



(11) **EP 1 831 257 B9**

(12) **CORRECTED EUROPEAN PATENT SPECIFICATION**

(15) Correction information:  
**Corrected version no 1 (W1 B1)**  
**Corrections, see**  
**Description Paragraph(s) 78**

(48) Corrigendum issued on:  
**04.05.2011 Bulletin 2011/18**

(45) Date of publication and mention  
of the grant of the patent:  
**23.02.2011 Bulletin 2011/08**

(21) Application number: **05823880.9**

(22) Date of filing: **29.12.2005**

(51) Int Cl.:  
**C07K 16/24** <sup>(2006.01)</sup> **A61K 39/395** <sup>(2006.01)</sup>  
**A61P 29/00** <sup>(2006.01)</sup>

(86) International application number:  
**PCT/KR2005/004634**

(87) International publication number:  
**WO 2006/071091 (06.07.2006 Gazette 2006/27)**

(54) **HUMANIZED ANTIBODY SPECIFIC FOR TUMOR NECROSIS FACTOR-ALPHA**  
TUMORNEKROSEFAKTOR-ALPHA SPEZIFISCHE HUMANISIERTE ANTIKÖRPER  
ANTICORPS HUMANISE SPECIFIQUE POUR LE FACTEUR ALPHA DE LA NECROSE TUMORALE

(84) Designated Contracting States:  
**AT BE BG CH CY CZ DE DK EE ES FI FR GB GR**  
**HU IE IS IT LI LT LU LV MC NL PL PT RO SE SI**  
**SK TR**

(30) Priority: **29.12.2004 KR 20040115709**

(43) Date of publication of application:  
**12.09.2007 Bulletin 2007/37**

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Remarks:

The file contains technical information submitted after the application was filed and not included in this specification

**Description**FIELD OF THE INVENTION

5     **[0001]** The present invention relates to a humanized antibody specific for human tumor necrosis factor- $\alpha$  defined in claim 1.

BACKGROUND OF THE INVENTION

10    **[0002]** Human tumor necrosis factor- $\alpha$  (hereinafter, referred to as "hTNF- $\alpha$ ") is a homotrimer consisting of three 17 kDa protein subunits (Eck M. J. et al., JBC, 267: 2119-2122, 1992; Smith R. A. et al., JBC, 262: 6951-6954, 1987). HTNF- $\alpha$  is an inflammatory cytokine secreted from macrophages and monocytes, and functions as a signal transmitter in several cellular reactions such as necrosis and apoptosis (Beyaert R. et al., FEBS Lett., 340: 9-16, 1994). hTNF- $\alpha$  causes a pro-inflammatory action leading to tissue destruction, such as breakdown of the cartilage and bone (Saklatvala, Nature, 322: 547-549, 1986), induction of an adhesion molecules, induction of procoagulation activity in vascular endothelial cells (Pober JS et al., J. Immunol., 136: 1680-1687, 1986), and increase in the adherence of neutrophils and lymphocytes (Pober et al., J. Immunol. 138: 3319-3324, 1987). In addition, it has been known that hTNF- $\alpha$  plays an important role in at defense mechanism against infectious disease and tumor (Fiers W., FEBS Lett., 285: 199-212, 1991).

15    **[0003]** hTNF- $\alpha$  is involved in inflammatory diseases, autoimmune diseases, bacterial infections, cancers and degenerative diseases. Among these diseases, hTNF- $\alpha$  has been regarded as a useful target protein for a specific physiological treatment of rheumatoid arthritis, Crohn's disease, psoriatic arthritis, and ankylosing spondylitis.

20    **[0004]** Meanwhile, it has been also suggested to use a hTNF- $\alpha$  inhibitor for the purpose of treating rheumatoid arthritis. It has been reported that hTNF- $\alpha$  is overexpressed in the synovial cells isolated from the early-stage rheumatoid joint (Buchan G. et al., Clin. Exp. Immunol., 73: 449-455, 1988), and cytokines relating to rheumatoid arthritis lesions are decreased when the above synovial cells are treated with an anti-hTNF- $\alpha$  monoclonal antibody (Butler D. M. et al., Eur. Cytokine Netw., 6: 225-230, 1995). Further, it has been found that an anti-hTNF- $\alpha$  antibody or a recombinant soluble hTNF- $\alpha$  receptor suppresses inflammation and destruction of a joint in a collagen induced mouse arthritis model (Piguet P. F. et al., Immunology, 77: 510-514, 1992; Wooley P. H. et al., J Immunol., 151: 6602-6607, 1993; Williams R. O. et al., Immunology, 84: 433-439, 1995). Moreover, it has been observed that inflammatory arthritis is induced in a transgenic mouse overexpressing hTNF- $\alpha$  (Keffer J. et al., EMBO J., 10: 4025-4031, 1991).

25    **[0005]** These results indicate that hTNF- $\alpha$  plays an important role as a direct or indirect regulator controlling inflammatory cytokines in rheumatoid arthritis. Accordingly, there has been a need to develop a monoclonal antibody having high selectivity and reactivity to hTNF- $\alpha$  for the purpose of treating rheumatoid arthritis.

30    **[0006]** Generally, an antibody having high selectivity and reactivity to a specific antigen is prepared through immunization of a mouse with the antigen (Kohler G. & Milstein C., Nature, 256: 495-497, 1975). A mouse monoclonal antibody is advantageous in that it is easy to prepare the antibody and to select an antibody having a high reactivity. However, it also has a problem that an anti-mouse antibody is formed in a human body when it is administered for a long time (Dimaggio J. J., et al., Cancer Chemother. Biol. Response Modif., 11: 177-203, 1990).

35    **[0007]** To overcome the undesirable properties of mouse monoclonal antibodies, a humanized antibody has been developed by replacing the framework regions except for the antigen-binding site with those of a human antibody. As a method for preparing such humanized antibody, a CDR (complementarity determining region) grafting method is currently employed, wherein only the CDRs of a mouse antibody are grafted to a human antibody. The humanized antibody prepared by CDR grafting has an advantage of reducing *in vivo* immune responses (Riechmann et al., Nature, 332: 323, 1988; Nakatani et al., Protein Engineering, 7: 435, 1994), however, it often loses the high selectivity and reactivity of the original mouse antibody (Carter P., et al., Proc. Natl. Acad. Sci. USA, 89: 4285-4289, 1992).

40    **[0008]** This is effectively the case for the humanized anti-TNF $\alpha$  mouse monoclonal antibody described by Wang et al. which, when composed to the original anti human TNF $\alpha$  monoclonal antibody has reduced neutralizing capabilities (Wang et al, Journal of immunological methods, 171-184, 2000)

45    **[0009]** The present inventors have endeavored to overcome such problems of the conventional humanized antibody, and developed a novel humanized antibody specifically binding to hTNF- $\alpha$  which shows an antigen binding affinity similar to that of the original mouse monoclonal antibody and minimized immunogenicity.

SUMMARY OF THE INVENTION

50    **[0010]** Accordingly, the present invention provides:

1. A humanized antibody specific for human tumor necrosis factor- $\alpha$  (hTNF- $\alpha$ ), comprising: a) a heavy chain variable region having the amino acid sequence of SEQ ID NO: 36, 37 or 38; b) a light chain variable region having the amino

acid sequence of SEQ ID NO: 39 or 40; c) a heavy chain constant region identical to that of a human antibody; and d) a light chain constant region identical to that of a human antibody.

2. The humanized antibody of 1, wherein the antigen binding affinity ( $K_d$ ) of the antibody ranges from  $1 \times 10^{-9}$  M to  $1 \times 10^{-13}$  M.

3. The humanized antibody of 1, wherein the dissociation constant ( $K_{off}$ ) of the antibody ranges from  $1 \times 10^{-6}$  s $^{-1}$  to  $1 \times 10^{-9}$  s $^{-1}$ , which is determined by surface plasmon resonance (SPR).

4. The humanized antibody of 1, wherein the heavy chain variable region of the antibody is encoded by a DNA having a nucleotide sequence of SEQ ID NO: 31, 32 or 33.

5. The humanized antibody of 1, wherein the light chain variable region of the antibody is encoded by a DNA having a nucleotide sequence of SEQ ID NO: 34 or 35.

6. An expression vector for the humanized antibody specific for hTNF- $\alpha$  of 1, which comprises a DNA encoding a heavy chain variable region having an amino acid sequence of SEQ ID NO: 36, 37 or 38 and a DNA encoding a light chain variable region having an amino acid sequence of SEQ ID NO: 39 or 40.

7. A pharmaceutical composition comprising the humanized antibody of any one of 1 to 5, for treating a hTNF- $\alpha$  related disease selected from the group consisting of: rheumatoid arthritis, Crohn's disease, psoriatic arthritis, psoriasis, septicemia, asthma, Wegener's granulomatosis, inflammation, and ankylosing spondylitis.

#### BRIEF DESCRIPTION OF THE DRAWING

**[0011]** The above and other objects and features of the present invention will become apparent from the following description of the invention, when taken in conjunction with the accompanying drawings, which respectively show:

Fig. 1: Comparison of the amino acid sequences of the heavy chain (TSK114 H) variable region of a mouse monoclonal antibody specifically binding to TNF- $\alpha$  (SEQ ID NO: 47), heavy chain subgroup 1 (Hh1) of a human antibody (SEQ ID NO: 48), and the heavy chains (TNHV2, TNHV2k and TNHV1k) of the humanized antibodies of the present invention (SEQ ID NOs: 36 to 38);

Fig. 2: Comparison of the amino acid sequences of the light chain (TSK114 L) of a mouse monoclonal antibody specifically binding to TNF- $\alpha$  (SBQ ID NO: 49), light chain subgroup 1 (Hk1) of a human antibody (SEQ ID NO: 50), and the light chains (TNLV2 and TNLV2d) of the humanized antibodies of the present invention (SEQ ID NOs: 39 and 40);

Fig. 3: Schematic diagram showing the process for preparing vector pHAB-TNHV2 HF expressing the heavy chain of the inventive humanized antibody;

Fig. 4: Schematic diagram showing the process for preparing vector pHAB-TNHV2k HF expressing the heavy chain of the inventive humanized antibody;

Fig. 5: Schematic diagram showing the process for preparing vector pHAB-TNHV1k HF expressing the heavy chain of the inventive humanized antibody;

Fig. 6: Schematic diagram showing the process for preparing vector pHAB-TNLV2 LF expressing the light chain of the inventive humanized antibody;

Fig. 7: Schematic diagram showing the process for preparing vector pHAB-TNLV2d LF expressing the light chain of the inventive humanized antibody;

Fig. 8: SDS-PAGE result of the inventive humanized antibody produced in an animal cell and purified thereafter; and

Fig. 9: Graph showing the binding affinities for TNF- $\alpha$  of a mouse monoclonal antibody and the inventive humanized antibodies.

#### DETAILED DESCRIPTION OF THE INVENTION

**[0012]** In the humanized antibodies of the present invention, the CDRs in the heavy and light chain variable regions thereof are derived from a mouse monoclonal antibody, and the framework regions except for the CDRs as well as the heavy and light chain constant regions are derived from a human antibody. Preferably, the inventive humanized antibody exhibits an antigen binding affinity ( $K_d$ ) ranging from  $1 \times 10^{-9}$  M to  $1 \times 10^{-13}$  M. Further, the dissociation constant ( $K_{off}$ ) of the inventive humanized antibody ranges from  $1 \times 10^{-6}$  s $^{-1}$  to  $1 \times 10^{-9}$  s $^{-1}$ , when determined by surface plasmon resonance (SPR).

**[0013]** The humanized antibody of the present invention can be prepared by a CDR grafting method from the mouse monoclonal antibody TSK114 (Deposit Accession Nos: KCTC 10514BP and KCTC 10515BP) specific for hTNF- $\alpha$ , wherein the heavy chain variable region of the mouse monoclonal antibody TSK114 comprises three CDRs of SEQ ID NOs: 41 to 43, and the light chain variable region, three CDRs of SEQ ID NOs: 44 to 46.

**[0014]** First, the amino acid sequences of the light chain and heavy chain variable regions of mouse monoclonal antibody are compared with human sequences in the GenBank database, and selected are the human heavy chain variable region subgroup 1 (Hh1) as defined by Kabat having the greatest sequence similarity to the mouse antibody heavy chain and the human light chain kappa variable region subgroup 1 (Hk1) having the most similarity to the mouse antibody light chain (Harris L. & Bajorath J., Protein Science, 4; 306-310, 1995).

**[0015]** The genes encoding heavy and light chains of the humanized antibody can be amplified by using these selected human genes as templates. In this procedure, each of the nucleotide sequences encoding the heavy and light chains of the humanized antibody may be modified based on the information of well-known genetic studies and antibody structural analyses. Further, if certain amino acid residues of the mouse monoclonal antibody affect the antigen binding affinity or they are important for maintaining the antibody structure, it is preferable to preserve the nucleotide sequences encoding the amino acid residues without modification (Kabat E.A. et al., *D.H.H.S. Publication number (NIH)* 91-3242, 1991; Chothia C. et al., Nature, 342; 877-883, 1989; Studnicka G.M. et al., Protein Engineering, 7; 805-814, 1994; Harris L. & Bajorath J., Protein Science, 4; 306-310, 1995).

**[0016]** A gene encoding the heavy chain variable region of the humanized antibody may be prepared by replacing the framework regions except for the antigen-binding site in the heavy chain variable region of the mouse monoclonal antibody TSK114 specifically binding to hTNF- $\alpha$  with the heavy chain subgroup 1 (Hh1) of a human antibody. Such a gene encoding the heavy chain variable region of the humanized antibody can be prepared by the steps of: designing primers for humanizing the mouse monoclonal antibody using the gene encoding the heavy chain of mouse monoclonal antibody TSK114 as a template; performing PCR (polymerase chain reaction) using the corresponding primers; and ligating the so-amplified PCR products to each other using restriction enzymes and DNA ligase. The genes encoding the heavy chain variable region of the humanized antibody thus prepared have been designated TNH2 (SEQ ID NO: 31), TNH2k (SEQ ID NO: 32), and TNH1k (SEQ ID NO: 33), which encode the polypeptides having the amino acid sequences of SEQ ID NOs: 36, 37 and 38, respectively (see Fig. 1).

**[0017]** In order to prepare a gene encoding the full-length heavy chain of the humanized antibody including the gene encoding the humanized heavy chain variable region, gene TNH2, TNH2k or TNH1k is inserted into an appropriate vector, e.g., TOPO vector (TOPO TA cloning kit, Invitrogen, US), to prepare expression vectors pCR-TNH2 Hv, pCR-TNH2k Hv and pCR-TNH1k Hv. A gene fragment encoding the heavy chain variable region of the humanized antibody is isolated from the expression vector pCR-TNH2 Hv, pCR-TNH2k Hv or pCR-TNH1k Hv, and inserted into vector pHAB-HC (Deposit Accession No: KCTC 10229BP; Korean Patent Laid-open Publication No: 2004-12266) containing the gene encoding the heavy chain constant region of a human antibody, to obtain expression vector of the humanized antibody heavy chain. Expression vectors thus prepared have been designated pHAB-TNH2 HF, pHAB-TNH2k HF, and pHAB-TNH1k HF.

**[0018]** *E. coli* TOP10F' transformants transformed with the expression vectors pHAB-TNH2 HF and pHAB-TNH2k HF were deposited on September 1, 2004 with the Korean Collection for Type Cultures (KCTC)(Address: Korea Research Institute of Bioscience and Biotechnology (KRIBB), #52, Oun-dong, Yusong-ku, Taejeon, 305-333, Republic of Korea) under the accession numbers KCTC 10691BP and KCTC 10692BP, respectively, and an *E. coli* TOP10F' transformant transformed with the expression vector pHAB-TNH1k HF was deposited on June 13, 2005 with KCTC under the accession number KCTC 10818BP, in accordance with the terms of Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purpose of Patent Procedure.

**[0019]** The genes encoding the full-length heavy chain of the humanized antibody can be isolated from the heavy chain expression vectors pHAB-TNH2 HF, pHAB-TNH2k HF, and pHAB-TNH1k HF, and have been designated TNH2 HF, TNH2k HF and TNH1k HF, respectively.

**[0020]** A gene encoding the light chain variable region of the humanized antibody may be prepared by replacing the framework regions except for the antigen-binding site in the light chain variable region of the mouse monoclonal antibody TSK114 specifically binding to hTNF- $\alpha$  with the light chain subgroup 1 (Hk1) of a human antibody. Such a gene encoding the light chain variable region of the humanized antibody can be prepared by the steps of: designing primers for humanizing the mouse monoclonal antibody using the gene encoding the light chain of mouse monoclonal antibody TSK114 as a template; performing PCR using the corresponding primers; and ligating the so-amplified PCR products to each other using restriction enzymes and DNA ligase. The genes encoding the light chain variable region of the humanized antibody thus prepared have been designated TNL2 and TNL2d, which encode the polypeptides having the amino acid sequences of SEQ ID NOs: 39 and 40, respectively (see Fig. 2).

**[0021]** In order to prepare a gene encoding the full-length light chain of the humanized antibody including the gene encoding the humanized light chain variable region, gene TNL2 or TNL2d is inserted into an appropriate vector, e.g., TOPO vector, to prepare expression vectors pCR-TNL2 Lv and pCR-TNL2d Lv. A gene fragment encoding the light

chain variable region of the humanized antibody is isolated from the expression vector pCR-TNLV2 Lv or pCR-TNLV2d Lv, and inserted into vector pHAB-KC (Deposit Accession No: KCTC 10230BP; Korean Patent Laid-open Publication No: 2004-12268) containing the gene encoding the light chain constant region of a human antibody, to obtain expression vector of the humanized antibody light chain. Expression vectors thus prepared have been designated pHAB-TNLV2 LF and pHAB-TNLV2d LF.

[0022] An *E. coli* TOP10F' transformant transformed with the expression vector pHAB-TNLV2 LF was deposited on September 1, 2004 with KCTC under the accession number KCTC 10690BP, and an *E. coli* TOP10F' transformant transformed with the expression vector pHAB-TNLV2d LF was deposited on June 13, 2005 with KCTC under the accession number KCTC 10817BP, in accordance with the terms of Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purpose of Patent Procedure.

[0023] The genes encoding the full-length light chain of the humanized antibody can be isolated from the light chain expression vectors pHAB-TNLV2 LF and pHAB-TNLV2d LF, and have been designated TNLV2 LF and TNLV2d LF, respectively.

[0024] CHO cell lines may be transformed with humanized heavy chain expression vector pHAB-TNHV2 HF and humanized light chain expression vector pHAB-TNLV2 LF by using an appropriate transformation solution such as GenePORTER (GTS, US) to obtain a transformant producing the humanized antibody specific for hTNF- $\alpha$ , and the transformant has been designated CHO-YHB1406. Further, another transformant may be prepared by using humanized heavy chain expression vector pHAB-TNHV2k HF and humanized light chain expression vector pHAB-TNLV2 LF or pHAB-TNLV2d LF according to the same method as described above. These transformants have been designated CHO-YHB1411 and CHO-YHB1411-2. In addition, another transformant may be prepared by using humanized heavy chain expression vector pHAB-TNHV1k HF and humanized light chain expression vector pHAB-TNLV2d LF according to the same method as described above, and has been designated CHO-YHB 1406-2.

[0025] To purify the humanized antibody of the present invention from the transformant cell lines, the transformant CHO-YHB 1406, CHO-YHB 1411, CHO-YHB1411-2 or CHO-YHB1406-2 may be cultured in an appropriate culture medium, and the culture supernatant may be subjected to column chromatography using Protein-A (Amersham Bioscience, Sweden) or goat anti-human immunoglobulin G (Zymed Laboratories Inc., USA). The humanized antibodies thus purified have been designated YHB1406, YHB1411, YHB1411-2 and YHB1406-2, respectively.

[0026] The antigen binding affinity of the humanized antibody may be determined by, e.g., competitive ELISA, solid phase ELISA or surface plasma resonance. Humanized antibodies YHB1406, YHB1411, YHB1411-2 and YHB1406-2 specifically binding to hTNF- $\alpha$  show antigen-binding affinities ranging from  $1 \times 10^{-9}$  M to  $1 \times 10^{-13}$  M, which demonstrates that the humanized antibodies of the present invention have antigen-binding activities similar to that of the original mouse monoclonal antibody, while showing significantly decreased immunogenicity, and therefore, may be effectively used for treating hTNF- $\alpha$  related diseases. Further, the dissociation constant ( $K_{off}$ ) of the inventive humanized antibody determined by SPR ranges from  $1 \times 10^{-6}$  s $^{-1}$  to  $1 \times 10^{-9}$  s $^{-1}$ , which reflects a very low degree of dissociation of the antigen-antibody complex.

[0027] For this purpose, the humanized antibody of the present invention may be used as an effective ingredient in a pharmaceutical composition to treat a hTNF- $\alpha$  related disease such as rheumatoid arthritis, Crohn's disease, psoriatic arthritis, psoriasis, septicemia, asthma, Wegener's granulomatosis, inflammation and ankylosing spondylitis.

[0028] The compositions of the present invention may be formulated so as to provide quick, sustained or delayed release of the effective ingredient after administration to a patient by employing any one of the procedures well known in the art. The composition may comprise pharmaceutically acceptable excipients, diluents, fillers, disintegrants, sweeteners, lubricants, and flavors. The pharmaceutical composition is preferably formulated for intravenous administration, either by bolus injection or sustained drip, or for release from an implanted capsule. A typical formulation for intravenous administration utilizes physiological saline as a diluent.

[0029] The dose of the antibody actually administered to a patient ought to be determined in light of various relevant factors including the specific antibody to be administered, administration time and formulation of the composition, route of administration, the disease to be treated, and body weight, age, gender, state of health and diet of a patient. A typical dose may range from 0.01 mg/kg/day to 1,000 mg/kg/day. More typically, the dose may range from 0.1 mg/kg/day to 10 mg/kg/day.

[0030] The following Examples are intended to further illustrate the present invention without limiting its scope.

#### **Example 1: construction of a gene encoding a heavy chain variable region of a humanized antibody**

[0031] PCR was conducted using a gene encoding the heavy chain variable region of the mouse monoclonal antibody TSK114 (Deposit Accession Nos: KCTC 10514BP and KCTC 10515BP) specific for TNF- $\alpha$  as a template and a primer pair of SEQ ID NOs: 1 and 14, to amplify gene A encoding the heavy chain variable region of the humanized antibody carrying a leader sequence. The amplified PCR product was electrophoresed on an agarose gel and recovered from the gel using QIAgel extraction kit (Qiagen, USA).

**[0032]** Gene A thus obtained was subjected to PCR with a primer pair of SEQ ID NOs: 1 and 7 or SEQ ID NOs: 6 and 14 to amplify DNA fragments, and overlapping PCR was performed using the resulting DNA fragments with a primer pair of SEQ ID NOs: 1 and 14. The amplified PCR product was incubated with TOPO vector (TOPO TA cloning kit, Invitrogen Inc., USA) at room temperature for 45 min to be cloned, and the cloned vector was transformed into *E. coli* TOP10F' (Invitrogen Inc., USA). After incubating the transformed *E. coli* in LB media containing 100 µg/ml of ampicillin, plasmid was isolated from the *E. coli*, and digested with EcoRI (BioLabs Inc., USA) to prepare about 450 bp gene B encoding the heavy chain variable region.

**[0033]** PCR was performed using gene B as a template and a primer pair of SEQ ID NOs: 1 and 9 or SEQ ID NOs: 8 and 14 to amplify DNA fragments, and overlapping PCR was performed using the resulting DNA fragments with a primer pair of SEQ ID NOs: 1 and 14. In the same manner as described above, the amplified PCR product was cloned in TOPO vector, and digested with EcoRI to prepare gene C encoding the heavy chain variable region.

**[0034]** In the same manner as described above, gene C was subjected to PCR with a primer pair of SEQ ID NOs: 1 and 11 or SEQ ID NOs: 10 and 14 to amplify DNA fragments, and overlapping PCR was performed using the resulting DNA fragments with a primer pair of SEQ ID NOs: 1 and 14. The PCR product thus amplified was cloned in TOPO vector to obtain gene D encoding the heavy chain variable region. Gene D was subjected to PCR with a primer pair of SEQ ID NOs: 4 and 14 or SEQ ID NOs: 1 and 5 to amplify DNA fragments, and overlapping PCR was performed using the resulting DNA fragments with a primer pair of SEQ ID NOs: 1 and 14. In the same manner as described above, the PCR product thus amplified was cloned in TOPO vector to obtain gene E encoding the heavy chain variable region.

**[0035]** Further, PCR was conducted using gene E as a template and a primer pair of SEQ ID NOs: 1 and 3 or SEQ ID NOs: 2 and 14 to amplify DNA fragments, and overlapping PCR was performed using the resulting DNA fragments with a primer pair of SEQ ID NOs: 1 and 14. In the same manner as described above, the PCR product was cloned in TOPO vector to obtain gene TNH2 (SEQ ID NO: 31) encoding the heavy chain variable region, and the TOPO vector inserted with TNH2 was designated pCR-TNH2 Hv.

**[0036]** In the same manner as described above, PCR was conducted using TNH2 as a template and a primer pair of SEQ ID NOs: 1 and 13 or SEQ ID NO: 12 and 14 to amplify DNA fragments, and overlapping PCR was performed using the resulting DNA fragments with a primer pair of SEQ ID NOs: 1 and 14. The PCR product was cloned in TOPO vector to obtain another gene encoding the heavy chain variable region. The gene was designated TNH2k (SEQ ID NO: 32), and the TOPO vector inserted with gene TNH2k was designated pCR-TNH2K Hv.

**[0037]** Further, in the same manner as described above, PCR was conducted using gene TNH2k as a template and a primer pair of SEQ ID NOs: 1 and 15, and the PCR product was cloned in TOPO vector to obtain still another gene encoding the heavy chain variable region. The gene was designated TNH1k (SEQ ID NO: 33), and the TOPO vector inserted with TNH1k was designated pCR-TNH1k Hv.

**[0038]** The primer pairs and reaction conditions employed in each PCR reaction are shown in Table 1. Each PCR reaction was conducted under the following conditions of 30 cycles as shown in Table 1 after initial denaturation of 7 min at 95°C, and final extension of 10 min at 72°C.

&lt;Table 1&gt;

Primer pair	Gene	PCR condition		
		Denaturation	Annealing	Extension
SEQ ID NOs: 1 and 14	A	95°C, 2 min	58°C, 1.5 min	72°C, 2 min
SEQ ID NOs: 6 and 14	B	95°C, 2 min	58°C, 1.5 min	72°C, 2 min
SEQ ID NOs: 1 and 7		95°C, 2 min	58°C, 1.5 min	72°C, 2 min
SEQ ID NOs: 8 and 14	C	95°C, 2 min	58°C, 1.5 min	72°C, 2 min
SEQ ID NOs: 1 and 9		95°C, 2 min	58°C, 1.5 min	72°C, 2 min
SEQ ID NOs: 10 and 14	D	95°C, 2 min	58°C, 1.5 min	72°C, 2 min
SEQ ID NOs: 1 and 11		95°C, 2 min	58°C, 1.5 min	72°C, 2 min
SEQ ID NOs: 4 and 14	E	95°C, 2 min	58°C, 1.5 min	72°C, 2 min
SEQ ID NOs: 1 and 5		95°C, 2 min	58°C, 1.5 min	72°C, 2 min
SEQ ID NOs: 2 and 14	TNH2	95°C, 2 min	58°C, 1.5 min	72°C, 2 min
SEQ ID NOs: 1 and 3		95°C, 2 min	58°C, 1.5 min	72°C, 2 min

(continued)

Primer pair	Gene	PCR condition		
		Denaturation	Annealing	Extension
SEQ ID NOs: 12 and 14	TNHV2k	95°C, 2 min	58°C, 1.5 min	72°C, 2 min
SEQ ID NOs: 1 and 13		95°C, 2 min	58°C, 1.5min	72°C, 2 min
SEQ ID NOs: 1 and 15	TNHV1k	95°C, 2 min	58°C, 1.5 min	72°C, 2 min

### **Example 2: Construction of a full-length heavy chain gene of a humanized antibody and an expression vector thereof**

**[0039]** In order to construct the gene encoding the humanized full-length heavy chain using the gene encoding the heavy chain variable region inserted in pCR-TNHV2 Hv vector prepared in Example 1, pHAB-HC (KCTC 10229BP, Korean Patent Laid-open Publication No. 2004-12266) vector containing the gene encoding the heavy chain constant region of the human antibody was employed.

**[0040]** First, in order to insert gene TNH2 encoding the heavy chain variable region of the humanized antibody into expression vector pHAB-HC, pCR- TNH2 Hv was treated with HindIII/ApaI (BioLabs, Inc., USA) at 37°C for 2 hrs, electrophoresed on a 1.5% agarose gel and stained with etidium bromide (EtBr), and an about 450 bp fragment of the gene was observed.

**[0041]** Also, pHAB-HC was treated with HindIII/ApaI, electrophoresed and stained, and an about 6.5 kb fragment of the digested vector was observed. After then, the gene fragments observed were recovered from the gels using a QIAgel extraction kit (Qiagen, USA), treated with T4 DNA ligase (BioLabs, Inc., USA) at 16°C overnight, and transformed into *E. coli* TOP10F' to obtain a transformant. The transformant was cultured in an LB medium supplemented with 100 µg/ml of ampicillin overnight, and a plasmid was isolated therefrom. The isolated plasmid was treated with HindIII/NotI and subjected to agarose gel electrophoresis, to obtain an about 1.4 kb full-length heavy chain gene of the humanized antibody.

**[0042]** The full-length heavy chain gene of the humanized antibody thus prepared was designated TNH2 HF. Further, the expression vector inserted with TNH2 HF was designated pHAB-TNH2 HF (Fig. 3), and transformed into *E. coli* TOP10F' to obtain a transformant, which was deposited on September 1, 2004 with the Korean Collection for Type Cultures (KCTC)(Address: Korea Research Institute of Bioscience and Biotechnology (KRIBB), #52, Oun-dong, Yusong-ku, Taejon, 305-333, Republic of Korea) under the accession number KCTC 10691BP.

**[0043]** In the same manner as described above, the full-length heavy chain gene of the humanized antibody was also prepared using TNH2k or TNH1k genes, and the full-length heavy chain genes of the humanized antibody thus prepared were designated TNH2k HF or TNH1k HF, respectively. Further, the heavy chain expression vector of the humanized antibody inserted with the full-length heavy chain gene, TNH2k HF or TNH1k HF, was designated pHAB-TNH2k HF or pHAB-TNH1k HF (Figs. 4 and 5), respectively, and the *E. coli* TOP10F' transformed with pHAB-TNH2k HF or pHAB-TNH1k HF was deposited on September 1, 2004 or June 13, 2005, respectively, with the Korean Collection for Type Cultures (KCTC) under the accession numbers KCTC 10692BP and KCTC 10818BP, respectively.

### **Example 3: Construction of a gene encoding a light chain variable region of a humanized antibody**

**[0044]** PCR was conducted using the gene encoding the light chain variable region of mouse monoclonal antibody TSK114 specifically recognizing TNF-α as a template and a primer pair of SEQ ID NOs: 16 and 30, to amplify gene A encoding the light chain variable region containing a leader sequence. Gene A was subjected to agarose gel electrophoresis and EtBr staining, and recovered from the gel using a QIAgel extraction kit.

**[0045]** Gene A thus purified was subjected to PCR with a primer pair of SEQ ID NOs: 16 and 25 or SEQ ID NOs: 24 and 30 to amplify DNA fragments, and overlapping PCR was performed using the resulting DNA fragments with a primer pair of SEQ ID NOs: 16 and 30. The amplified PCR product was incubated with TOPO vector (TOPO TA cloning kit, Invitrogen Inc., USA) at room temperature for 45 min, and the cloned vector was transformed into *E. coli* TOP10F' to obtain a transformant. After incubating the transformant in LB media containing 100 µg/ml of ampicillin overnight, plasmid was isolated from the *E. coli*, and digested with EcoRI (BioLabs Inc., USA) to obtain about 400 bp gene B encoding the light chain variable region.

**[0046]** PCR was conducted using gene B as a template and a primer pair of SEQ ID NOs: 26 and 30 or SEQ ID NOs: 16 and 27 to amplify DNA fragments, and overlapping PCR was performed using the resulting DNA fragments with a primer pair of SEQ ID NOs: 16 and 30. In the same manner as described above, the amplified PCR product was cloned in TOPO vector, and digested with EcoRI to prepare gene, C encoding the light chain variable region.

**[0047]** In the same manner as described above, gene C was subjected to PCR with a primer pair of SEQ ID NOs: 16



and 23 or SEQ ID NOs: 22 and 30 to amplify DNA fragments, and overlapping PCR was performed using the resulting DNA fragments with a primer pair of SEQ ID NOs: 16 and 30. In the same manner as described above, the PCR product thus amplified was cloned in TOPO vector to obtain gene D encoding the light chain variable region.

