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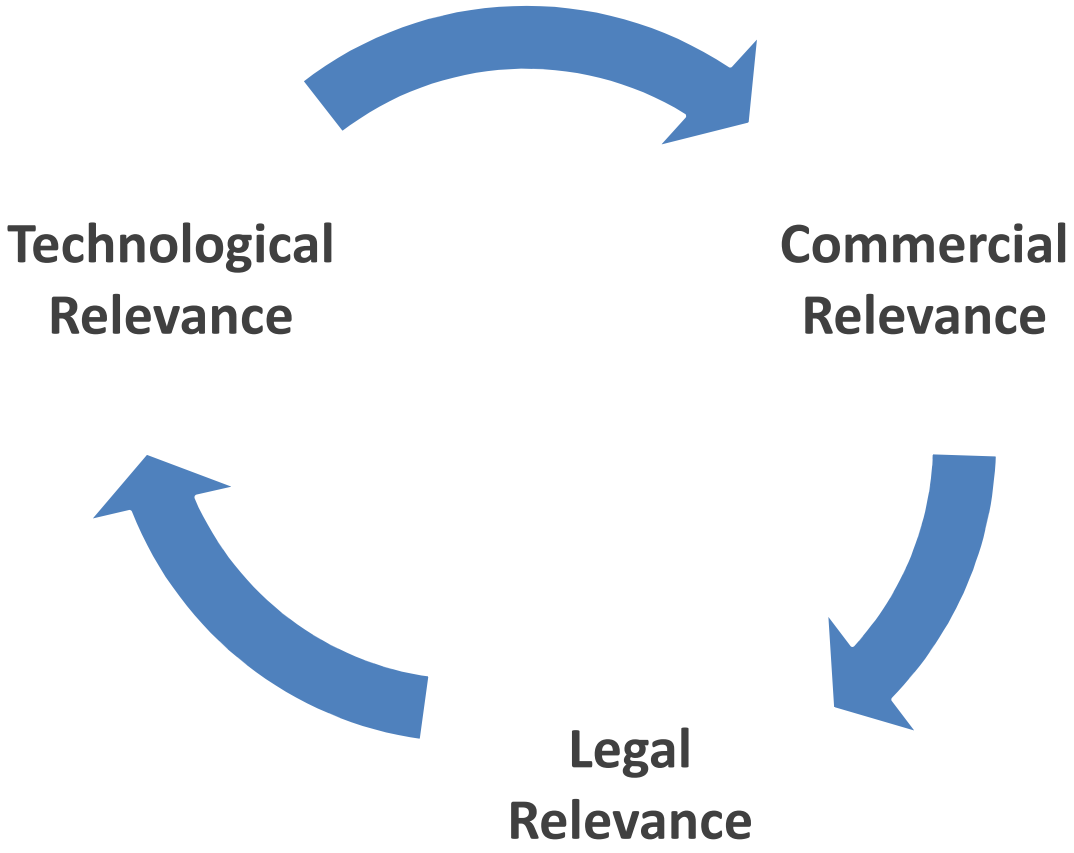
Patent Information

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Examples of Biosimilar Patent Documents

1. US5225539: Recombinant altered antibodies and methods of making altered antibodies
2. US5656272: Methods of treating TNF-α-mediated Crohn's disease using chimeric anti-TNF antibodies
3. US6054297: Humanized antibodies and methods for making them
4. US6090382: Human antibodies that bind human TNFα
5. US6217866: Monoclonal antibodies specific to human epidermal growth factor receptor and therapeutic methods employing same
6. US6331415: Methods of producing immunoglobulins, vectors and transformed host cells for use therein
7. US8048854: Amidated insulin glargine
8. WO2011024024: A process for recovering darbepoeitin alfa isoforms

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Reference: www.espacenet.com

1. US Patent No. **5225539** titled "Recombinant altered antibodies and methods of making altered antibodies" teaches a process for production of **an altered antibody in which Complementarity Determining Regions (CDRs) in the light or heavy chain variable domains of the antibody has been replaced by the analogous CDRs from an antibody of different specificity using recombinant DNA techniques**.

The process comprises constructing a gene encoding a variable domain HuVNP, comprising the B1-8 CDRs and the NEWM framework regions.

Then, the oligonucleotides are assembled by phosphorylating 5' end with T4 polynucleotide kinase and mixed together with 5 pmole of the terminal oligonucleotide which has been phosphorylated with 5 µCi [γ-32 p] ATP.

The oligonucleotides are annealed by heating (80°C) and cooling over 30 minutes to room temperature, with unkinased oligonucleotides as splints (in 150 µl of 50 mM Tris.Cl, pH 7.5, 10 mM MgCl2). Then ligated by adding ATP (1 mM) and DTT (10 mM) with 50 U T4 DNA ligase and incubated at RT (30 minutes), 10mM EDTA is added and the sample is extracted with phenol, precipitated from ethanol, dissolved in 20 µl water and boiled for 1 minute with an equal volume of formamide dyes.

The sample is loaded onto and run on a 0.3 mm 8 m-urea 10% polyacrylamide gel.

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After the extraction, the blocks are annealed and cloned into a vector (M13mp18) cut with cut with PstI and Hind III and the gene is sequenced using the dideoxy technique.

The MoVNP gene constructed is transferred as a Hind III-BamHI fragment from the vector pSV-VNP to the vector M13mp8. MoVNP coding sequences is replaced by the synthetic HuVNP fragment, by removing three Hind II sites from the 5' non-coding sequence by site directed mutagenesis and a new Hind II site is introduced near the end of the fourth framework region.

The Hind III-Bam HI fragment, now carrying the HuVNP gene, is excised from M13 and cloned back into pSV-VNP to replace the MoVNP gene and produce a vector pSV-HuVNP.HE.

This vector is transfected into the myeloma line J558 L by spheroplast fusion.

The plasmid pSV-HuVNP.HE contains the gpt marker, hence, stably transfected myeloma cells can be selected in a medium containing mycophenolic acid. