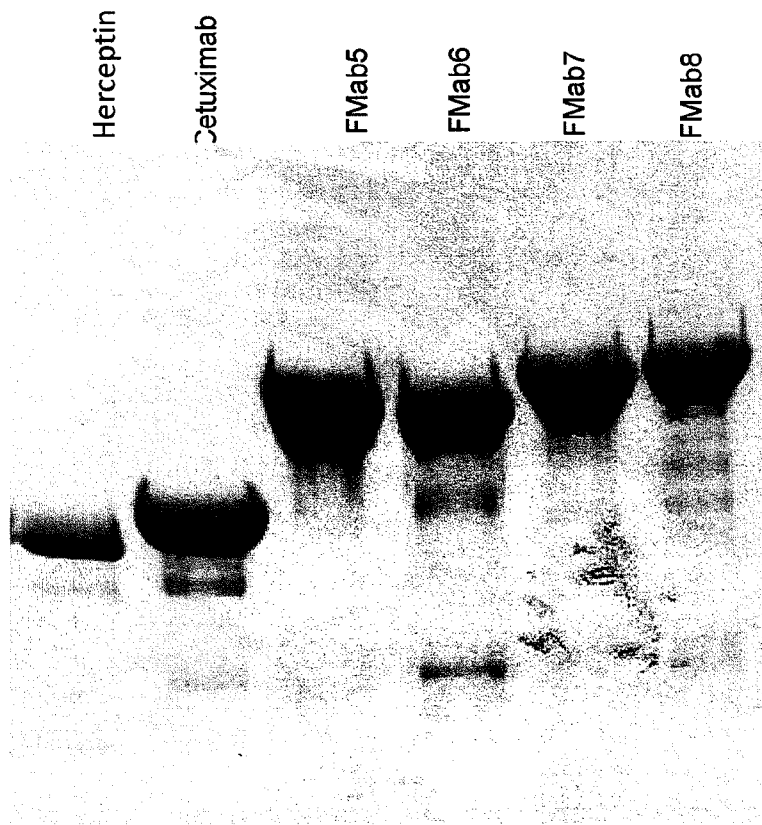


Figure 21

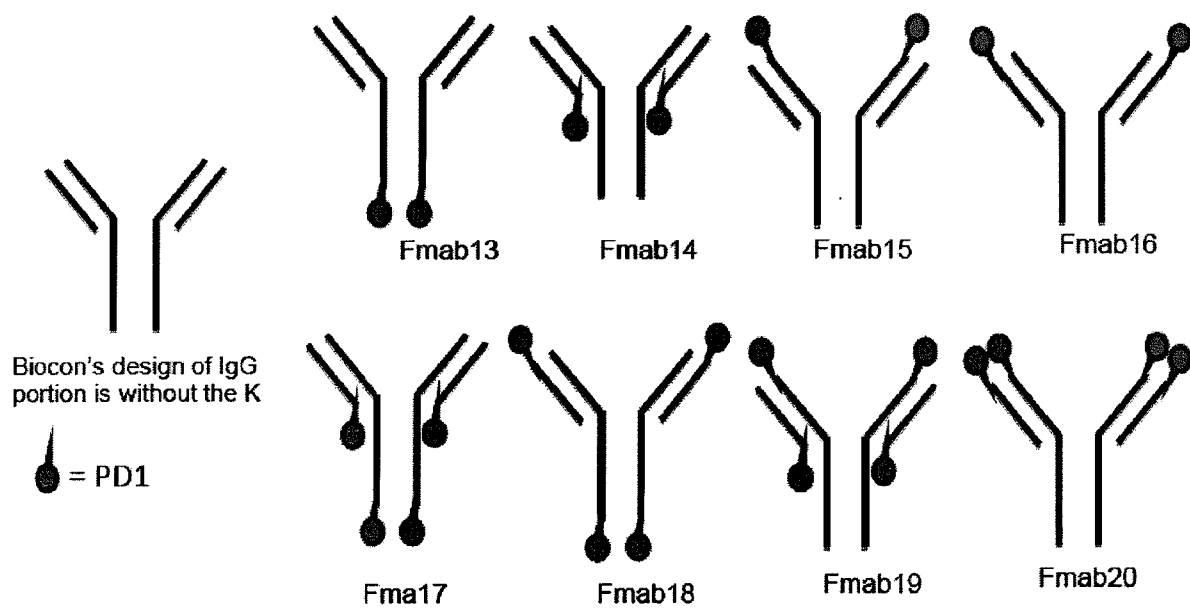
24/66

**Figure 22**

25/66

**Figure 23**

26/66

**Figure 24**

Anti-CTLA4-PD1 (HC-C-terminus):

Amino acid sequence of Anti-CTLA4 HC constant-PD1 fusion protein: SEQ ID NO: 32

QVQLVESGGGVVQPGRSLRLSCAASGFTFSSYTMHWVRQAPGKGLEWVTFISYDGNKYYAD
SVKGRFTISRDN SKNTLYLQMNSLR AEDTAIYYCARTGWLGPFDYWGQGLVTVSSASTKGP
SVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSV
VTVPSSSLGTQTYICNVNHKPSNTKVDKRVEPKSCDKTHTCPPCPAPELLGGPSVFLPPPKP
KDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVL
HQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKG
FYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNV FSCSVMEALH
NHYTQKSLSLSPGGGGGSGGGGSGGGGSPGWFLDSPDRPWNPPTFSPALLV VTEGDNATFTC
SFSNTSESFVLNWMSPSNQTDKLAAPEDRSQPGQDCRFRVTQLPNGRDFHMSVVRARRN
DSGTYLCGAISLAPKAQIKESLRAELRVTERRAEVPTAHPSPSRPAGQFQTLV

Amino acid sequence of Anti-CTLA4 light chain: SEQ ID NO: 14

EIVLTQSPGTLISLSPGERATLSCRASQSVGSSYLAWYQQKPGQAPRLLIYGAFSRATGIPDR
FSGSGSGTDFTLTISRLEPEDFAVYYCQQYGSSPWTFGQGTKVEIKRTVAAPSVFIFPPSDE
QLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSYSTYLSSTLTLSKAD
YEKHKVYACEVTHQGLSSPVTKSFNRGEC

Figure 25

Anti-CTLA4-PD1 (LC-C-terminus):

Amino acid sequence of Anti-CTLA4 heavy chain: SEQ ID NO: 13

QVQLVESGGGVVQPGRSLRLSCAASGFTFSSYTMHWVRQAPGKGLEWVTFISYDGNKYYAD
SVKGRFTISRDN SKNTLYLQMNSLRAEDTAIYYCARTGWLGPFDYWGQGLVTVSSASTKGP
SVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSV
VTVPSSSLGTQTYICNVNHKPSNTKVDKRVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKP
KDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVL
HQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKG
FYPSDIAVEWESNGQPENNYKTTTPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALH
NHYTQKSLSLSPG

Amino acid sequence of Anti-CTLA4 LC constant-PD1 fusion
protein: SEQ ID NO: 33

EIVLTQSPGTLSLSPGERATLSCRASQSVGSSYLAWYQQKPGQAPRLLIYGAFSRATGIPDR
FSGSGSGTDFTLTISRLEPEDFAVYYCQQYGSSPWTFGQGTKVEIKRTVAAPSVFIFPPSDE
QLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDYSLSTLTLSKAD
YEKHKVYACEVTHQGLSPVTKSFNRGECGGGGGGGGGGGGGGSPGWFLDSPDRPWNPPPTFS
PALLVVTEGDNATFTCSFSNTSESFVLNWRMSPSNQTDKLAAPFEDRSQPGQDCRFRTQL
PNGRDFHMSVVRARRNDSGTLYLCAISLAPKAQIKESLRAELRVTERRAEVPTAHPSPSPRP
AGQFQTLV

Figure 26

Anti-CTLA4-PD1 (HC-N-terminus) :

Amino acid sequence of PD1-Anti-CTLA4 HC variable fusion
protein: SEQ ID NO: 34

PGWFLDSPDRPWNPPTFSPALLVVTEGDNATFTCSFSNTSESFVLNWYRMSPSNQTDKLAAF
PEDRSQPGQDCRFRVTQLPNGRDFHMSVVRARRNDSTYLCAISLAPKAQIKESLRAELRV
TERRAEVPTAHPSPSPRAGQFQTLVGGGGSGGGSGGGGSQVQLVESGGGVVQPGRSLRLS
CAASGFTFSSYTMHWVRQAPGKGLEWVTFISYDGNMKYYADSVKGRFTISRDNKNTLYLQM
NSLRAEDTAIYYCARTGWLGPFDYWGQGTLLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGC
LVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVTVPSSSLGTQTYICNVNHKP
SNTKVDKRVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSH
EDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPA
PIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYK
TTPFVLDSDGSFFLYSKLTVDKSRWQQGNVFSQSVMEALHNHYTQKSLSLSPG

Amino acid sequence of Anti-CTLA4 light chain: SEQ ID NO: 14

EIVLTQSPGTLSSLSPGERATLSCRASQSVGSSYLAWYQQKPGQAPRLLIYGAFSRATGIPDR
FSGSGSGTDFTLTISRLEPEDFAVYYCQQYGSSPWTFGQGTKVEIKRTVAAPSVFIFPPSDE
QLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSSTYSLSSTLTLSKAD
YEKHKVYACEVTHQGLSPVTKSFNRGEC

Figure 27

Anti-CTLA4-PD1 (LC-N-terminus):

Amino acid sequence of Anti-CTLA4 heavy chain: SEQ ID NO: 13

QVQLVESGGGVVQPGRSLRLSCAASGFTTFSSYTMHWVRQAPGKGLEWVTFISYDGNNKYYAD
 SVKGRFTISRDNKNTLYLQMNSLRAEDTAIYYCARTGWLGPFDYWGQGTILVTVSSASTKGP
 SVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSV
 VTPSSSLGTQTYICNVNHKPSNTKVDKRVKVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKP
 KDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVL
 HQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKG
 FYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSQSVMHREALH
 NHYTQKSLSLSPG

Amino acid sequence of PD1-Anti-CTLA4 LC variable fusion protein: SEQ ID NO: 35

PGWFLDSPDRPWNPPTFSPALLVVTEGDNATFTCSFSNTSESFVLNWMSPSNQTDKLAAP
 PEDRSQPGQDCRFRVTQLPNGRDFHMSVVRARRNDSTYLCGAISLAPKAQIKESLRAELRV
 TERRAEVPTAHPSPPRPAGQFQTLVGGGGSGGGSGGGGSEIVLTQSPGTLSTLSPGERATL
 SCRASQSVGSSYLAWYQQKPGQAPRLLIYGAFSRATGIPDRFSGSGSGTDFTLTISRLEPED
 FAVYYCQQYGSSPWTFGQGTKVEIKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREA
 KVQWKVDNALQSGNSQESVTEQDSKDSSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSPVT
 KSFNRGEC

Figure 28

Anti-CTLA4-PD1 (HC-C-terminus & LC- C-terminus):

Amino acid sequence of Anti-CTLA4 HC constant-PD1 fusion
protein: SEQ ID NO: 32

QVQLVESGGGVVQPGRSLRLSCAASGFTFSSYTMHWVRQAPGKGLEWVTFISYDGNKYYAD
SVKGRFTISRDN SKNTLYLQMNSLRAEDTAIYYCARTGWLGPFDYWGQGLVTVSSASTKGP
SVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSV
VTVPSSSLGTQTYICNVNHKPSNTKVDKRVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKP
KDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVL
HQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKG
FYPSDIAVEWESNGQPENNYKTTTPVLDSDGSFFLYSKLTVDKSRWQQGNVFSVSMHEALH
NHYTQKSLSLSPGGGGGSGGGGSGGGGSPGWFLDSPDRPWNPTTFSPALLVVTEDGNATFTC
SFSNTSESFVLNWRMSPSNQTDKLAAPFEDRSQPGQDCRFRVTQLPNGRDFHMSVVRARRN
DSGTYLCGAISLAPKAQIKESLRAELRVTERRAEVPTAHPSPSPRPAGQFQTLV

Amino acid sequence of Anti-CTLA4 LC constant-PD1 fusion
protein: SEQ ID NO: 33

EIVLTQSPGTLSSLSPGERATLSCRASQSVGSSYLAWYQQKPGQAPRLLIYGAFSRATGIPDR
FSGSGSGTDFTLTISRLEPEDFAVYYCQQYGSSPWTFGQGTKVEIKRTVAAPSVFIFPPSDE
QLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSSTYLSLSSTLTLSKAD
YEKHKVYACEVTHQGLSSPVTKSFNRGECGGGGSGGGGSGGGGSPGWFLDSPDRPWNPTFS
PALLVVTEDGNATFTCSFSNTSESFVLNWRMSPSNQTDKLAAPFEDRSQPGQDCRFRVTQL
PNGRDFHMSVVRARRNDSGTYLCGAISLAPKAQIKESLRAELRVTERRAEVPTAHPSPSPRP
AGQFQTLV

Figure 29

Anti-CTLA4-PD1 (HC-C-terminus & LC- N-terminus):

Amino acid sequence of Anti-CTLA4 HC constant-PD1 fusion
protein: SEQ ID NO: 32

QVQLVESGGGVVQPGRSLRLSCAASGFTFSYTMHWVRQAPGKGLEWVTFISYDGNKYYAD
SVKGRFTISRDNKNTLYLQMNSLRAEDTAIYYCARTGWLGPFDYWGQGTLVTVSSASTKGP
SVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSV
VTVPSSSLGTQTYICNVNHKPSNTKVDKRVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKP
KDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKFREEQYNSTYRVVSVLTVL
HQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKG
FYPSDIAVEWESNGQPENNYKTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSVMHEALH
NHYTQKSLSLSPGGGGSGGGGSGGGGSPGWFLDSPDRPWNPTTFSPALLVTEGDNATFTC
SFSNTSESFVLNWRMSPSNQTDKLAAPFEDRSQPGQDCRFRVTQLPNGRDFHMSVVRARRN
DSGTYLCGAISLAPKAQIKESLRAELRVTERRAEVPTAHPSPSRPAGQFQTLV

Amino acid sequence of PD1-Anti-CTLA4 LC variable fusion
protein: SEQ ID NO: 35

PGWFLDSPDRPWNPTTFSPALLVTEGDNATFTCSFSNTSESFVLNWRMSPSNQTDKLAAP
PEDRSQPGQDCRFRVTQLPNGRDFHMSVVRARRNDSGTYLCGAISLAPKAQIKESLRAELRV
TERRAEVPTAHPSPSRPAGQFQTLVGGGGSGGGGSGGGGSEIVLTQSPGTLSPGERATL
SCRASQSVGSSYLAWYQQKPGQAPRLLIYGAFSRATGIPDRFSGSGSGTDFTLTISRLEPED
FAVYYCQYQSSPWTFGQGTKVEIKRTVAAPSVFIFPPSDEQLKSGTASVVCILNNFYPREA
KVQWKVDNALQSGNSQESVTEQDSKDSSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVT
KSFNRGEC