**[0048]** Gene E encoding the light chain variable region was obtained by performing PCR using gene D as a template with a primer pair of SEQ ID NOs: 18 and 30 or SEQ ID NOs: 16 and 19 to amplify DNA fragments, and overlapping PCR was performed using the resulting DNA fragments with a primer pair of SEQ ID NOs: 16 and 30. In the same manner as described above, the PCR product thus amplified was cloned in TOPO vector.

**[0049]** Gene F encoding the light chain variable region was obtained by performing PCR using gene E as a template with a primer pair of SEQ ID NOs: 20 and 30 or SEQ ID NOs: 16 and 21 to amplify DNA fragments, and overlapping PCR was performed using the resulting DNA fragments with a primer pair of SEQ ID NOs: 16 and 30. In the same manner as described above, the PCR product thus amplified was cloned in TOPO vector.

**[0050]** Further, PCR was conducted using gene F as a template and a primer pair of SEQ ID NOs: 28 and 30 or SEQ ID NOs: 16 and 29 to amplify DNA fragments, and overlapping PCR was performed using the resulting DNA fragments with a primer pair of SEQ ID NOs: 16 and 30. In the same manner as described above, the PCR product was cloned in TOPO vector to obtain gene TNLV2 (SEQ ID NO: 34) encoding the light chain variable region, and the TOPO vector inserted with gene TNLV2 was designated pCR-TNLV2 Lv.

**[0051]** Further, in the same manner as described above, PCR was conducted using TNLV2 as a template and a primer pair of SEQ ID NOs: 16 and 17 to amplify DNA fragment, and the fragment was subjected to PCR using a primer of SEQ ID NOs: 30 as a megaprimer to obtain a gene encoding the light chain variable region of the humanized antibody. The gene was designated TNLV2d (SEQ ID NO: 35), and the TOPO vector inserted with TNLV2d was designated pCR-TNLV2d Lv.

**[0052]** The primer pairs and PCR conditions employed in the above PCR reactions are described in the following Table 2. Each of the PCR reactions was conducted under the conditions of 30 cycles as shown in Table 2 after initial denaturation of 7 min at 95°C, and final extension of 10 minutes at 72°C.

<Table 2>

Primer pair	Gene	PCR condition		
		Denaturation	Annealing	Extension
SEQ ID NOs: 16 and 30	A	95°C, 2 min	58°C, 1.5 min	72°C, 2 min
SEQ ID NOs: 24 and 30	B	95°C, 2 min	58°C, 1.5 min	72°C, 2 min
SEQ ID NOs: 25 and 16		95°C, 2 min	58°C, 1.5 min	72°C, 2 min
SEQ ID NOs: 26 and 30	C	95°C, 2 min	58°C, 1.5 min	72°C, 2 min
SEQ ID NOs: 27 and 16		95°C, 2 min	58°C, 1.5 min	72°C, 2 min
22 and 30 22 and 30	D	95°C, 2 min	58°C, 1.5 min	72°C, 2 min
SEQ ID NOs: 23 and 16		95°C, 2min	58°C, 1.5 mm	72°C, 2 min
SEQ ID NOs: 18 and 30	E	95°C, 2 min	58°C, 1.5 min	72°C, 2 min
SEQ ID NOs: 19 and 16		95°C, 2 min	58°C, 1.5 min	72°C, 2 min
SEQ ID NOs: 20 and 30	F	95°C, 2 min	58°C, 1.5 min	72°C, 2 min
SEQ ID NOs: 21 and 16		95°C, 2 min	58°C, 1.5 min	72°C, 2 min
SEQ ID NOs: 28 and 30	TNLV2	95°C, 2 min	58°C, 1.5 min	72°C, 2 min
SEQ ID NOs: 29 and 16		95°C, 2 min	58°C, 1.5 min	72°C, 2 min
SEQ ID NOs: 16 and 17	TNLV2d	95°C, 2 min	58°C, 1.5 min	72°C, 2 min
SEQ ID NO: 30		95°C, 2 min	58°C, 1.5 min	72°C, 2 min

#### **Example 4: Construction of a full-length light chain gene of a humanized antibody and an expression vector thereof**

**[0053]** In order to construct the gene encoding the humanized full-length light chain using the gene encoding the light chain variable region inserted in pCR-TNLV2 Lv vector prepared in Example 3, pHAB-KC (KCTC 10230BP, Korean Patent Laid-open Publication No. 2004-12268) expression vector containing the gene encoding the light chain constant

region of the human antibody was employed.

[0054] First, in order to insert gene TNLV2 encoding the light chain variable region of the humanized antibody into expression vector pHAB-KC, pCR- TNLV2 Lv was treated with *HindIII/BsiI* (BioLabs, Inc., USA) at 37°C for 2 hrs, electrophoresed on a 1.5% agarose gel and stained with etidium bromide (EtBr), and an about 400 bp fragment of the gene was observed.

[0055] In the same manner as described above, expression vector pHAB-KC was also treated with *HindIII/BsiI*, and an about 6.5 kb fragment of the digested vector was observed. After then, the gene fragments observed were recovered from the gels using QIAgel extraction kit (Qiagen, USA), treated with T4 DNA ligase (BioLabs, Inc., USA) at 16°C overnight, and transformed into *E. coli* TOP10F' to obtain a transformant. The transformant was cultured in an LB medium supplemented with 100 µg/ml of ampicillin overnight, and a plasmid was isolated from the culture medium. The isolated plasmid was treated with *HindIII/NotI* (BioLabs, Inc., USA) and subjected to agarose gel electrophoresis, to obtain an about 0.7 kb full-length light chain gene of the humanized antibody.

[0056] The full-length light chain gene of the humanized antibody thus prepared was designated TNLV2 LF. Further, the expression vector inserted with TNLV2 LF was designated pHAB-TNLV2 LF (Fig. 6), and transformed into *E. coli* TOP10F' to obtain a transformant, which was deposited on September 1, 2004 with the Korean Collection for Type Cultures (KCTC) under the accession number KCTC 10690BP.

[0057] In the same manner as described above, a full-length light chain gene of the humanized antibody was prepared using TNLV2d gene, and the full-length light chain gene of the humanized antibody thus prepared was designated TNLV2d LF. Further, the expression vectors inserted with TNLV2d LF was designated pHAB-TNLV2d LF (Fig. 7), and transformed into *E. coli* TOP10F' to obtain a transformant, which was deposited on June 13, 2005, with the Korean Collection for Type Cultures (KCTC) under the accession number KCTC 10817BP.

#### **Example 5: Transformation of a humanized antibody into CHO cell lines**

[0058] In order to measure the humanized antibody activity in animal cells, heavy chain expression vector pHAB-TNHV2 HF prepared in Example 2 and light chain expression vector pHAB-TNLV2 LF prepared in Example 4 were transfected into CHO cell line (ATCC CRL-9096, USA) as follows.

[0059] First, CHO cells were grown in DMEM/F12 medium (JRH Inc., USA) supplemented with 2.0 g/l of sodium bicarbonate (Sigma, USA) and 10% heat inactivated FBS (Gibco BRL, USA) in a 37°C humidified CO<sub>2</sub> incubator for 2 to 3 days. The cultured cells were harvested by centrifuging the culture solution at 1,200 rpm and room temperature (25°C) for 5 min, stained with 0.4% trypan blue (Gibco BRL, USA), and counted with a hemacytometer. The cells were seeded to a T-75 flask in an amount of about  $3 \times 10^5$  cells to be sub-cultured.

[0060] The seeded CHO cells were allowed to proliferate until they occupied the flask's surface by a density of 60 to 90%. 10 µg of pHAB-TNHV2 HF, 10 µg of pHAB-TNLV2 LF and 80 µl of GenePORTER solution (GTS, USA) for transfection were mixed with 5 ml of DMEM/F12 (serum-free) medium. After the mixture was kept at room temperature for 10 to 45 min, the culture medium was removed from the flask, and the sub-cultured CHO cells were incubated with the mixture in a 37°C humidified CO<sub>2</sub> incubator for about 3 to 5 hrs, to obtain a transfectant designated CHO-YHB 1406.

[0061] In accordance with the same manner as described above, heavy chain expression vector pHAB-TNHV2k HF and light chain expression vector pHAB-TNLV2 LF, heavy chain expression vector pHAB-TNHV2k HF and light chain expression vector pHAB-TNLV2d LF, or heavy chain expression vector pHAB-TNHV1k HF and light chain expression vector pHAB-TNLV2d LF, which were prepared in Examples 2 and 4, respectively, were transfected into CHO cells to obtain transfectants, which were designated CHO-YHB1411, CHO-YHB1411-2, and CHO-YHB 1406-2, respectively.

#### **Example 6: Purification of a humanized antibody**

[0062] The transfectant CHO-YHB 1406 cells obtained in Example 5 were seeded to a T-75 flask containing a DMEM/F12 medium supplemented with 10% fetal bovine serum in an amount of  $2 \times 10^5$  cells, and the flask was incubated in a 37°C humidified CO<sub>2</sub> incubator for 7 days to express the humanized antibody. The culture medium was harvested, centrifuged and filtered to obtain a cell-free culture solution comprising antibodies.

[0063] In order to purify the humanized antibody from the solution, sepharose 4 Fast flow columns conjugated with protein-A (Pharmacia) and goat anti-human Immunoglobulin G (Zymed Laboratories Inc., USA), respectively, were employed. The culture medium comprising antibodies was applied to protein-A conjugated sepharose 4 Fast flow column to allow the humanized antibody to bind with protein-A, and a glycine buffer (pH 2.5) was introduced into the column to elute the humanized antibody. Then, the eluate was neutralized by mixing with 1 M Tris-Cl (pH 8.0) at a volume ratio of 1:10, and subjected to sepharose 4 Fast flow column conjugated with goat anti-human IgG to allow the column to specifically capture the antibody protein.

[0064] The humanized antibody was eluted according to the same method as described above, and the purified humanized antibody was designated YHB 1406.

[0065] In accordance with the same manner as described above, the transfectants, CHO-YHB1411, CHO-YHB1411-2 and CHO-YHB 1406-2, prepared in Example 5 were cultured and the produced antibodies therefrom were purified, respectively, to obtain the purified humanized antibodies designated YHB1411, YHB1411-2 and YHB1406-2, respectively.

[0066] As a result of analyzing the purified humanized antibodies with SDS-PAGE, bands of about 50 kDa and about 25 kDa were observed, which were identified as the heavy chain and light chain of the humanized antibody, respectively (Fig. 8).

#### **Example 7: Measurement of antigen binding affinity of a humanized antibody to hTNF- $\alpha$**

[0067] The humanized antibodies purified in Example 6 were quantified with competitive ELISA, and their antigen-binding activities were analyzed by using a recombinant hTNF- $\alpha$  (Biosource, USA). For antibody quantification, 100 ng of goat anti-human immunoglobulin G.A.M (Zymed Laboratories Inc., USA) was distributed into each well of a microplate (Dynatech Laboratories Inc., USA) and the well plate was kept at 4°C overnight to coat it with the immunoglobulin. At this time, mouse monoclonal antibody TSK114 was used as a control.