Figure 30

Anti-CTLA4-PD1 (HC-N-terminus & LC- C-terminus):

Amino acid sequence of PD1-Anti-CTLA4 HC variable fusion
protein: SEQ ID NO: 34

PGWFLDSPDRPWNPPFTFSPALLVVTEDGNATFTCSFSNTSESFVLNWYRMSPSNQTDKLAAP
PEDRSQPGQDCRFRVTQLPNGRDFHMSVVRARRNDSGTYLCGAISLAPKAQIKESLRAELRV
TERRAEVPTAHPSPPRPAGQFQTLVGGGSGGGSGGGSGVQLVESGGGVVQGRSLRLS
CAASGFTFSSYTMHWVRQAPGKGLEWVTFISYDGMNKYYADSVKGRFTISRDNKNTLYLQM
NSLRAEDTAIYYCARTGWLGPFDYWGGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGC
LVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVTVPSSSLGTQTYICNVNHKP
SNTKVDKRVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSH
EDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPA
PIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYK
TTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFCFSVMHEALHNHYTQKSLSLSPG

Amino acid sequence of Anti-CTLA4 LC constant-PD1 fusion
protein: SEQ ID NO: 33

EIVLTQSPGTLSSLSPGERATLSCRASQSVGSSYLAWYQQKPGQAPRLLIYGAFSRATGIPDR
FSGSGSGTDFTLTISRLEPEDFAVYYCQQYGSSPWTFCGQTKVEIKRTVAAPSVFIFPPSDE
QLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKSTYSLSTLTLSKAD
YEKHKVYACEVTHQGLSSPVTKSFNRGECGGGGSGGGSGGGSGPGWFLDSPDRPWNPPFTS
PALLVVTEDGNATFTCSFSNTSESFVLNWYRMSPSNQTDKLAAPFEDRSQPGQDCRFRVTQL
PNGRDFHMSVVRARRNDSGTYLCGAISLAPKAQIKESLRAELRVTERRAEVPTAHPSPPRP
AGQFQTLV

Figure 31

Anti-CTLA4-PD1(HC-N-terminus & LC- N-terminus):

Amino acid sequence of PD1-Anti-CTLA4 HC variable fusion
protein:SEQ ID NO: 34

PGWFLDSPDRPWNPPTFSPALLVVTEGDNATFTCSFSNTSESFVLNWYRMSPSNQTDKLAAF
PEDRSQPGQDCRFRVTQLPNGRDFHMSVVRARRNDSTYLCGAISLAPKAQIKESLRAELRV
TERRAEVPTAHPSPPRPAGQFQTLVGGGSGGGGSGGGGSGVQLVESGGGVVQPGRSLRLS
CAASGFTTFSSYTMHWVRQAPGKGLEWVTFISYDGNKYYADSVKGRFTISRDN SKNTLYLQM
NSLRAEDTAIYYCARTGWLGPFDYWGQGTLLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGC
LVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVTVPSSSLGTQTYICNVNHKP
SNTKVDKRVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSH
EDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPA
PIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYK
TTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSQSVMEALHNHYTQKSLSLSPG

Amino acid sequence of PD1-Anti-CTLA4 LC variable fusion
protein: SEQ ID NO: 35

PGWFLDSPDRPWNPPTFSPALLVVTEGDNATFTCSFSNTSESFVLNWYRMSPSNQTDKLAAF
PEDRSQPGQDCRFRVTQLPNGRDFHMSVVRARRNDSTYLCGAISLAPKAQIKESLRAELRV
TERRAEVPTAHPSPPRPAGQFQTLVGGGSGGGGSGGGGSGEIVLTQSPGTLSLSPGERATL
SCRASQSVGSSYLAWYQQKPGQAPRLLIYGAFSRATGIPDRFSGSGSGTDFTLTISRLEPED
FAVYYCQQYGSSPWTFCGQGTKVEIKRTVAAPSVFIFPPPSDEQLKSGTASVVCLLNNFYPREA
KVQWKVDNALQSGNSQESVTEQDSKDSSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSPVT
KSFNRGEC

Figure 32

Expression constructs developed using the cDNAs:

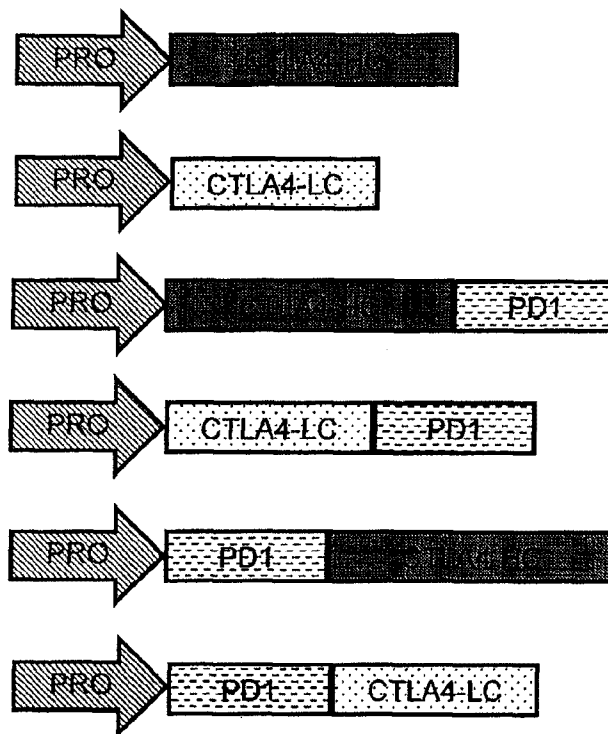
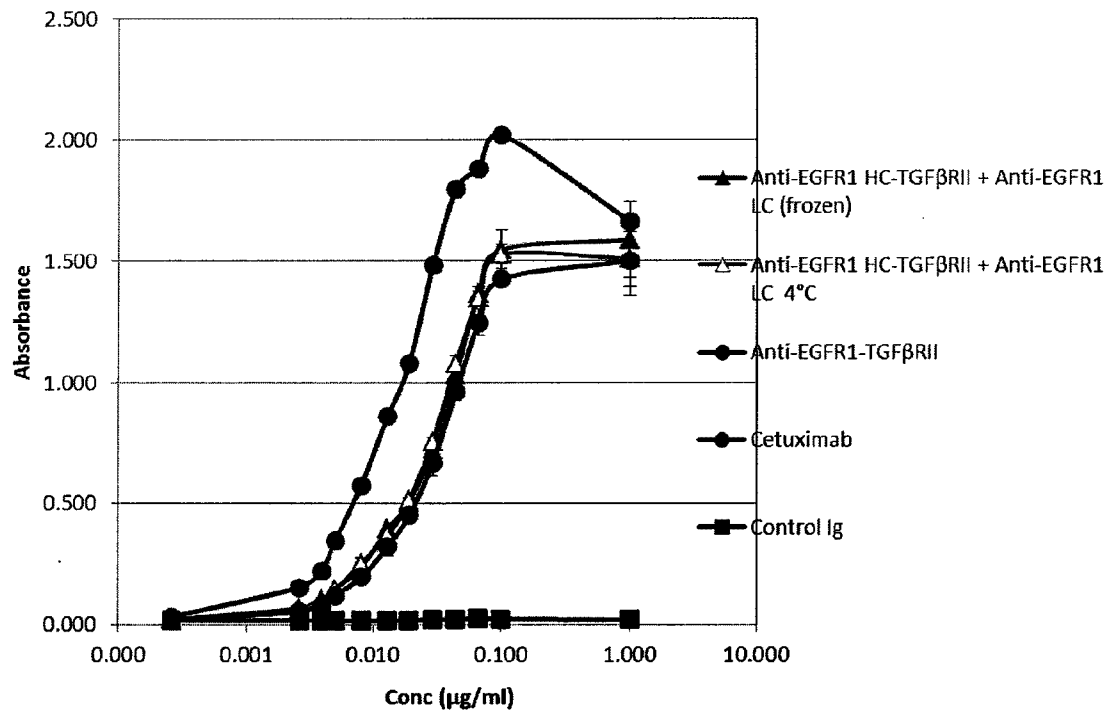
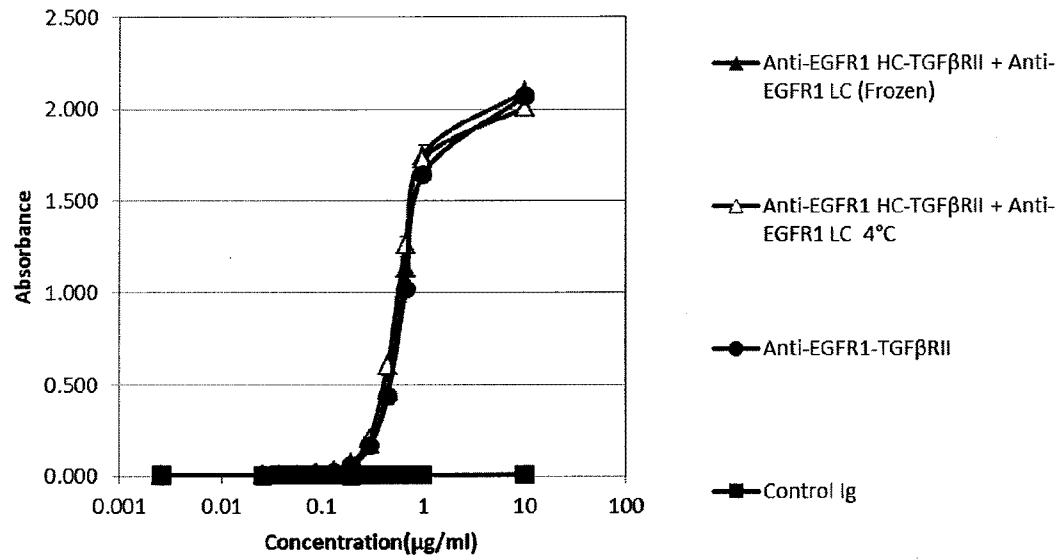


Figure 33

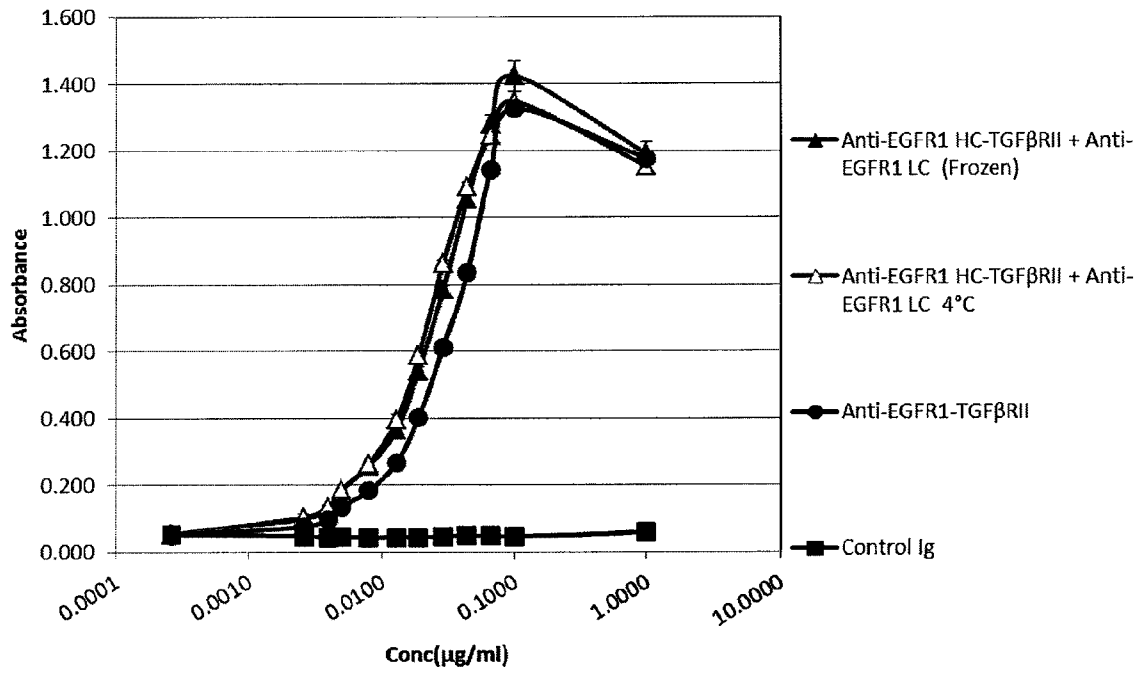
36/66

**Figure 34**

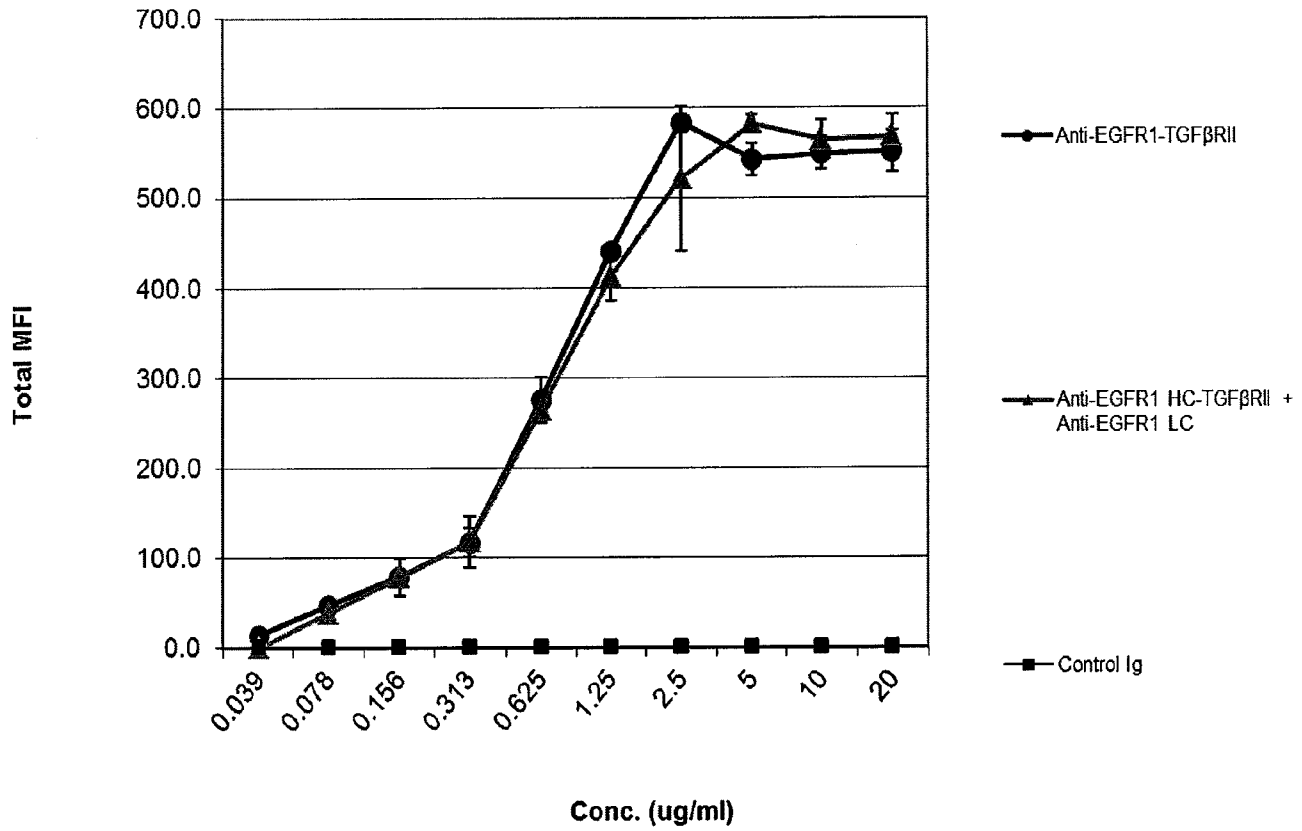
37/66

**Figure 35**

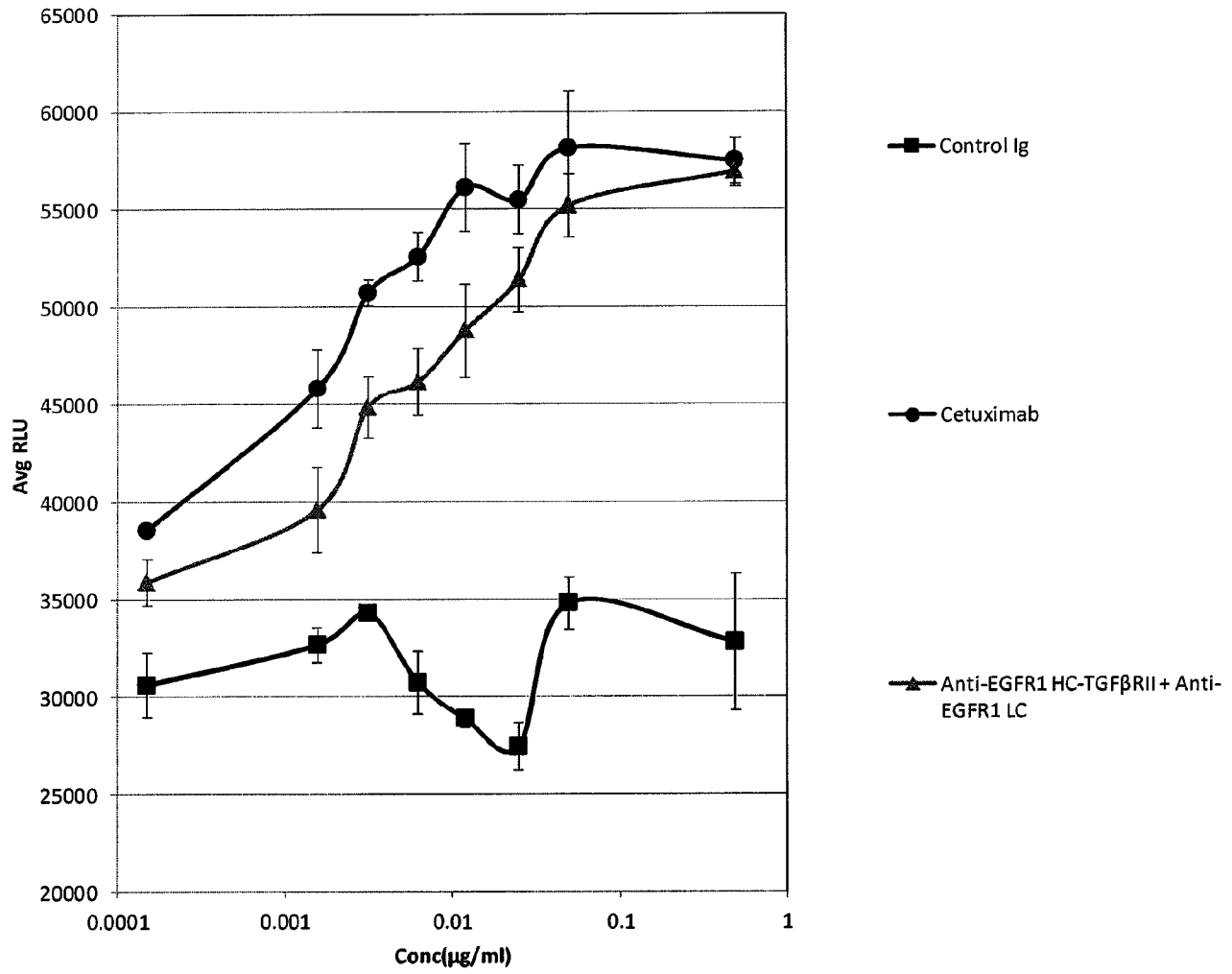
38/66

**Figure 36**

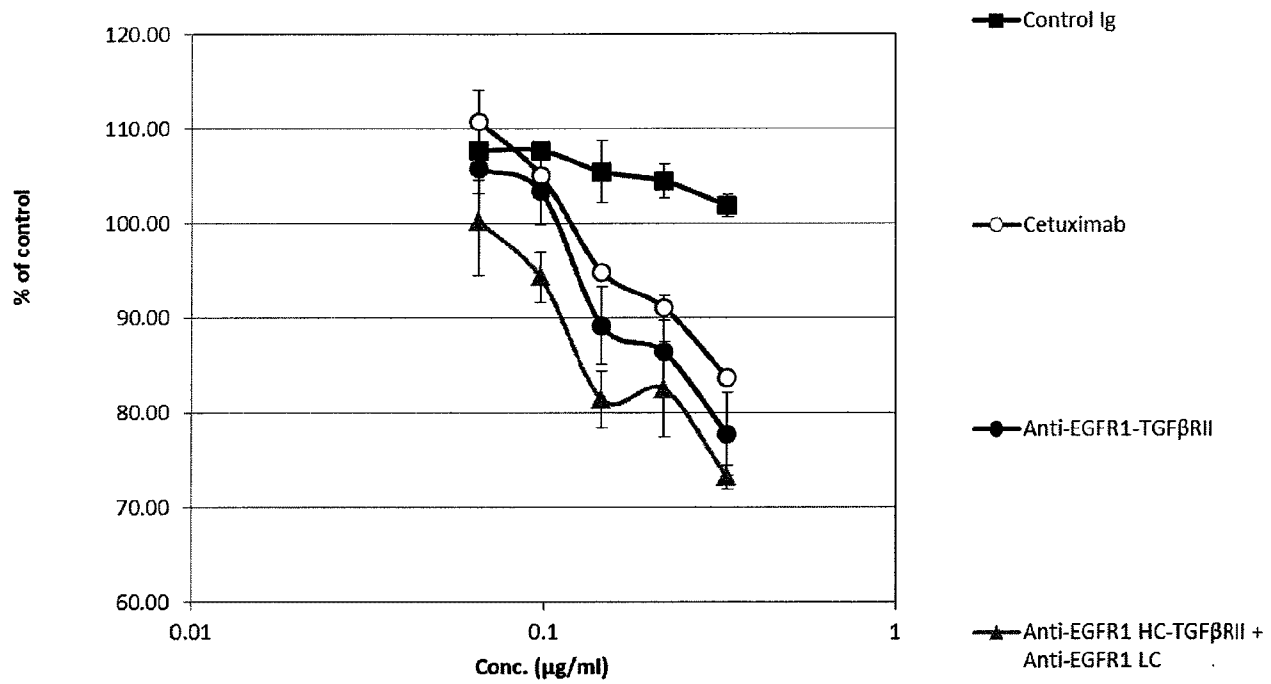
39/66

**Figure 37**

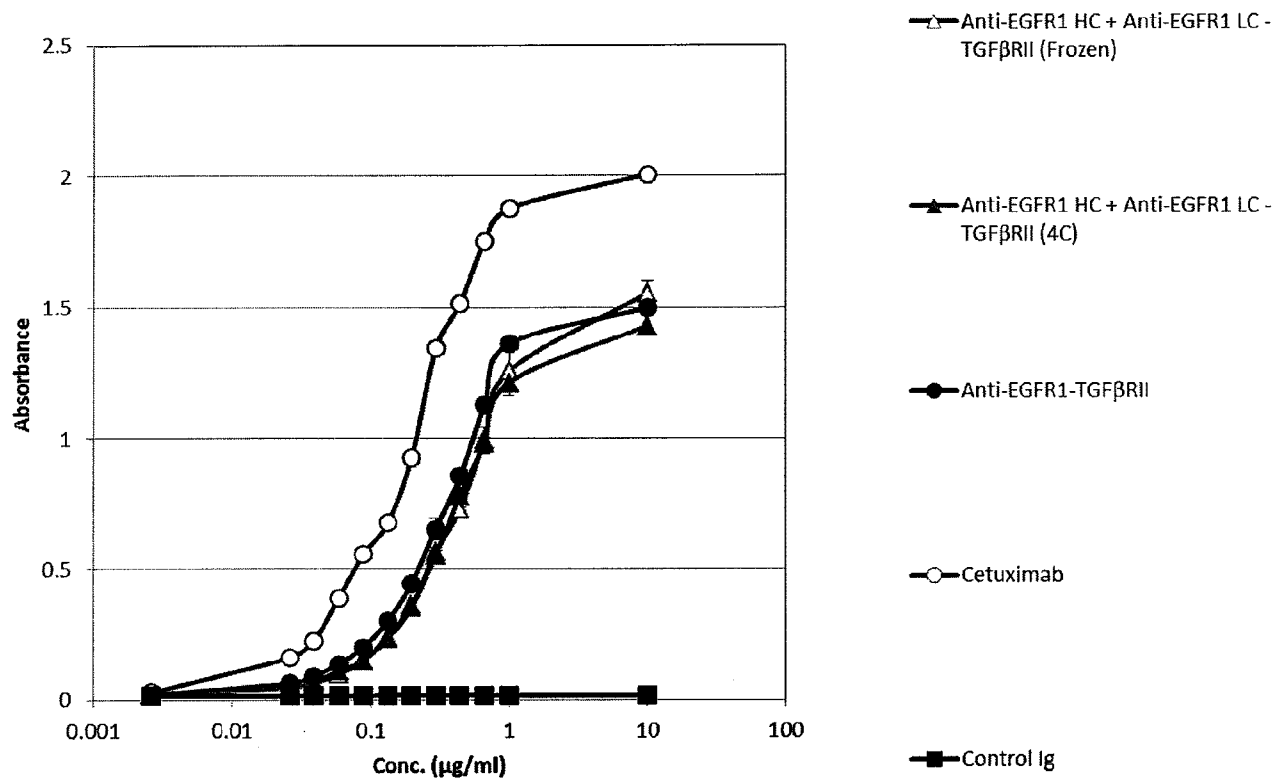
40/66

**Figure 38**

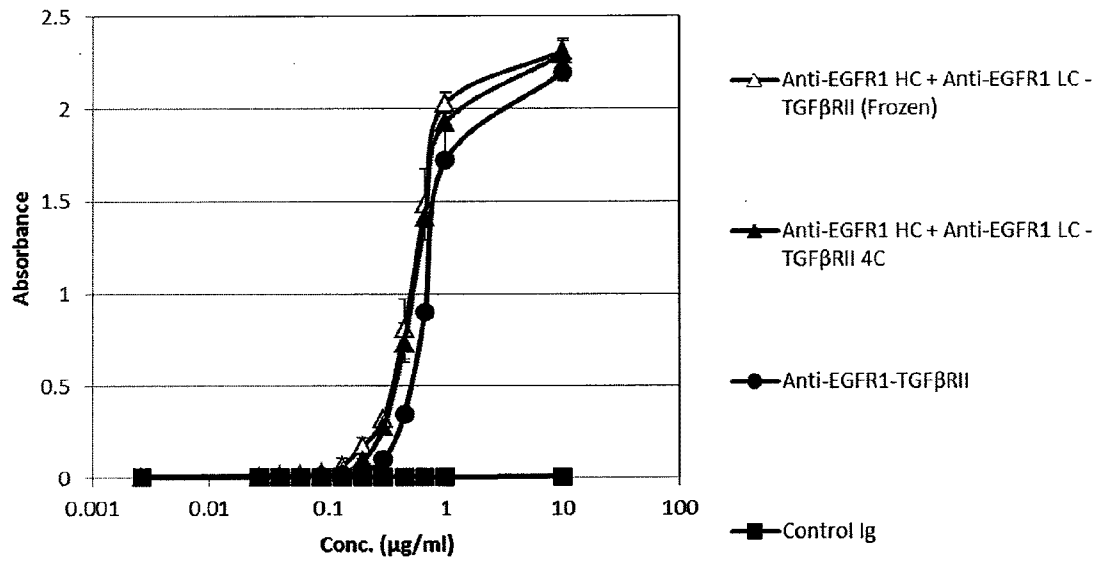