[0068] In order to measure an antigen-binding affinity of the humanized antibody to recombinant hTNF- $\alpha$  antigen, antigen solutions (100  $\mu$ l each) having various concentrations ranging from  $10^{-11}$  to  $10^{-7}$  M were mixed with 5 ng of mouse monoclonal antibody TSK114, humanized antibodies YHB1406, YHB1411, YHB 1406-2, and YHB1411-2, respectively, and reacted at 37 °C for 2 hrs. Each of the reactants was added to the well plate coated with 0.4  $\mu$ g of the antigen and the well plate was kept at 37°C for about 1.5 hrs. A goat anti-human polyclonal antibody (BioRad, USA) conjugated with horseradish peroxidase was diluted at a ratio of 1:1000 and 100  $\mu$ l of the diluted antibody solution was added to each well. The well plate was kept at 37°C for 1 hr. After the reaction was completed, the optical density of each well was measured using a horseradish peroxidase substrate kit (BioRad, USA).

[0069] Concentrations of the antibody bound to the antigen and the unbound antibody were calculated from the measured optical density, and antigen-binding affinity of the humanized antibodies were determined therefrom (Friguet B. et al., J. Immunol. Meth., 77: 305-319, 1985). The results are shown in Table 3 and Fig. 9.

<Table 3>

Antigen	Antigen binding affinity (Kd)
Mouse antibody TSK114	$1.4 \times 10^{-11}$ M
Humanized antibody YHB 1406	$2.2 \times 10^{-11}$ M
Humanized antibody YHB1411	$2.0 \times 10^{-10}$ M
Humanized antibody YHB1411-2	$2.0 \times 10^{-10}$ M
Humanized antibody YHB 1406-2	$2.9 \times 10^{-10}$ M

[0070] As can be seen in Table 3 and Fig. 9, the antigen binding affinities of humanized antibodies YHB1406, YHB1411, YHB1411-2, and YHB1406-2 were similar to that of mouse monoclonal antibody TSK114.

#### **Example 8: Test of *in vitro* antigen neutralization capability of the humanized antibody**

[0071] The capabilities of the inventive humanized antibodies for neutralizing hTNF- $\alpha$  antigen *in vitro* were determined by employing WHEI 164 cell line (ATCC CRL-1751, USA), as follows (Khabar KSA et al., Immunol. Lett. 46; 107-110, 1995).

[0072] Mouse monoclonal antibody TSK 114, and humanized antibodies YHB 1406, YHB1411, YHB1411-2 and YHB1406-2 were diluted in PBS buffer to various concentrations ranging from 0.024 ng/mL to 1.0  $\mu$ g/mL, respectively, and each 100  $\mu$ l of the resulting dilutions was added to the wells of a 96-well culture plate. 50  $\mu$ l of hTNF- $\alpha$  solution (100 pg/mL) was added to each well and a reaction was carried out at 37°C for 1 hr. Then, 50  $\mu$ l of WHEI 164 cell lines ( $1 \times 10^6$  cells/ml) treated with 2  $\mu$ g/ml actinomycin D (Sigma, USA) was added to each well.

[0073] The cells were cultured at 37°C for 24 hrs, and 100  $\mu$ l of the culture solution was removed. 20  $\mu$ l of a solution of methyl thiazole tetrazolium (MTT, Sigma, USA) diluted in PBS buffer to a concentration of 5 mg/mL was added to each well of the culture plate, and the plate was incubated at 37°C for 4 hrs for color development. Then, 100  $\mu$ l of 0.1N HCl-10% SDS solution (Sigma, USA) was added to each well to completely dissolve the colored precipitate, and the absorbance of each well was measured at 595 nm.

[0074] From the absorbance data obtained above, a curve of survival rate of WHEI 164 cell lines versus antibody

concentration was drawn. The concentration of the antibodies inhibiting cell death by 50 % ( $IC_{50}$ ) was calculated from the curve, and the result is shown in Table 4.

<Table 4> Antigen neutralization capabilities of the antibodies

Antigen	$IC_{50}$ (ng/mL)
Mouse monoclonal antibody TSK114	4.7
Humanized antibody YHB 1406	9.2
Humanized antibody YHB 1411	6.7
Humanized antibody YHB1411-2	5.5
Humanized antibody YHB 1406-2	6.5

[0075] As shown in Table 4,  $IC_{50}$  of mouse monoclonal antibody (TSK114) was 4.7 ng/mL, and those of the inventive humanized antibodies YHB1406, YHB1411, YHB1411-2 and YHB 1406-2 were 9.2 ng/mL, 6.7 ng/mL, 5.5 ng/mL and 6.5 ng/mL, respectively. This result shows that the humanized antibodies have *in vitro* antigen neutralization capabilities similar to the mouse monoclonal antibody.

#### **Example 9: Measurement of antigen binding affinity of a humanized antibody to hTNF- $\alpha$ by SPR**

[0076] The binding affinities to hTNF- $\alpha$  of a mouse monoclonal antibody and a humanized antibody were measured by surface plasmon resonance (SPR) method. Specifically, hTNF- $\alpha$  (Biosource, USA) was immobilized on a sensor chip CM5 (Biacore, Sweden) at a concentration of 200 ug/ml, and reacted with 25, 50, 100, 200 and 400 nM of mouse monoclonal antibody TSK 114 or humanized antibody YHB1411-2, respectively. Then, association and dissociation constants of the reactions were measured by Biacore2000 (Biacore, Sweden).

[0077] From the association and dissociation constants measured above, antigen binding affinities of the antibodies were calculated, and the results are shown in Table 5.

<Table 5> Comparison of the binding affinities to hTNF- $\alpha$  of the mouse monoclonal antibody and humanized antibody

Antibody	Association constant ( $k_{on}$ , $M^{-1}s^{-1}$ )	Dissociation constant ( $k_{off}$ , $s^{-1}$ )	Antigen binding affinity ( $K_d$ , M)
TSK 114	$4.12 \times 10^4$	$2.39 \times 10^{-7}$	$5.79 \times 10^{-12}$
YHB1411-2	$3.96 \times 10^4$	$6.80 \times 10^{-8}$	$1.71 \times 10^{-12}$

[0078] As shown in Table 5, the inventive humanized antibody YHB1411-2 exhibited a high antigen binding affinity of picomole level ( $10^{-12}M$ ) and a low dissociation constant. Further, the antigen binding affinity of humanized antibody YHB1411-2 was higher by about 3 folds than that of mouse monoclonal antibody TSK114.

#### SEQUENCE LISTING

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5	ccaggacagg gtttagagtg gatgggctgg ataaacacca aacttgaga gccaagatat	180
	gatgaagagt tcaagggacg ggttaccatg actagggaca cctctgccag cactgcctat	240
	atggagctca gcagcctcag aagtgaggac acggctgtat attactgtgc aagatatgat	300
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	ccaggaaagg gtttagagtg gatgggctgg ataaacacca aacttgaga gccaagatat	180
	gatgaagagt tcaagggacg ggttaccatg actagggaca cctctgccag cactgcctat	240
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	ccaggatccg cccccaaact ctggatttat agctcatcca atctggcttc tggagtccca	180
35	tctcgttca gtggcagtgg gtctgggacc gatttcactt tcacaatcag cagcctgcag	240
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Lys Gly Arg Val Thr Met Thr Arg Asp Thr Ser Ala Ser Thr Ala Tyr  
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Met Glu Leu Ser Ser Leu Arg Ser Glu Asp Thr Ala Val Tyr Tyr Cys  
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Ile Tyr Ser Ser Ser Asn Leu Ala Ser Gly Val Pro Ser Arg Phe Ser  
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 Ile Tyr Ser Ser Ser Asn Leu Ala Ser Gly Val Pro Ser Arg Phe Ser  
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 Pro Glu Asp Ile Ala Thr Tyr Tyr Cys His Gln Tyr Glu Arg Ser Pro

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 Gly Trp Ile Asn Thr Asn Thr Gly Glu Pro Arg Tyr Asp Glu Glu Phe  
 Lys Gly Arg Phe Ala Phe Ser Leu Glu Thr Ser Ala Ser Thr Ala Tyr  
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 Tyr Met His Trp Val Arg Gln Ala Pro Gly Gln Gly Leu Glu Trp Met  
 Gly Trp Ile Asn Pro Asn Ser Gly Gly Thr Asn Tyr Ala Gln Lys Phe  
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 50 55 60  
 15 Gly Ser Gly Ser Gly Thr Ser Tyr Ser Leu Thr Ile Ser Ser Met Glu  
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 Ser Gly Ser Gly Thr Asp Phe Thr Phe Thr Ile Ser Ser Leu Gln Pro  
 65 70 75 80  
 45 Glu Asp Ile Ala Thr Tyr Tyr Cys Gln Gln Tyr Asp Asn Leu Pro  
 85 90 95

## Claims

- 50
1. A humanized antibody specific for human tumor necrosis factor- $\alpha$  (hTNF- $\alpha$ ), comprising:
    - a) a heavy chain variable region having the amino acid sequence of SEQ ID NO: 36, 37 or 38;
    - b) a light chain variable region having the amino acid sequence of SEQ ID NO: 39 or 40;
    - c) a heavy chain constant region identical to that of a human antibody; and
    - d) a light chain constant region identical to that of a human antibody.
  2. The humanized antibody of claim 1, wherein the antigen binding affinity ( $K_d$ ) of the antibody ranges from  $1 \times 10^{-9}$
- 55

M to  $1 \times 10^{-13}$  M.

3. The humanized antibody of claim 1, wherein the dissociation constant ( $K_{off}$ ) of the antibody ranges from  $1 \times 10^{-6} \text{ s}^{-1}$  to  $1 \times 10^{-9} \text{ s}^{-1}$ , which is determined by surface plasmon resonance (SPR).
4. The humanized antibody of claim 1, wherein the heavy chain variable region of the antibody is encoded by a DNA having a nucleotide sequence of SEQ ID NO: 31, 32 or 33.
5. The humanized antibody of claim 1, wherein the light chain variable region of the antibody is encoded by a DNA having a nucleotide sequence of SEQ ID NO: 34 or 35.
6. An expression vector for the humanized antibody of claim 1, which comprises a DNA encoding a heavy chain variable region having an amino acid sequence of SEQ ID NO: 36, 37 or 38 and a DNA encoding a light chain variable region having an amino acid sequence of SEQ ID NO: 39 or 40.
7. A pharmaceutical composition comprising the humanized antibody of any one of claims 1 to 5, for treating a hTNF- $\alpha$  related disease selected from the group consisting of: rheumatoid arthritis, Crohn's disease, psoriatic arthritis, psoriasis, septicemia, asthma, Wegeners granulomatosis, inflammation, and ankylosing spondylitis.