41/66

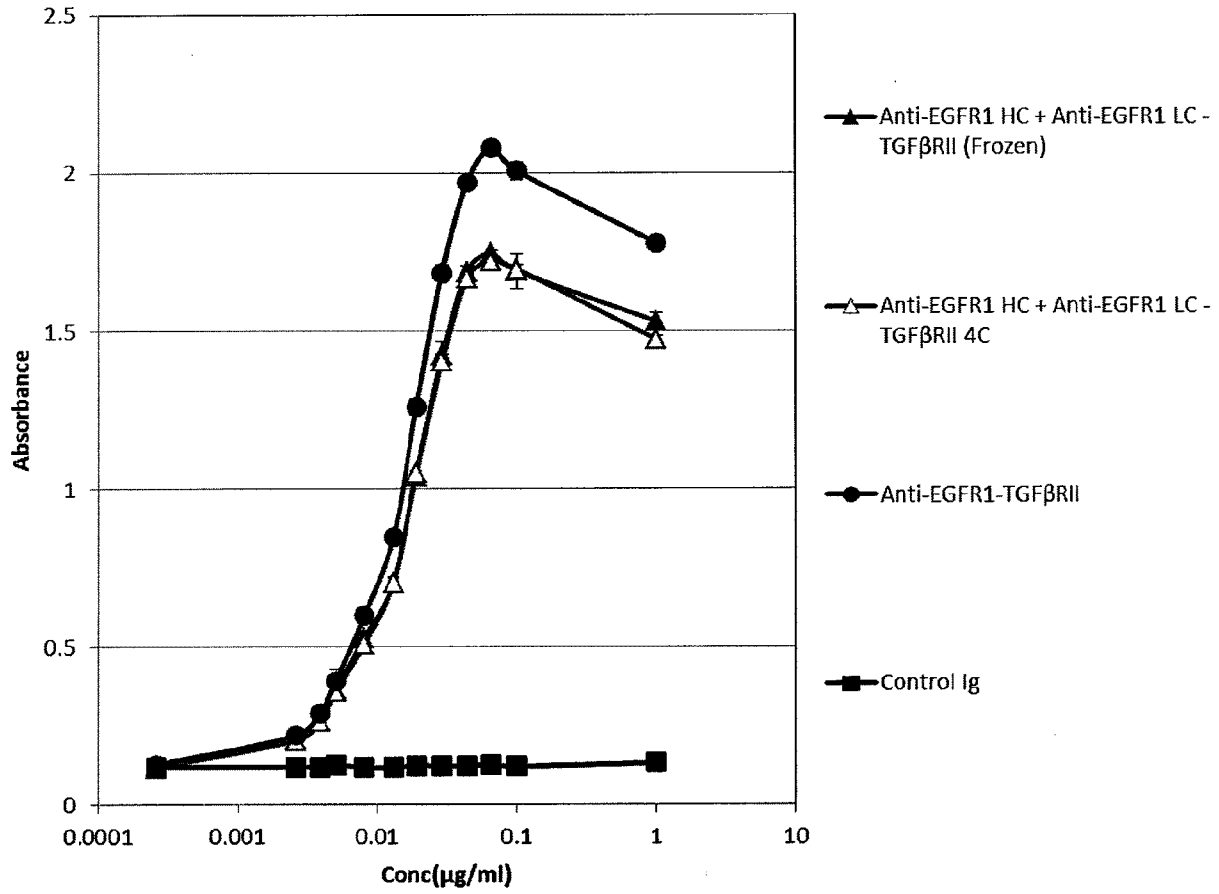
**Figure 39**

42/66

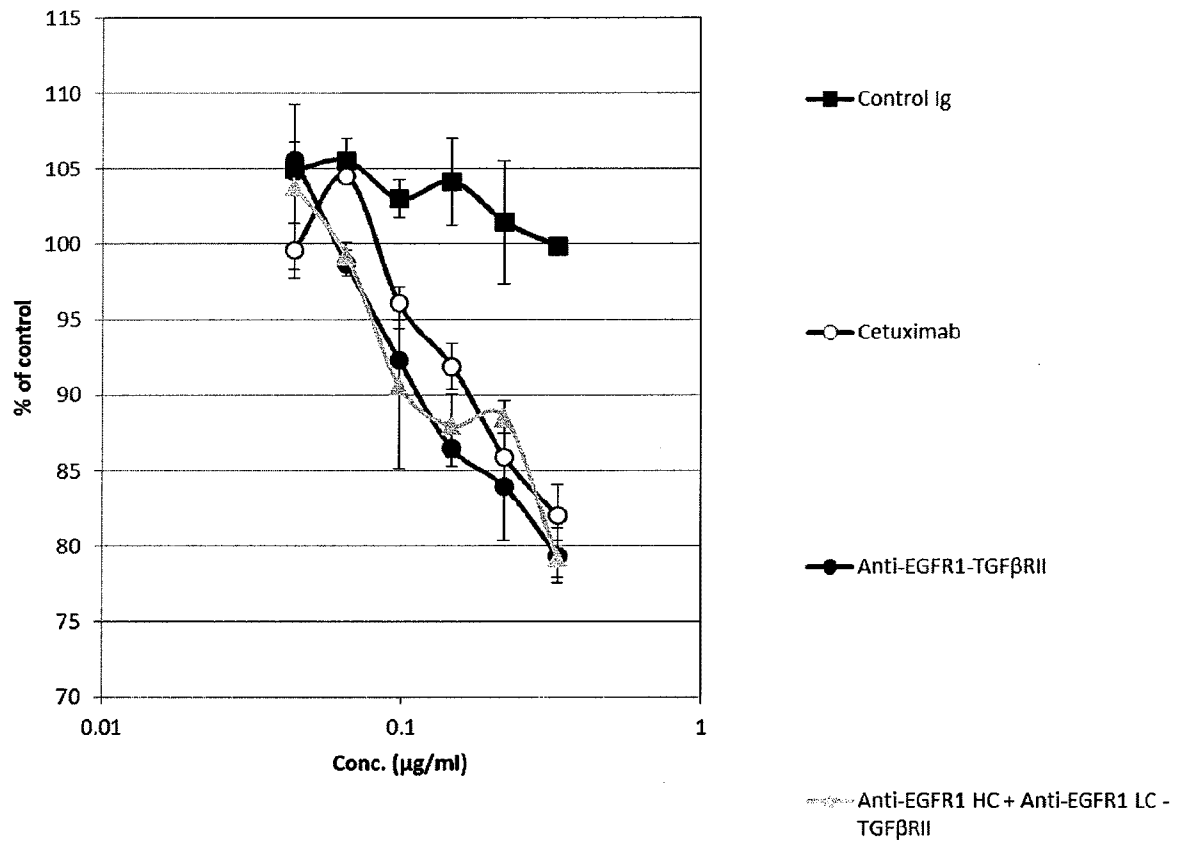
**Figure 40**

43/66

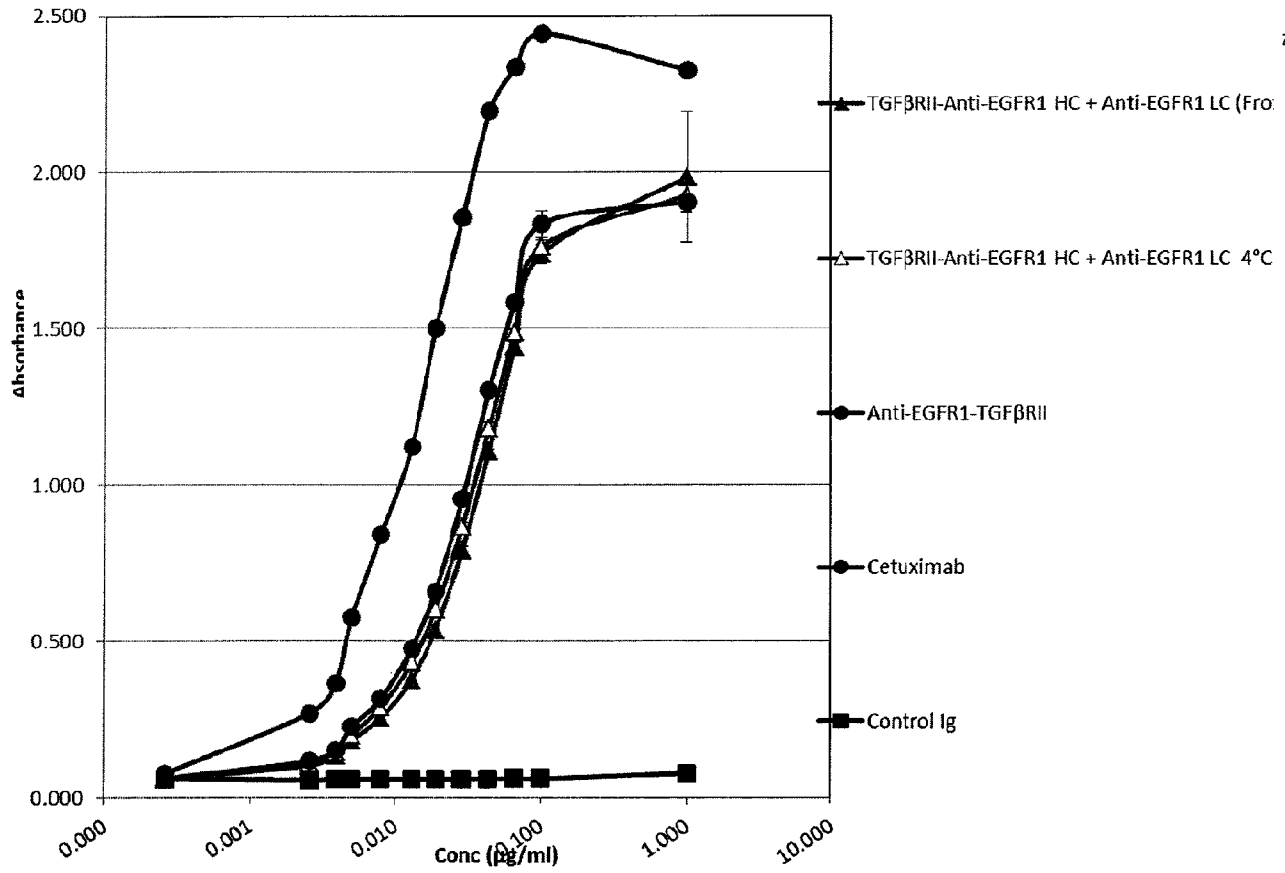
**Figure 41**

**Figure 42**

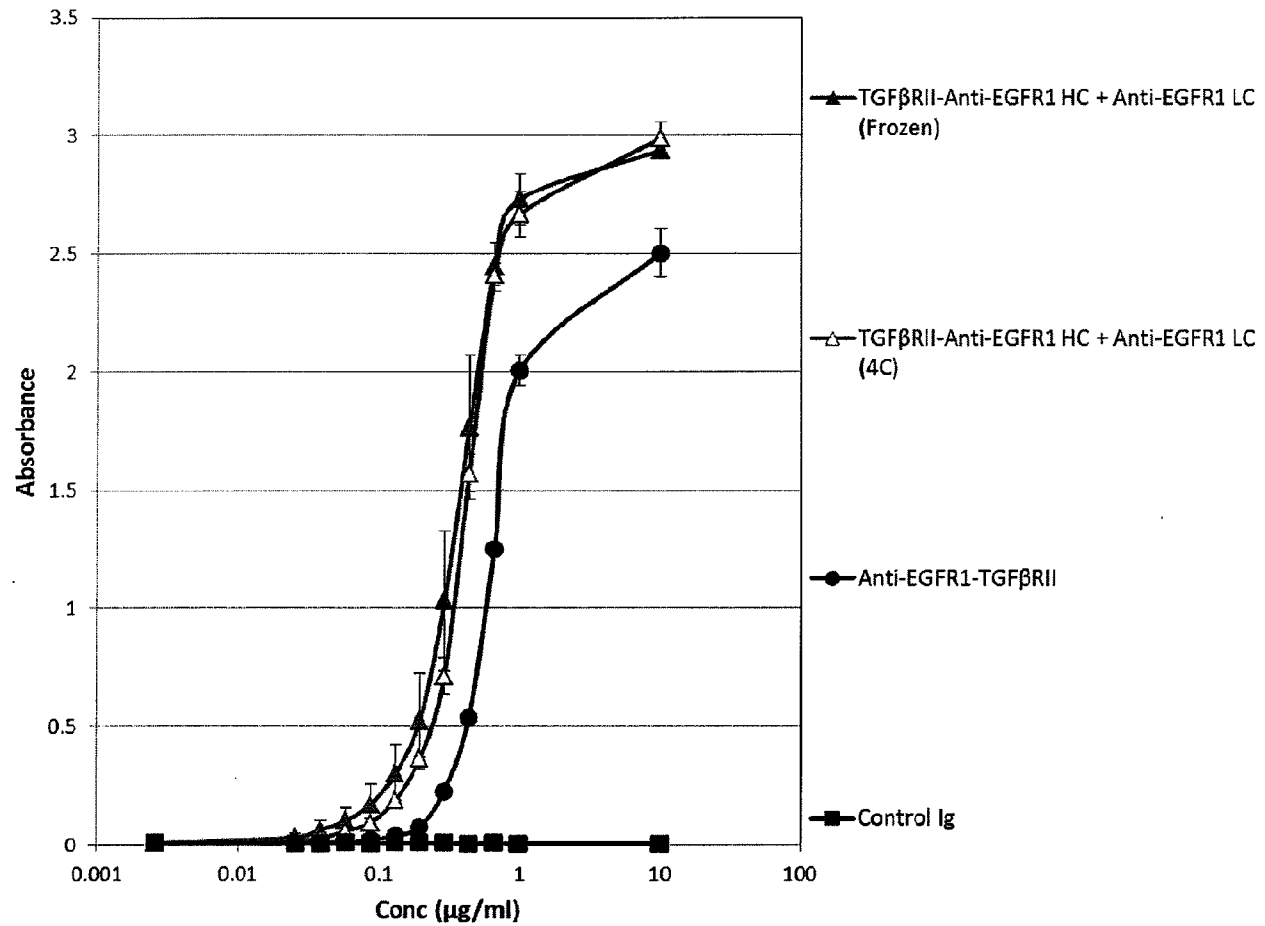
45/66

**Figure 43**

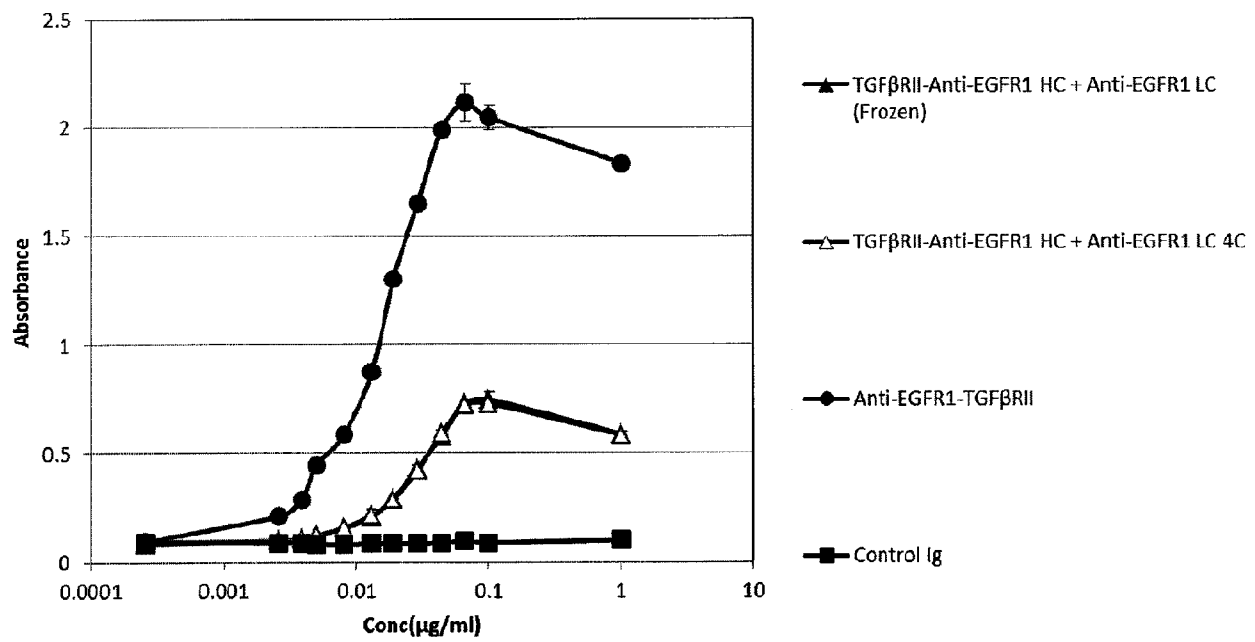
46/66