## Patentansprüche

1. Humanisierter Antikörper, der für humanen Tumornekrosefaktor- $\alpha$  (hTNF- $\alpha$ ) spezifisch ist, umfassend:
  - a) eine variable Region der schweren Kette mit der Aminosäuresequenz von SEQ ID NO: 36, 37 oder 38;
  - b) eine variable Region der leichten Kette mit der Aminosäuresequenz von SEQ ID NO: 39 oder 40;
  - c) eine konstante Region der schweren Kette, die mit derjenigen eines humanen Antikörpers identisch ist, und
  - d) eine konstante Region der leichten Kette, die mit derjenigen eines humanen Antikörpers identisch ist.
2. Humanisierter Antikörper nach Anspruch 1, wobei die Antigenbindungsaffinität ( $K_d$ ) des Antikörpers von  $1 \times 10^{-9}$  M bis  $1 \times 10^{-13}$  M reicht.
3. Humanisierter Antikörper nach Anspruch 1, wobei die Dissoziationskonstante ( $K_{off}$ ) des Antikörpers von  $1 \times 10^{-6} \text{ s}^{-1}$  bis  $1 \times 10^{-9} \text{ s}^{-1}$  reicht, was durch Oberflächenplasmonresonanz bestimmt wird.
4. Humanisierter Antikörper nach Anspruch 1, wobei eine DNA mit der Nukleotidsequenz von SEQ ID NO: 31, 32 oder 33 für die variable Region der schweren Kette des Antikörpers codiert.
5. Humanisierter Antikörper nach Anspruch 1, wobei eine DNA mit der Nukleotidsequenz von SEQ ID NO: 34 oder 35 für die variable Region der leichten Kette des Antikörpers codiert.
6. Expressionsvektor für den humanisierten Antikörper nach Anspruch 1, welcher eine DNA, die für eine variable Region der schweren Kette mit einer Aminosäuresequenz von SEQ ID NO: 36, 37 oder 38 codiert, und eine DNA, die für eine variable Region der leichten Kette mit einer Aminosäuresequenz von SEQ ID NO: 39 oder 40 codiert, umfasst.
7. Pharmazeutische Zusammensetzung, welche den humanisierten Antikörper nach einem der Ansprüche 1 bis 5 umfasst, zum Behandeln einer Krankheit, die mit hTNF- $\alpha$  in Beziehung steht und aus der Gruppe bestehend aus rheumatoider Arthritis, Morbus Crohn, Psoriasis-Arthritis, Psoriasis, Septikämie, Asthma, Wegener-Granulomatose, Entzündung und Spondylitis ankylosans ausgewählt ist.

## Revendications

1. Anticorps humanisé spécifique du facteur  $\alpha$  humain de nécrose tumorale (hTNF- $\alpha$ ), comprenant :
  - a) une région variable de chaîne lourde ayant la séquence d'acides aminés de la SEQ ID NO : 36, 37 ou 38 ;
  - b) une région variable de chaîne légère ayant la séquence d'acides aminés de la SEQ ID NO : 39 ou 40 ;

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- c) une région constante de chaîne lourde identique à celle d'un anticorps humain ; et
- d) une région constante de chaîne légère identique à celle d'un anticorps humain.

2. Anticorps humanisé selon la revendication 1, dans lequel l'affinité de liaison à un antigène ( $K_d$ ) de l'anticorps est située dans la plage allant de  $1 \times 10^{-9}$  M à  $1 \times 10^{-13}$  M.
3. Anticorps humanisé selon la revendication 1, dans lequel la constante de dissociation ( $K_{off}$ ) de l'anticorps, laquelle est déterminée par résonance plasmonique de surface (SPR), est située dans la plage allant de  $1 \times 10^{-6}$  s<sup>-1</sup> à  $1 \times 10^{-9}$  s<sup>-1</sup>.
4. Anticorps humanisé selon la revendication 1, dans lequel la région variable de chaîne lourde de l'anticorps est codée par un ADN ayant une séquence de nucléotides de la SEQ ID NO : 31, 32 ou 33.
5. Anticorps humanisé selon la revendication 1, dans lequel la région variable de chaîne légère de l'anticorps est codée par un ADN ayant une séquence de nucléotides de la SEQ ID NO : 34 ou 35.
6. Vecteur d'expression, pour l'anticorps humanisé de la revendication 1, qui comprend un ADN codant une région variable de chaîne lourde ayant une séquence d'acides aminés de la SEQ ID NO : 36, 37 ou 38 et un ADN codant une région variable de chaîne légère ayant une séquence d'acides aminés de la SEQ ID NO : 39 ou 40.
7. Composition pharmaceutique comprenant l'anticorps humanisé de l'une quelconque des revendications 1 à 5, pour le traitement d'une maladie liée au hTNF- $\alpha$  choisie dans l'ensemble constitué par : la polyarthrite rhumatoïde, la maladie de Crohn, le rhumatisme psoriasique, le psoriasis, la septicémie, l'asthme, la granulomatose de Wegener, une inflammation, et la spondylarthrite ankylosante.

FIG. 1

**TSK114H:** QVQLVQSGPE LKKPGETVKI SCKASGYTFT HYGMNWVKQA PGGLKWMGW INTNTGEP  
**Hh1:** QVQLVQSGAE VKKPGASVKV SCKASGYTFT GYMHWVRQA PGQGLEWMGW INPNSGGTNY  
**TNHV2:** QVQLVQSGPE LKKPGASVKV SCKASGYTFT HYGMNWVRQA PGQGLEWMGW INTNTGEP  
**TNHV2k:** QVQLVQSGPE LKKPGASVKV SCKASGYTFT HYGMNWVRQA PGKGLEWMGW INTNTGEP  
**TNHV1k:** QVQLVQSGPE LKKPGASVKV SCKASGYTFT HYGMNWVRQA PGKGLEWMGW INTNTGEP

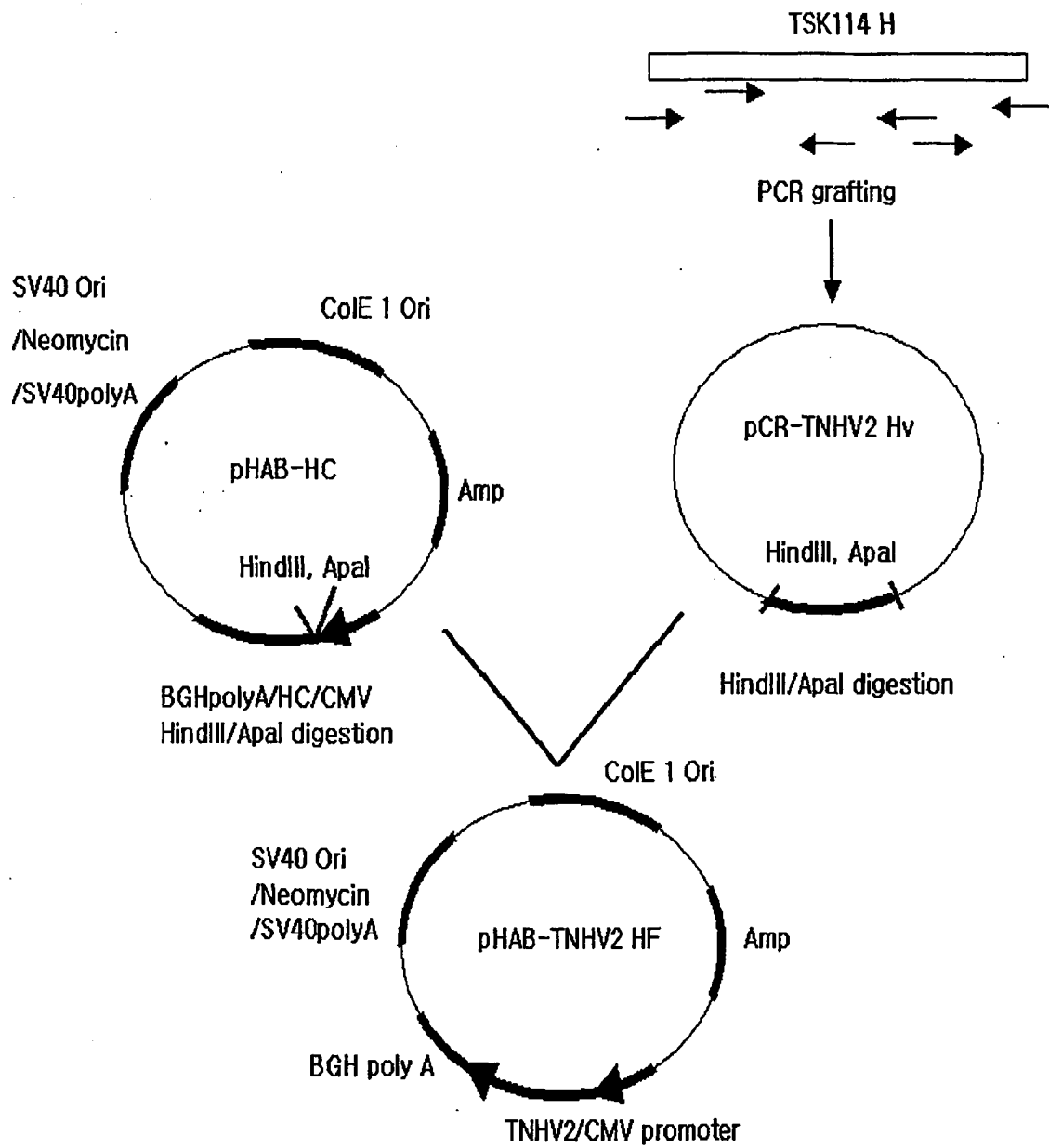
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**Hh1:** AQKFQGRVTM TRDTSASTAY MELSSLRSED TAVYYCAR-- ---- (SEQ ID NO: 48)  
**TNHV2:** DEEFKGRVTM TRDTSASTAY MELSSLRSED TAVYYCARYD SRGEDCWGQG TTVTVSS (SEQ ID NO: 36)  
**TNHV2k:** DEEFKGRVTM TRDTSASTAY MELSSLRSED TAVYYCARYD SRGEDCWGQG TTVTVSS (SEQ ID NO: 37)  
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FIG. 2