**Figure 44**

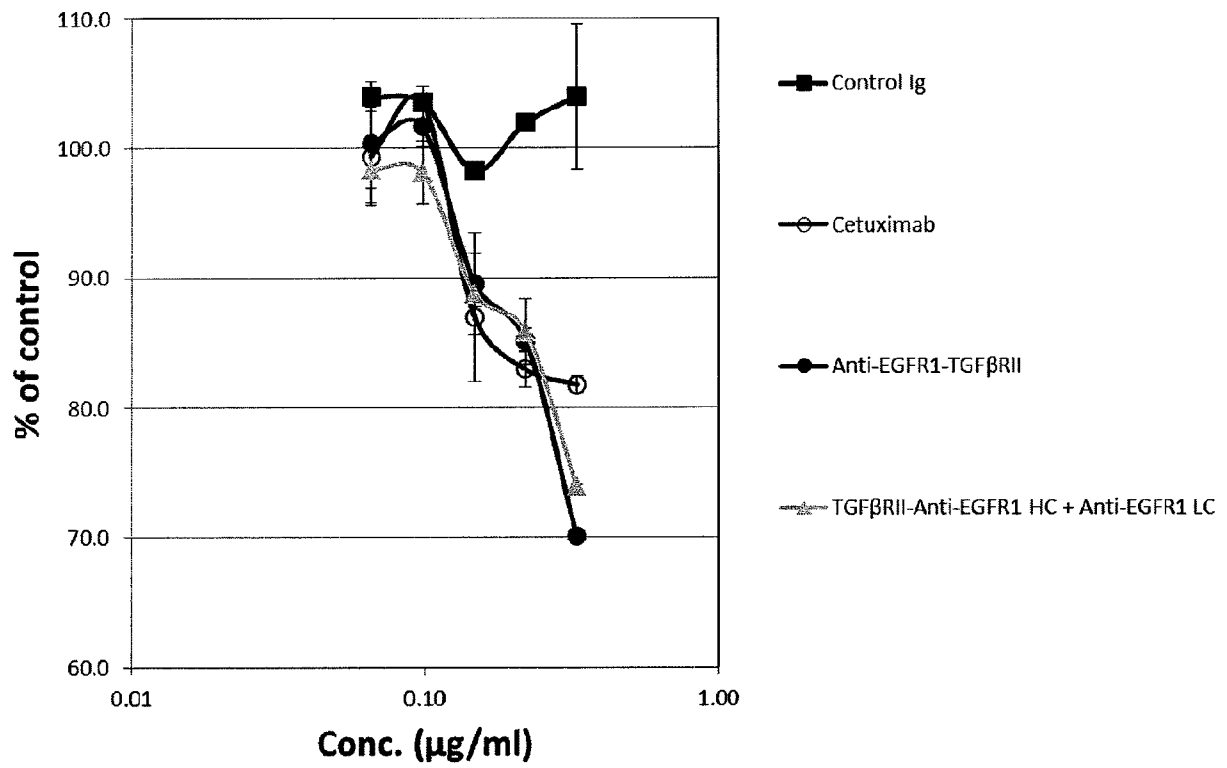
47/66

**Figure 45**

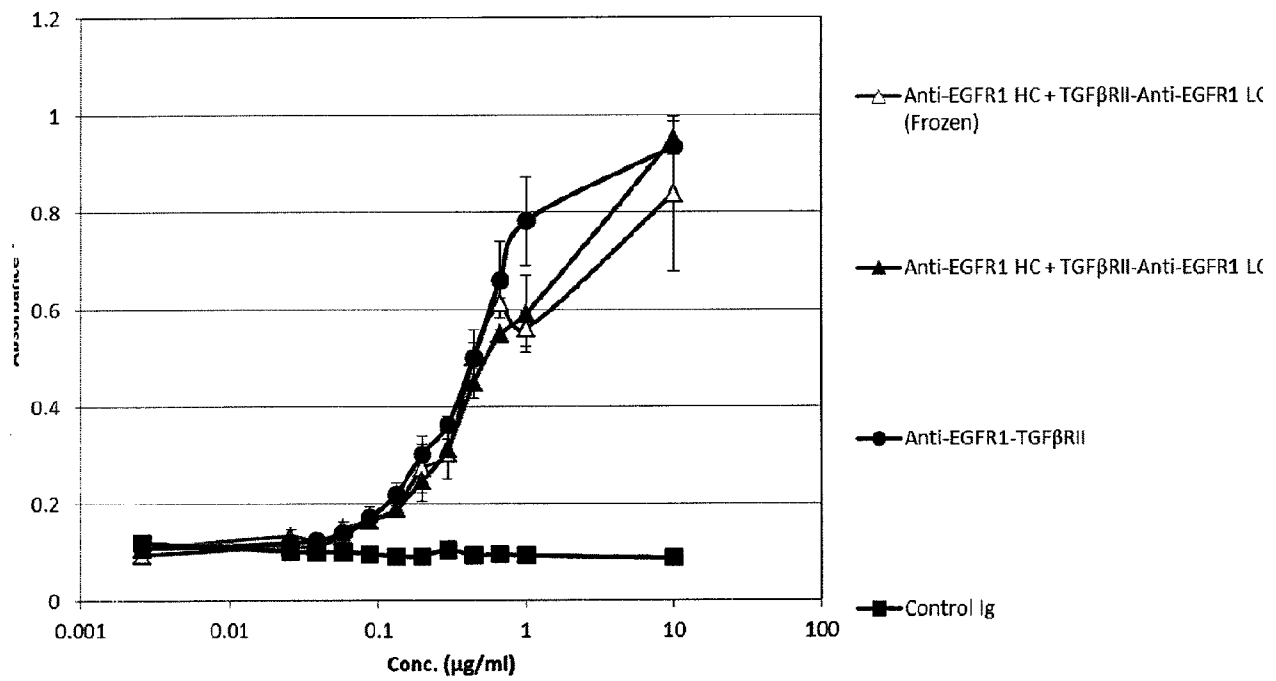
48/66

**Figure 46**

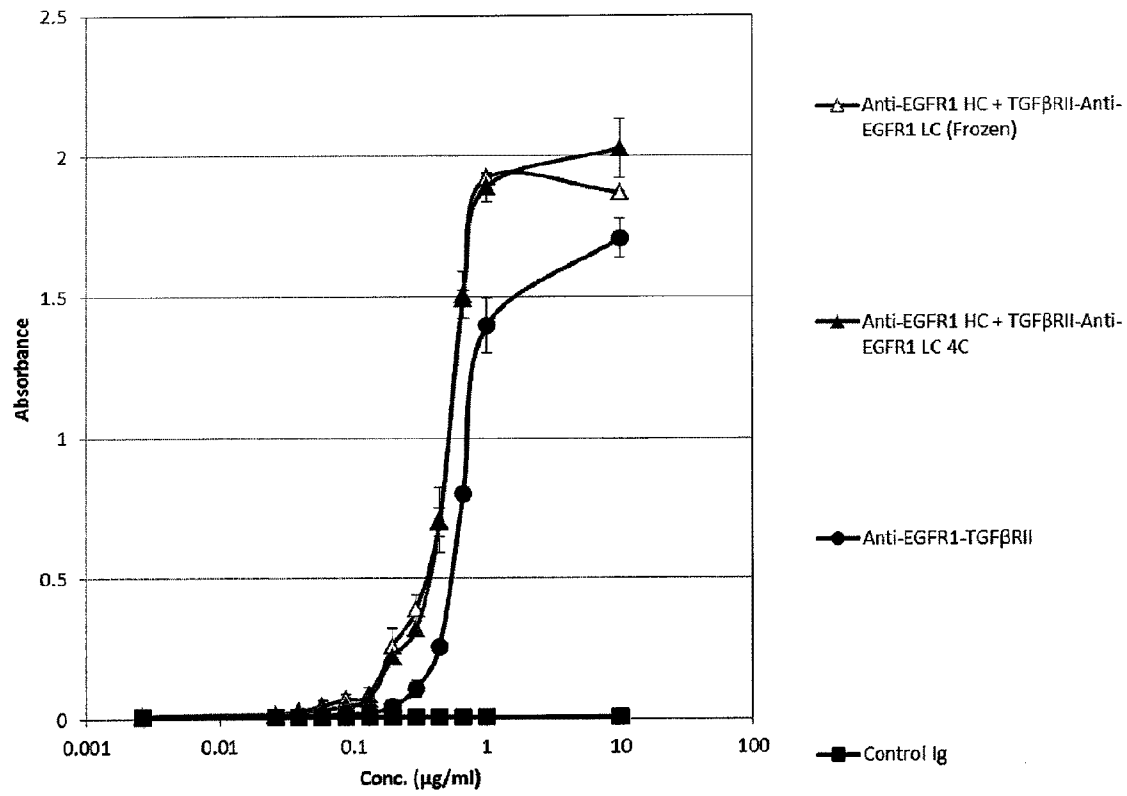
49/66

**Figure 47**

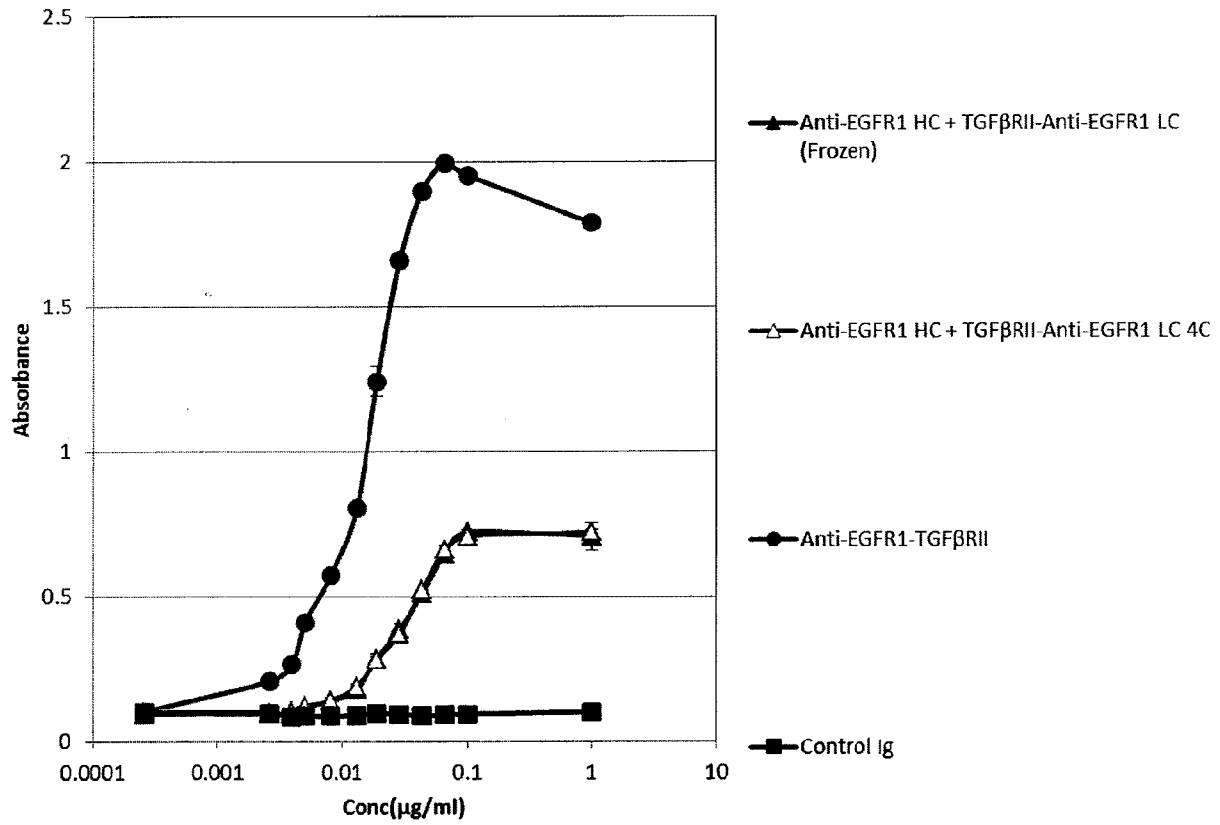
50/66

**Figure 48**

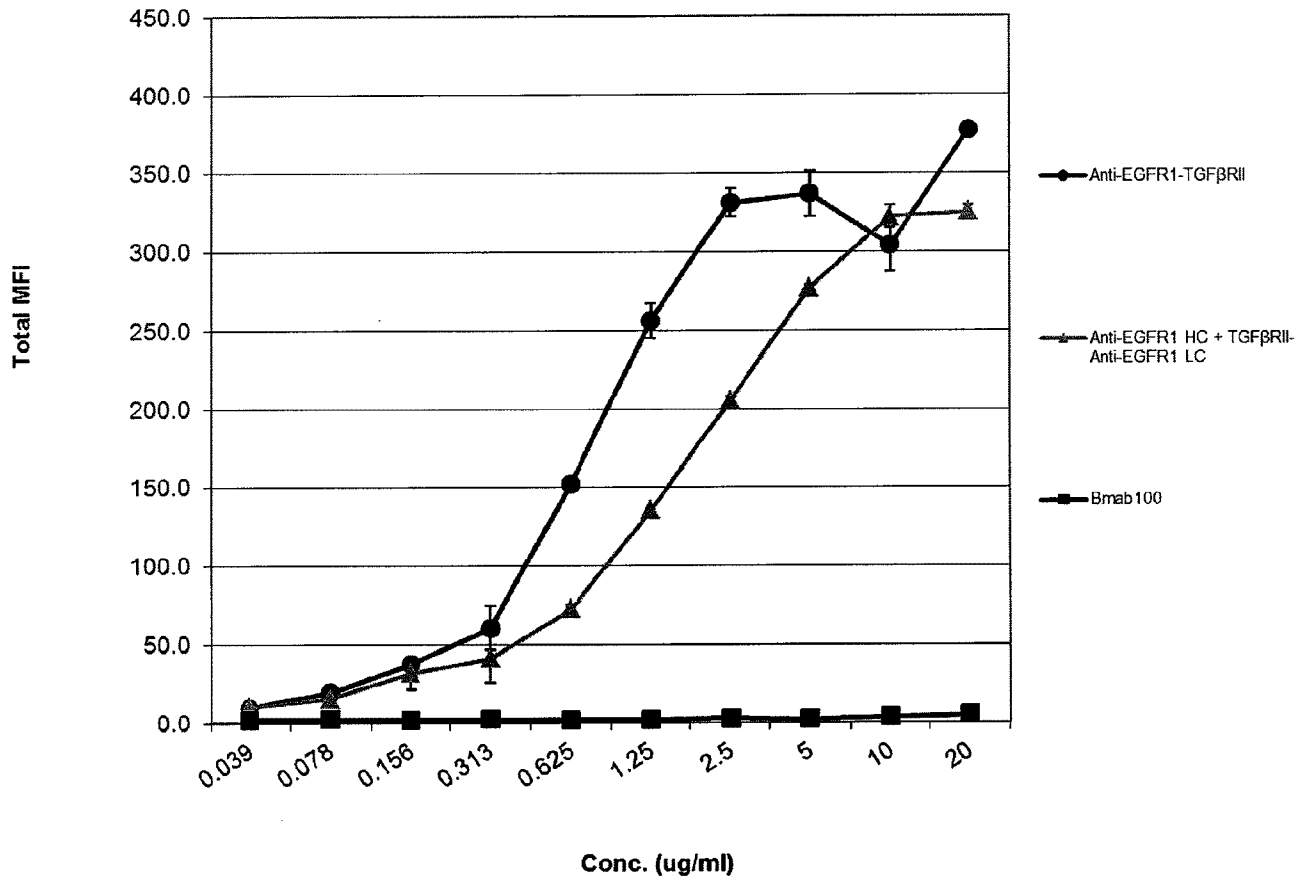
51/66

**Figure 49**

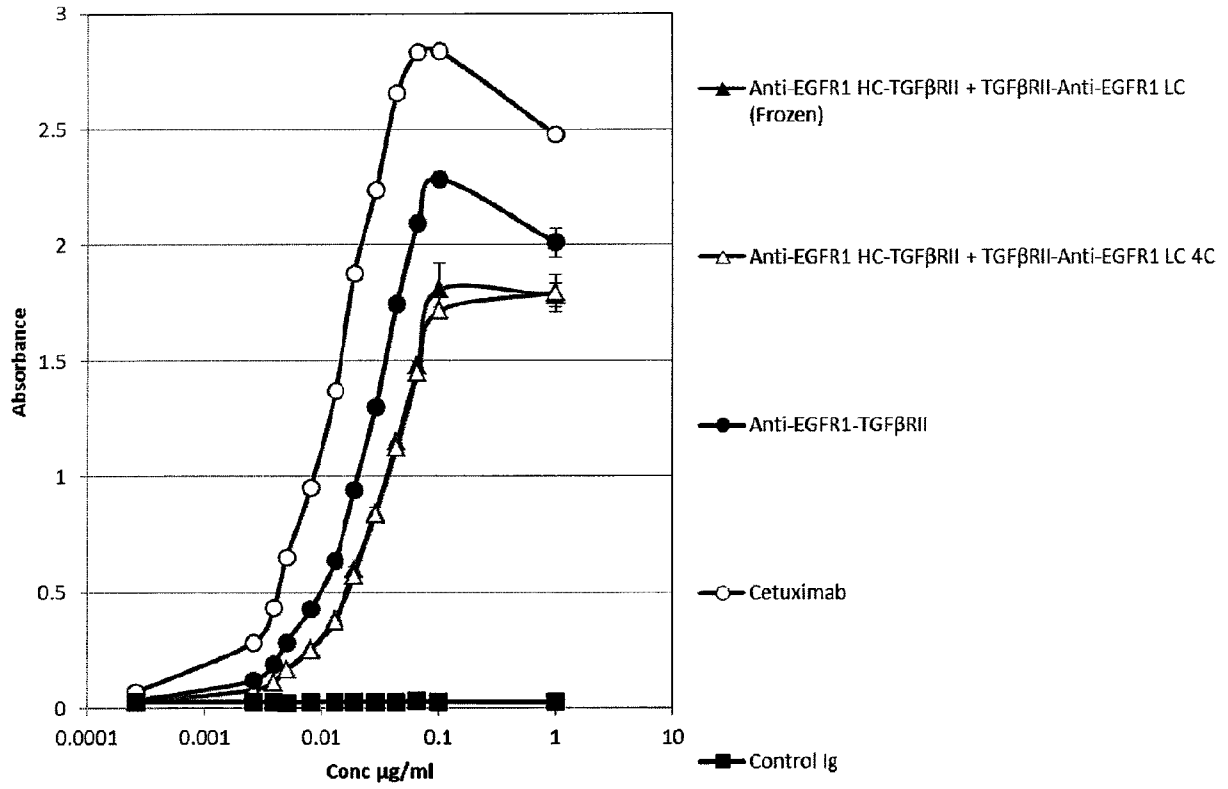