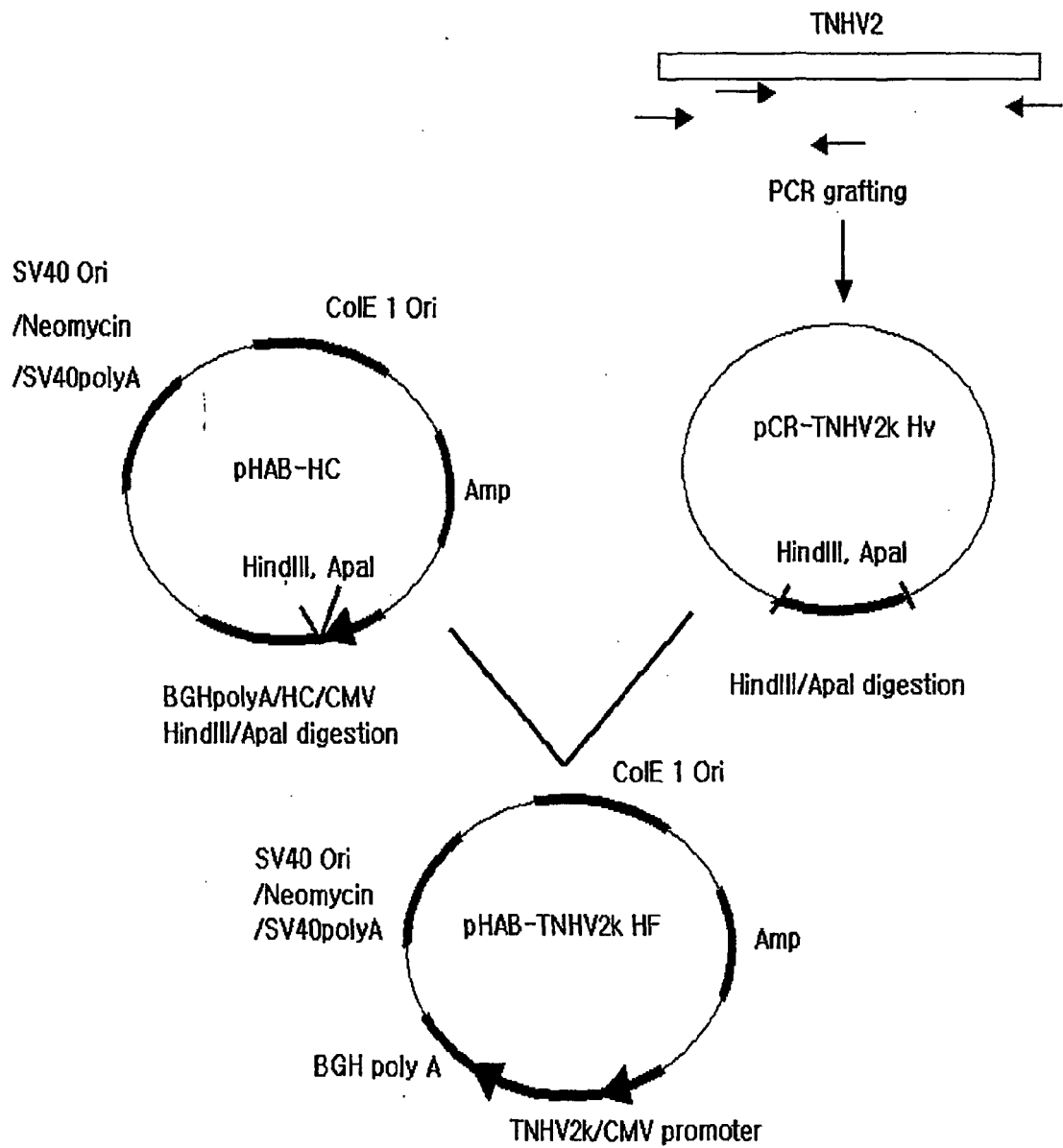
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**Hk1:** QIQMTQSPSS LSASVGDRVT ITCQASQDIS -NYLNWYQQK PGKAPKLLIY DASNLETGVP  
**TNLV2:** QIVMTQSPSS LSASVGERVT ITCTASSSIS YNYFHWYQQK PGSAPKLWIY SSSNLASGVP  
**TNLV2d:** DIVMTQSPSS LSASVGERVT ITCTASSSIS YNYFHWYQQK PGSAPKLWIY SSSNLASGVP

**TSK114L:** PRISGSGSGT SYSLTISSME AEDAATYCH OYERSPWTFG GGTKLEIKR (SEQ ID NO: 49)  
**Hk1:** SRFSGSGSGT DFTFTISSLQ PEDIATYCYCQ QYDNLP- (SEQ ID NO: 50)  
**TNLV2:** SRFSGSGSGT DFTFTISSLQ PEDIATYCH OYERSPWTFG GGTKVEIKR (SEQ ID NO: 39)  
**TNLV2d:** SRFSGSGSGT DFTFTISSLQ PEDIATYCH OYERSPWTFG GGTKVEIKR (SEQ ID NO: 40)

**FIG. 3**



**FIG. 4**



**FIG. 5**

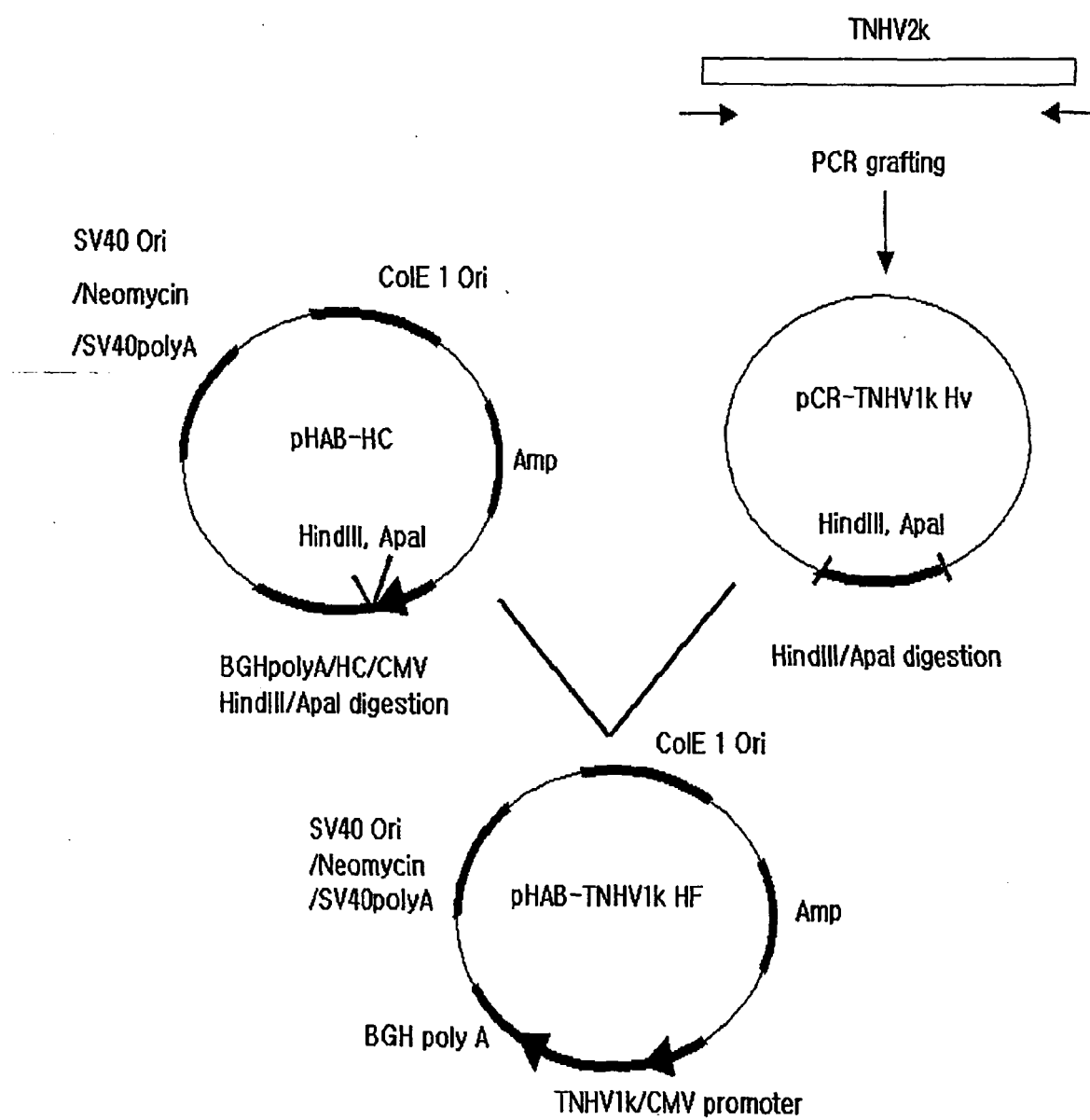
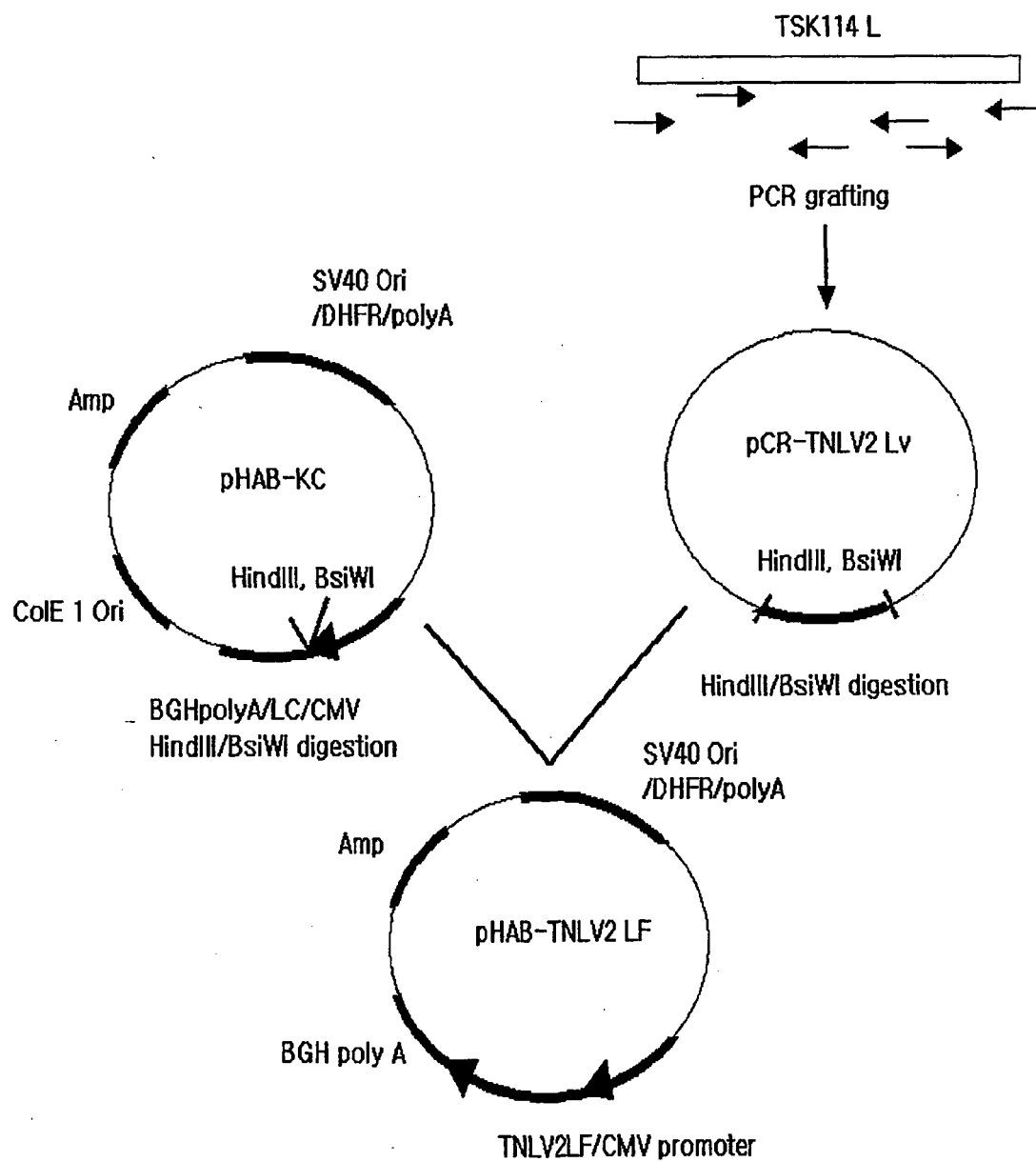
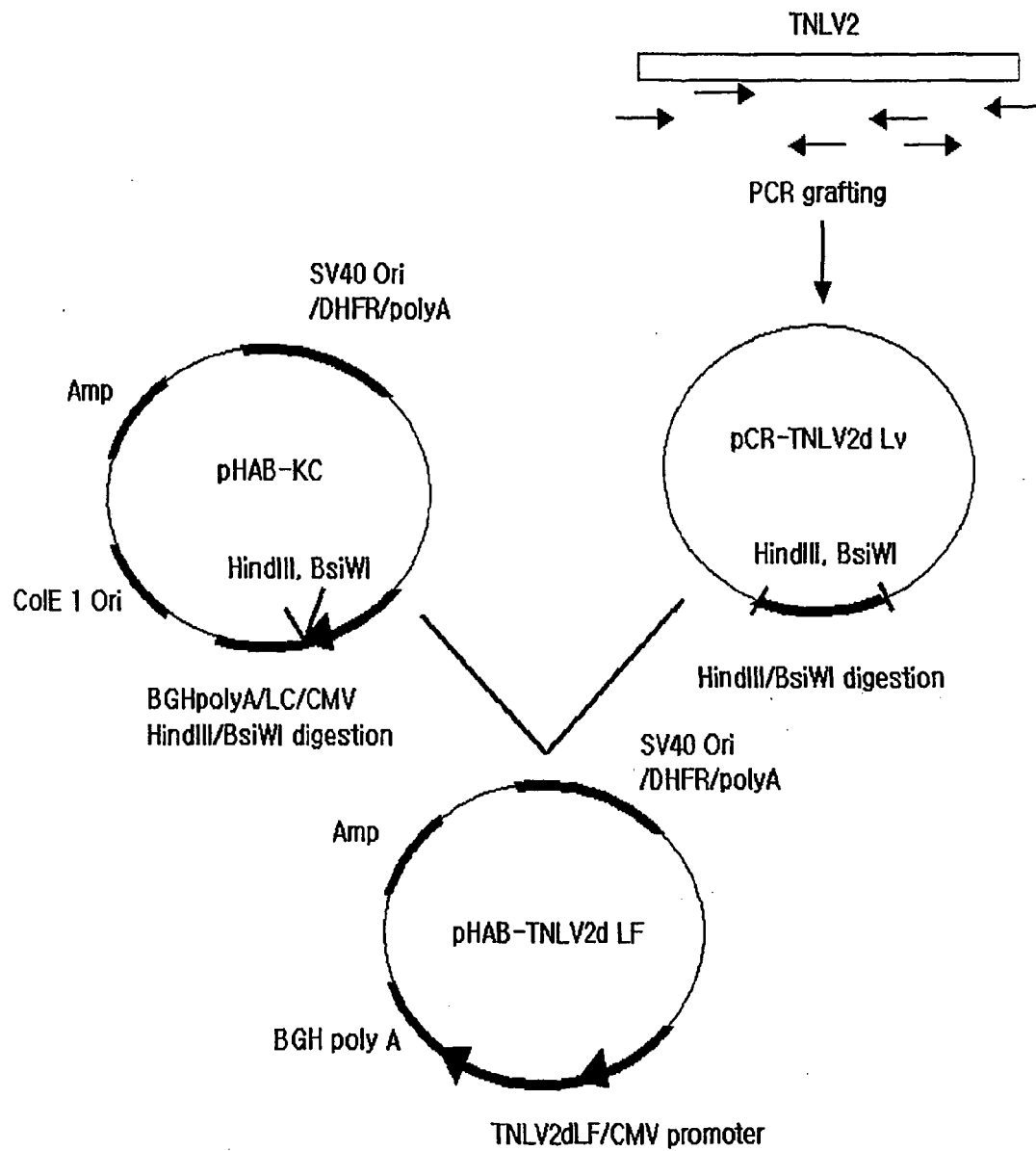




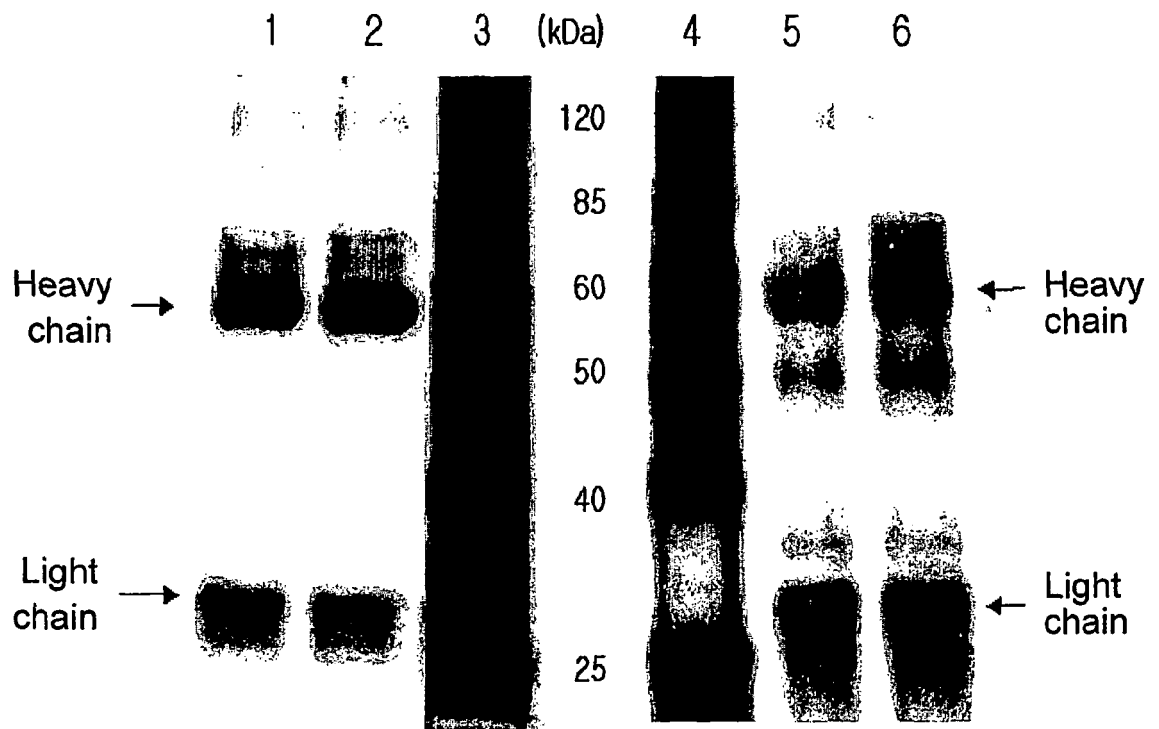
FIG. 6



**FIG. 7**



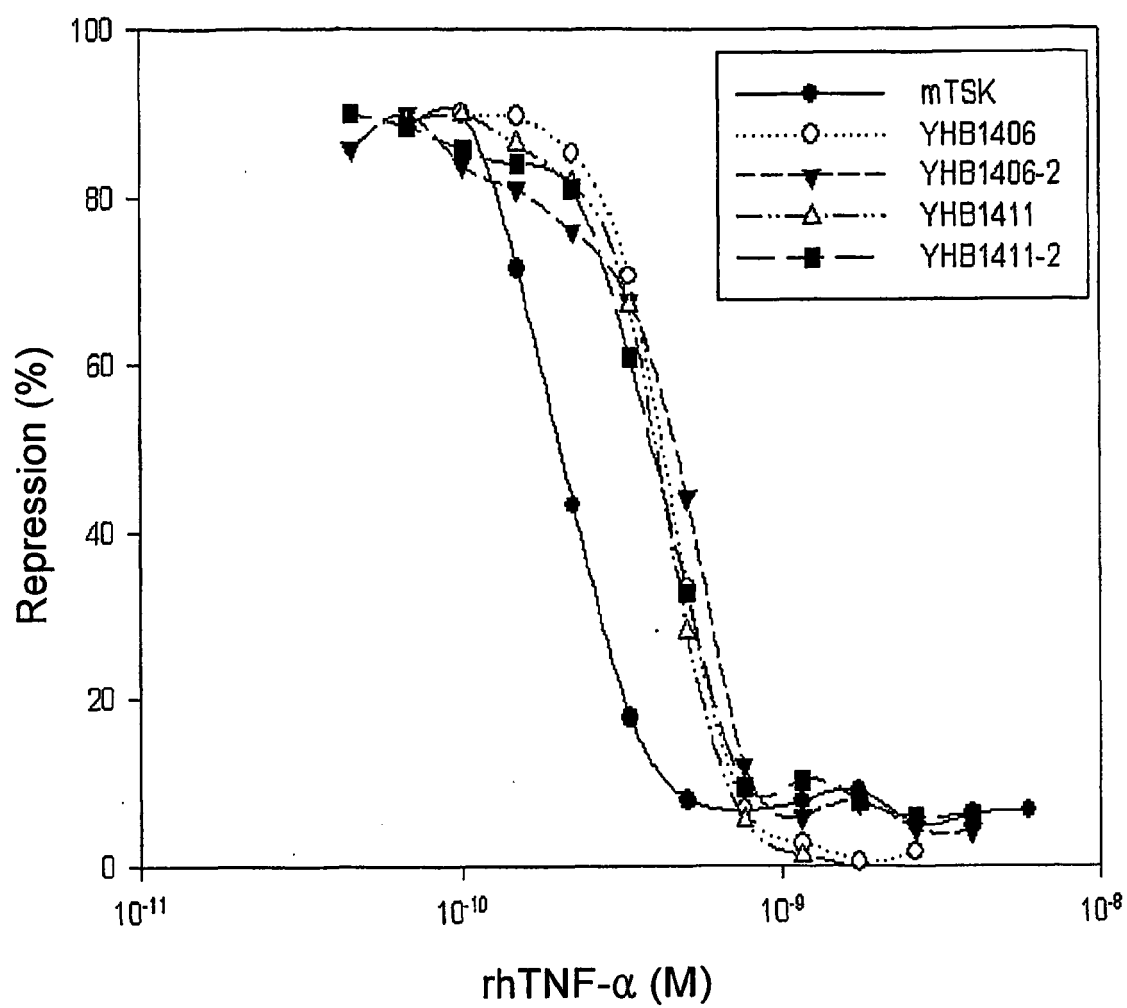
**FIG. 8**



1. Humanized Ab YHB1406
2. Humanized Ab YHB1411
3. Protein size marker

4. Protein size marker
5. Humanized Ab YHB1406-2
6. Humanized Ab YHB1411-2

FIG. 9



## REFERENCES CITED IN THE DESCRIPTION

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