52/66

**Figure 50**

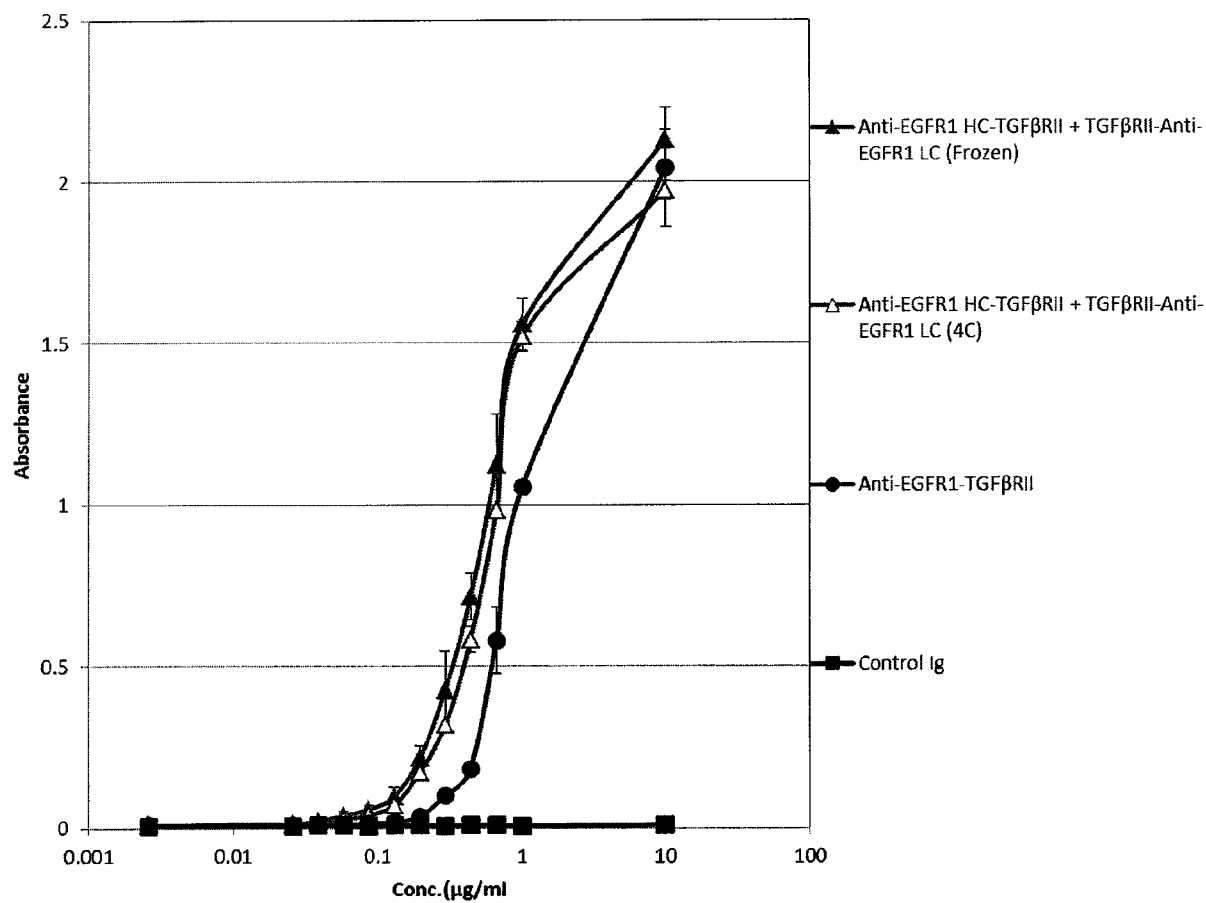
53/66

**Figure 51**

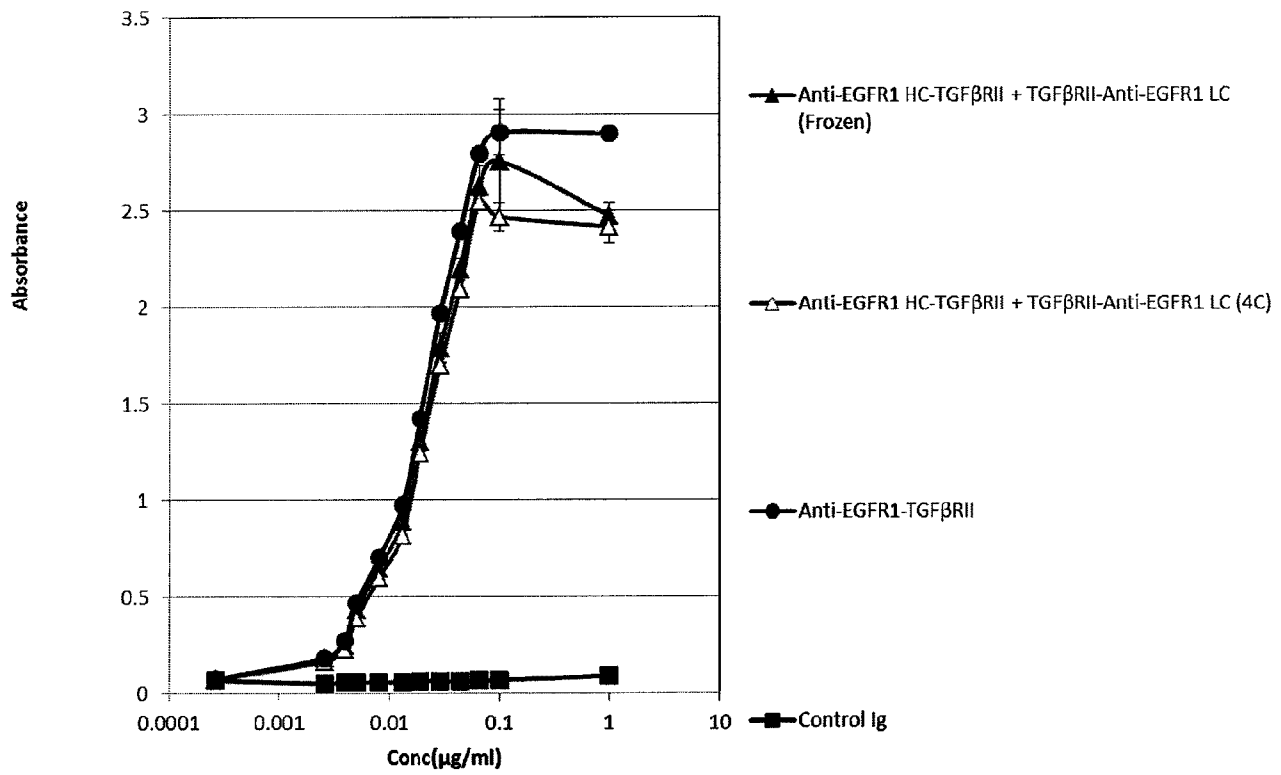
54/66

**Figure 52**

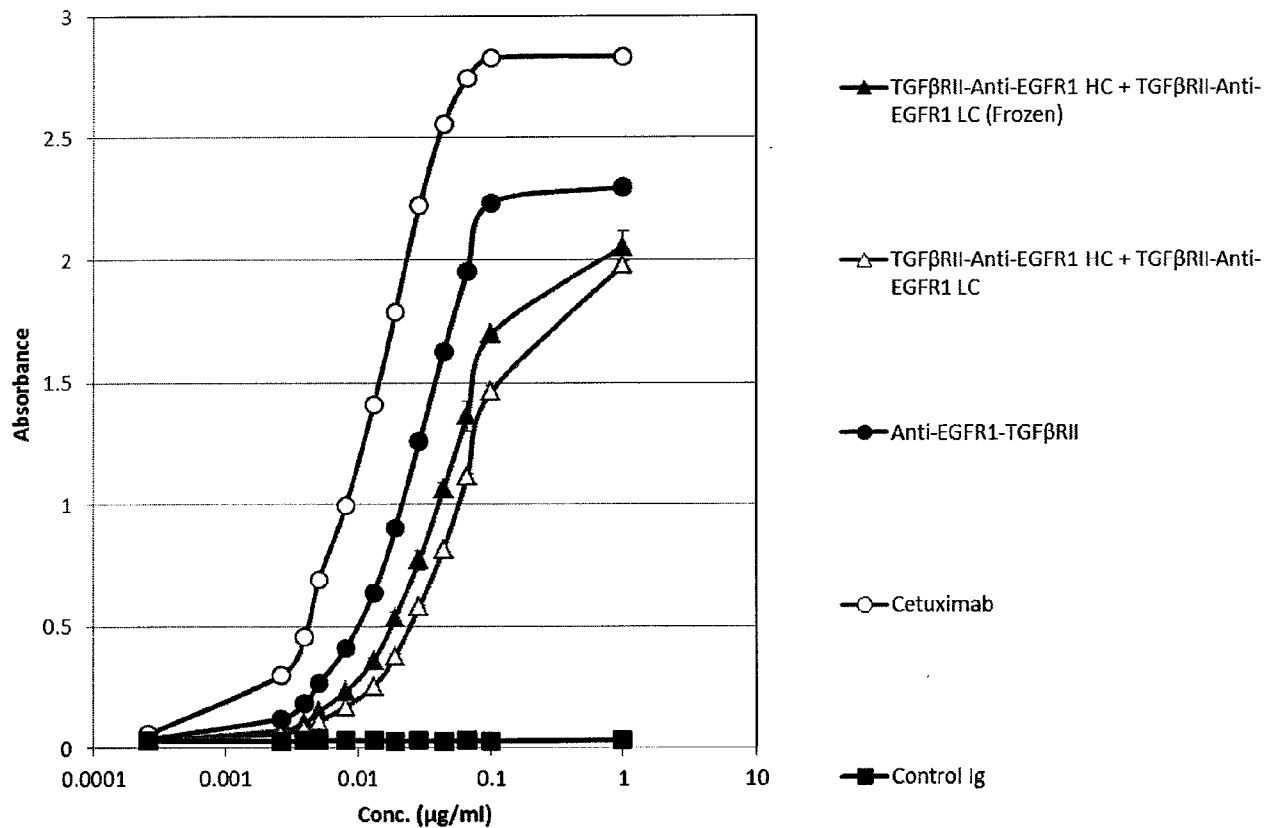
55/66

**Figure 53**

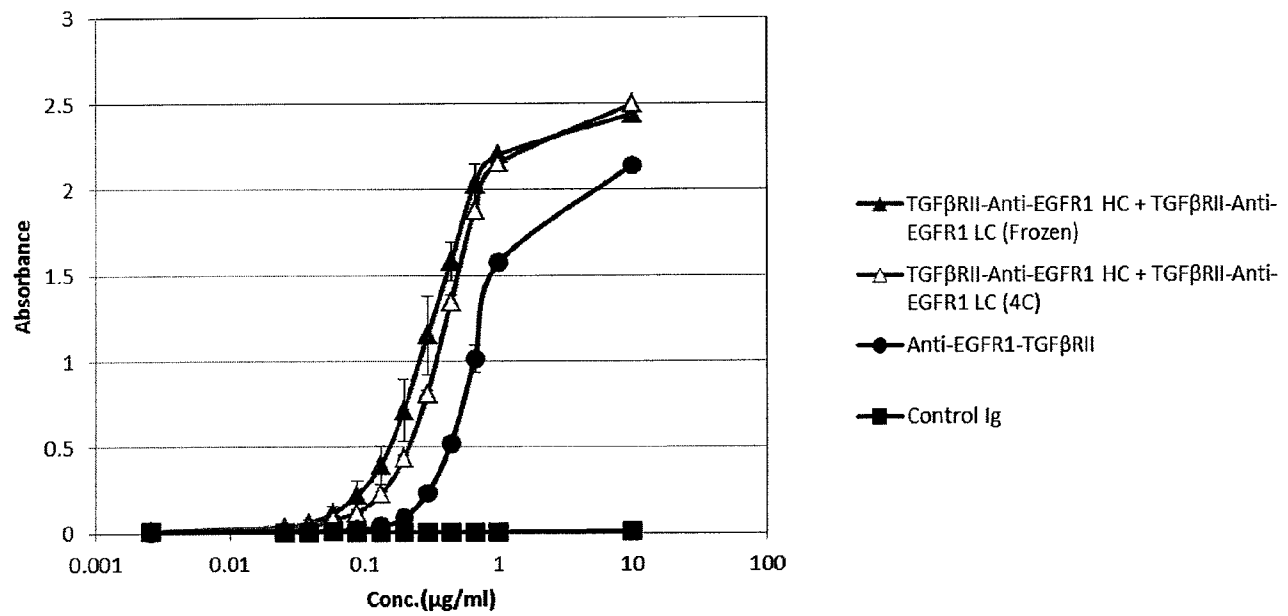
56/66

**Figure 54**

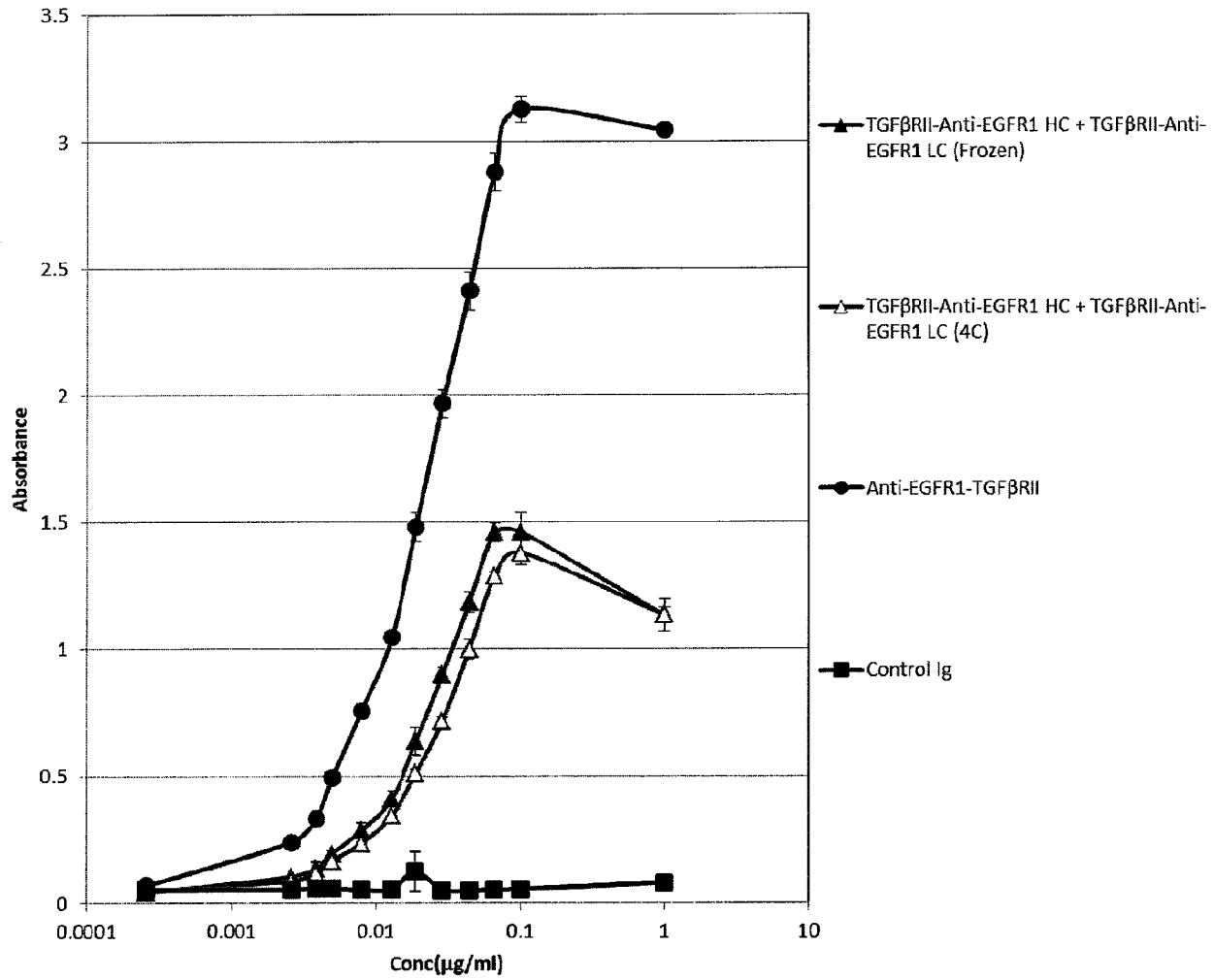
57/66

**Figure 55**

58/66

**Figure 56**

59/66

**Figure 57**

60/66

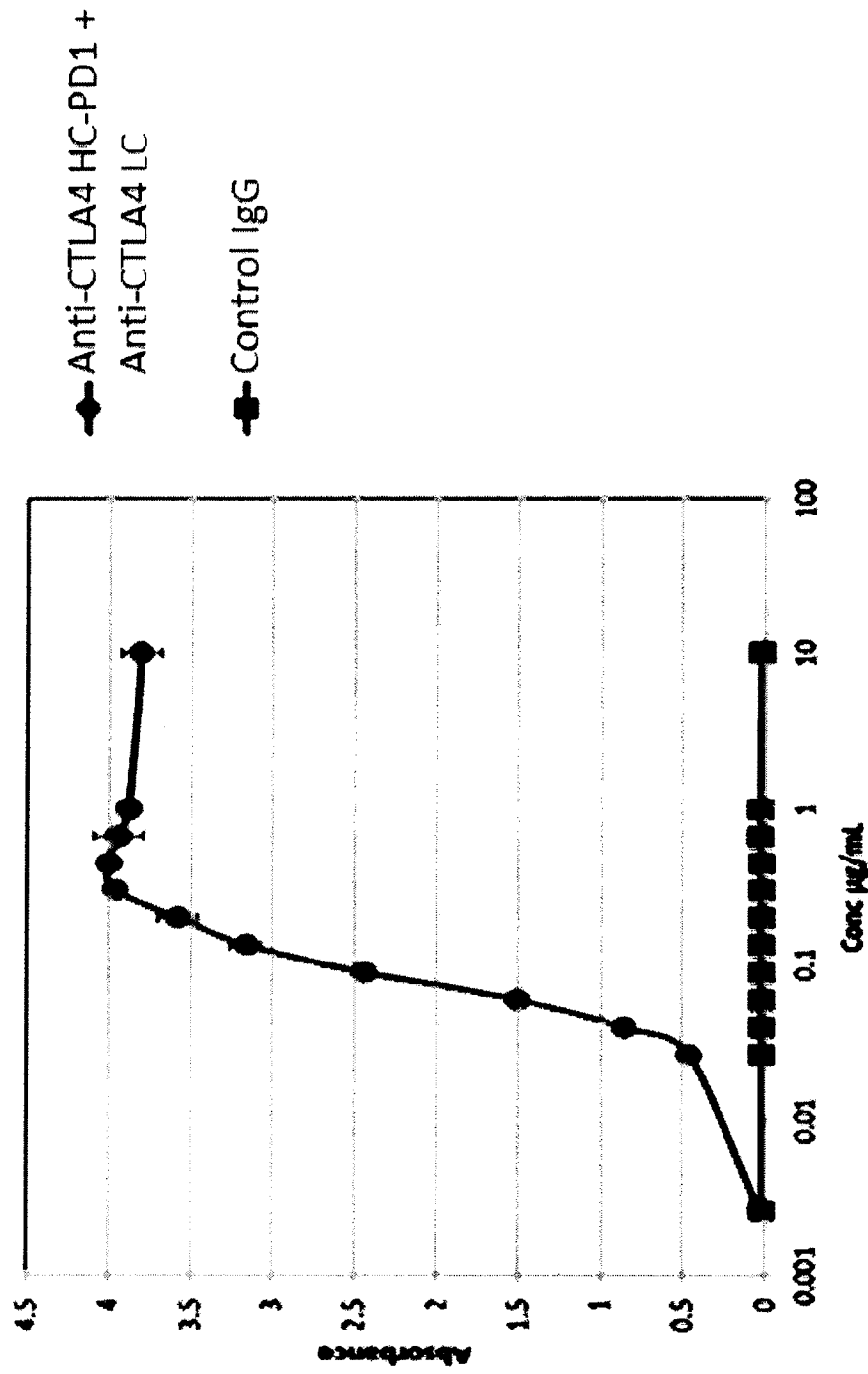


Figure 58

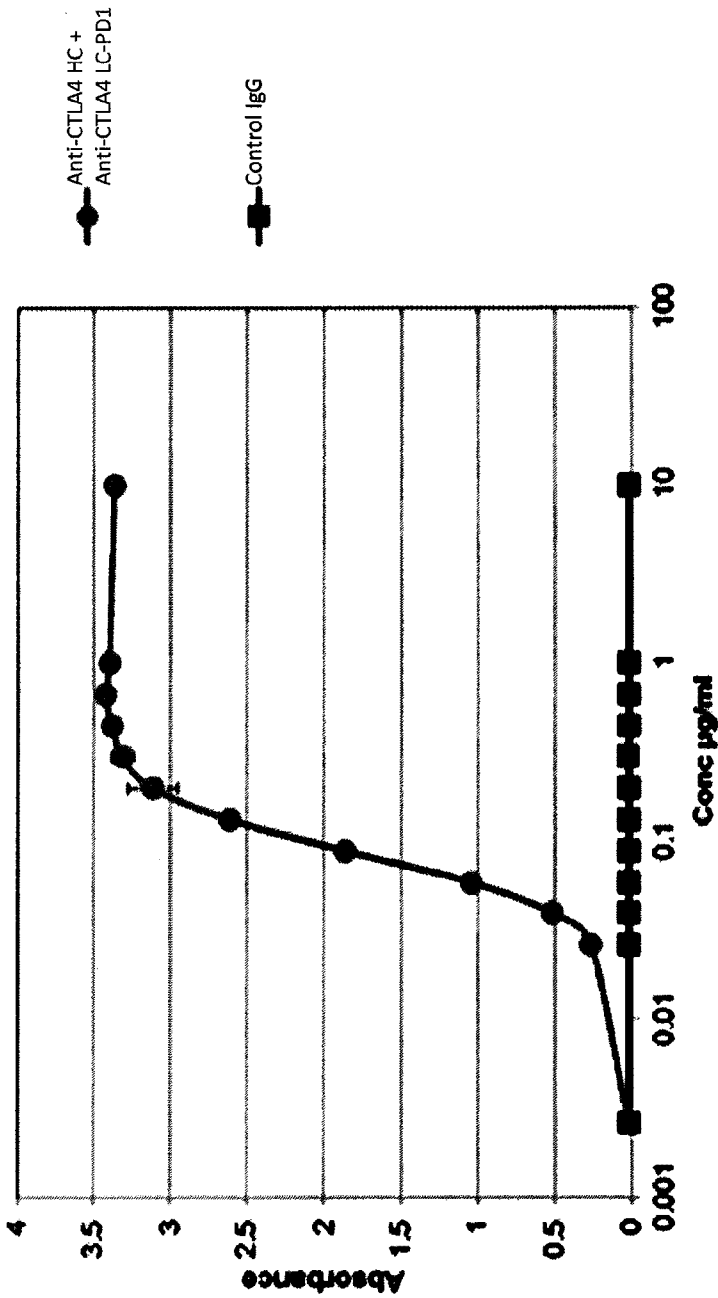


Figure 59

62/66

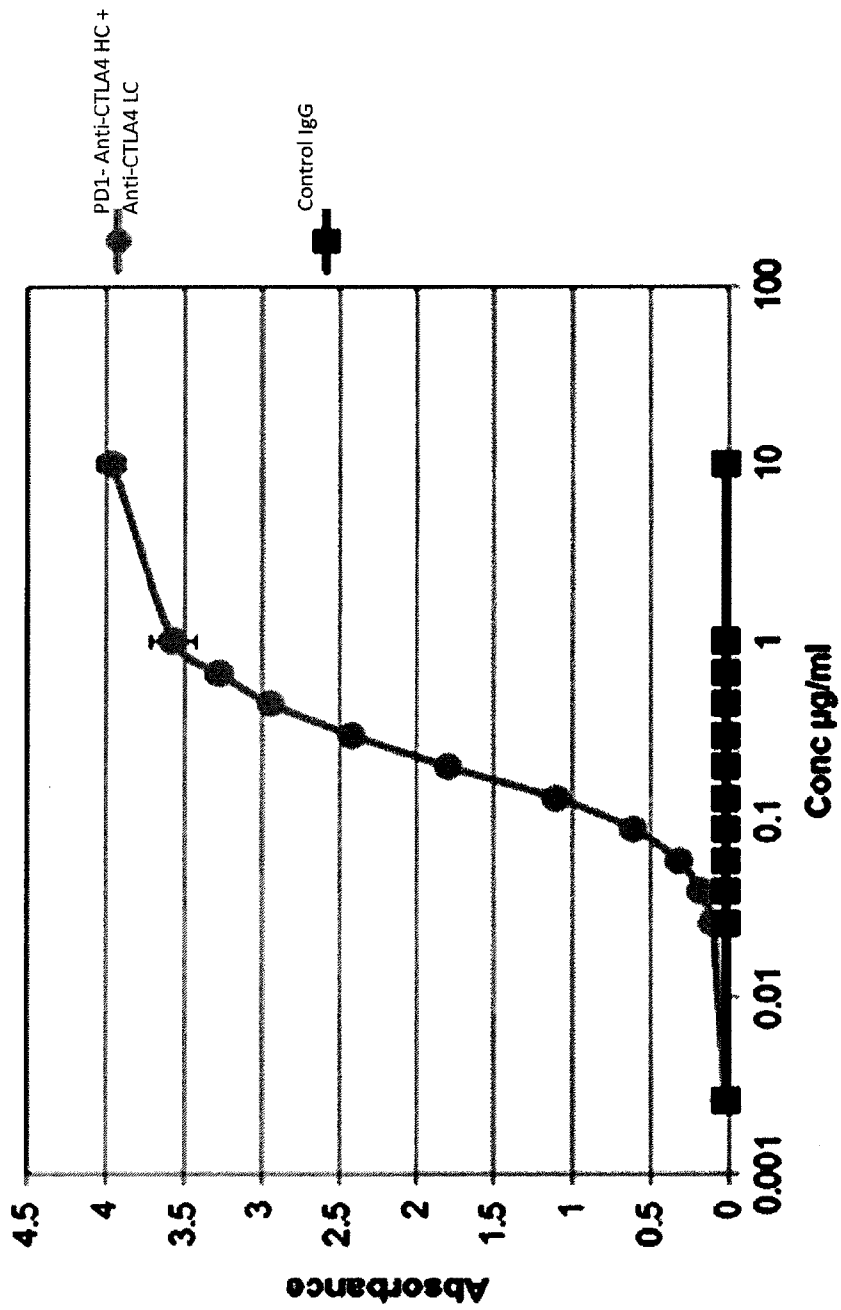


Figure 60

63/66

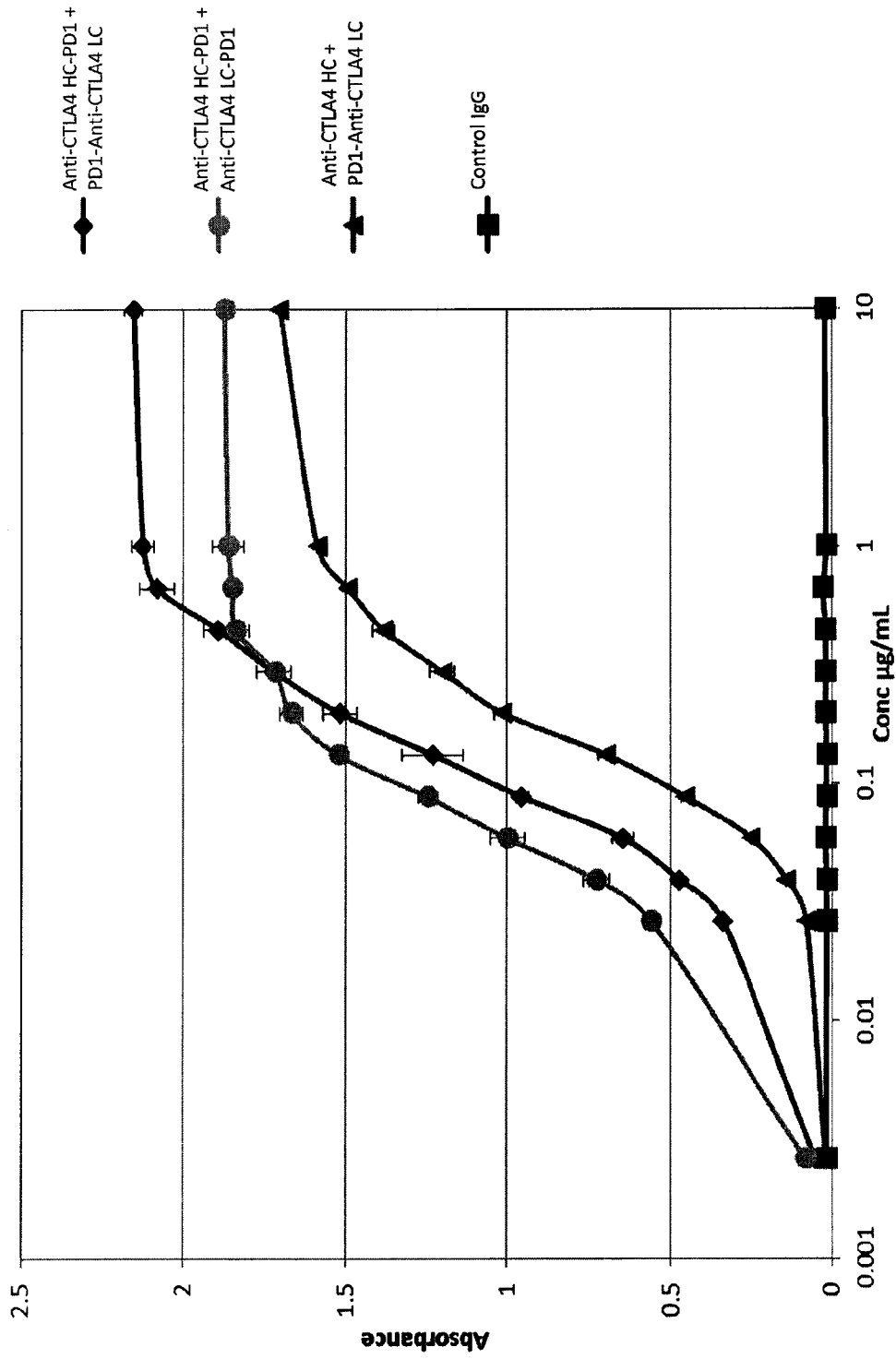


Figure 61

64/66

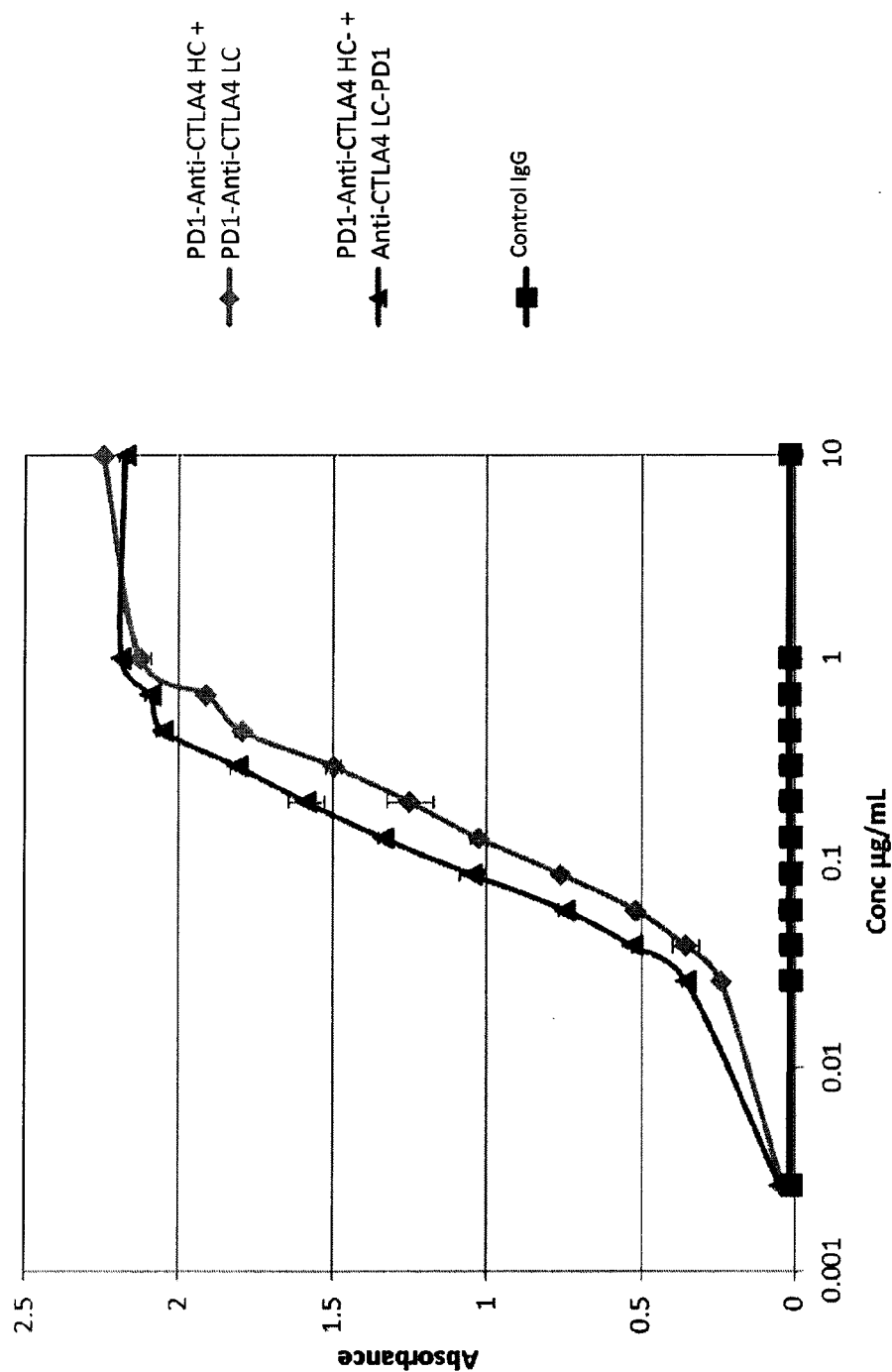


Figure 62

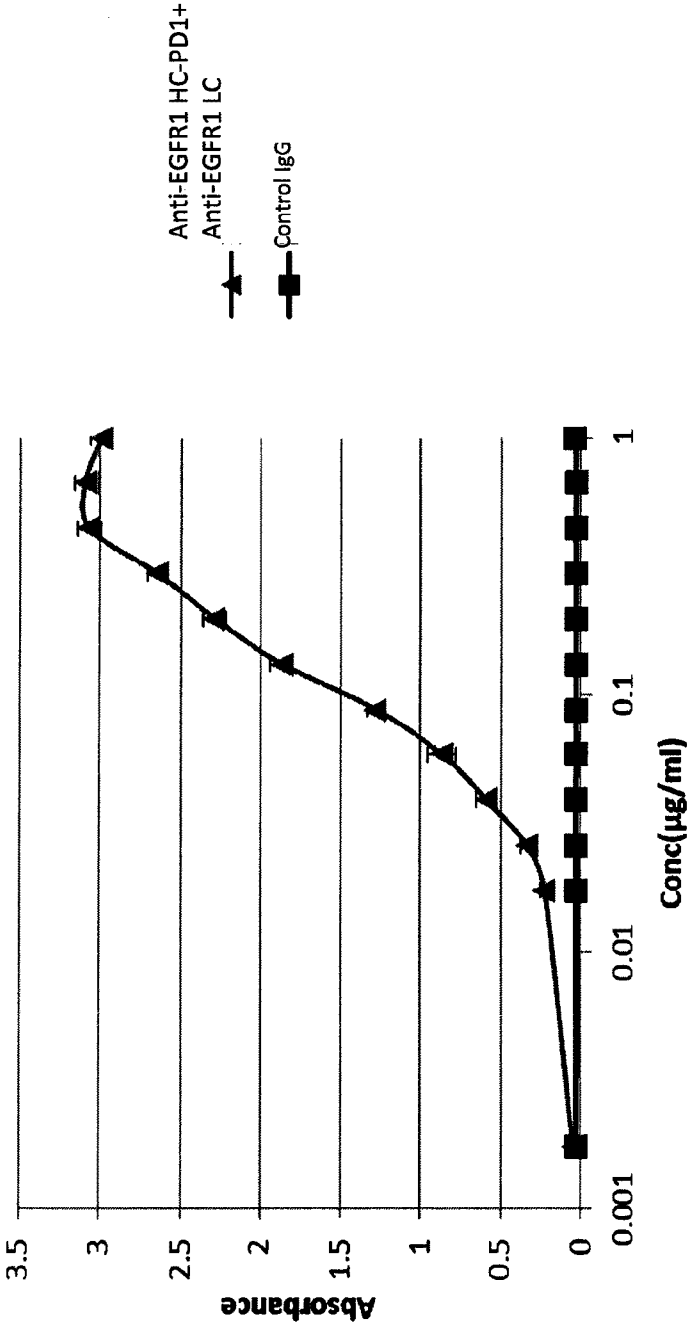


Figure 63

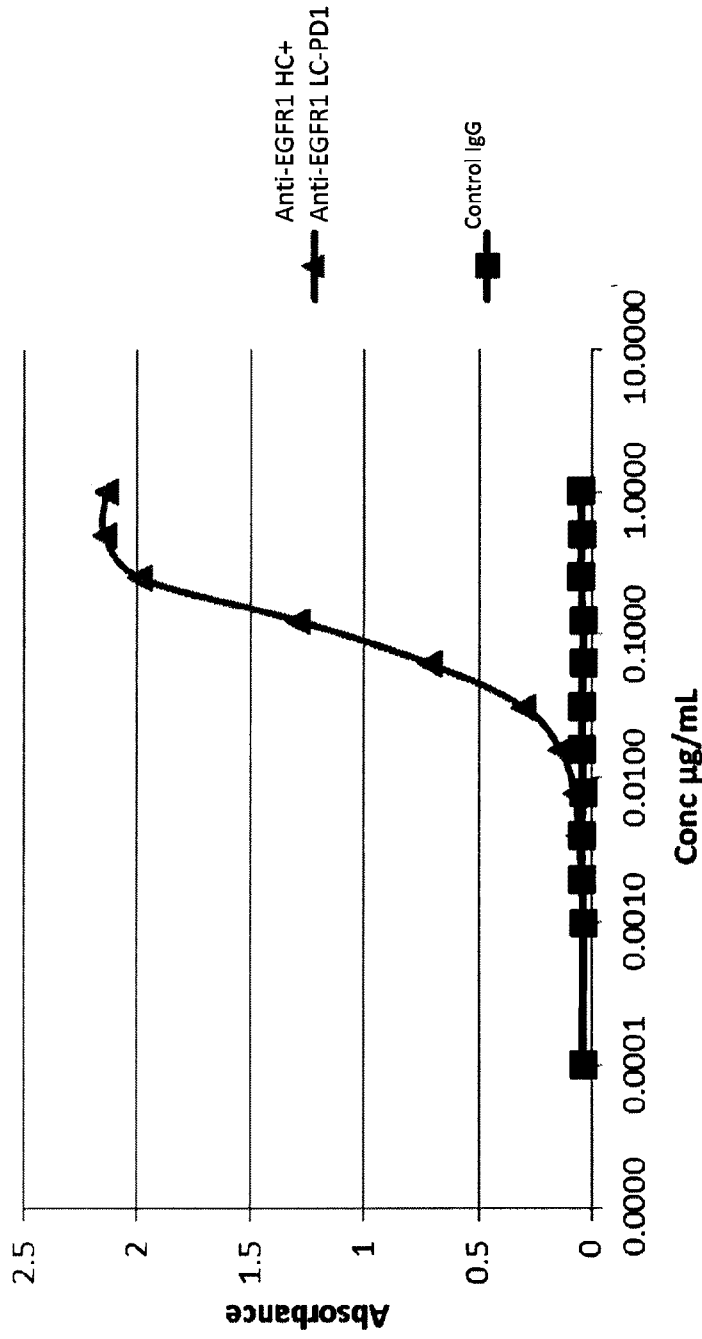
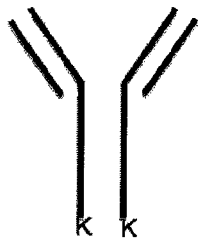
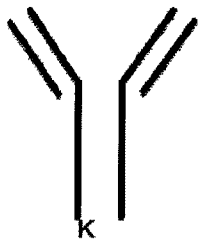


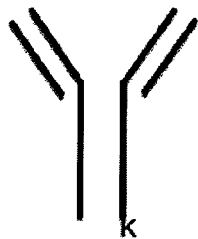
Figure 64



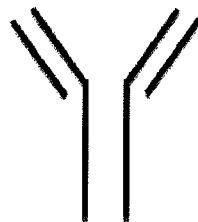
1. Mab designed/secreted



2. Mab secreted



3. Mab secreted



4. Mab commercial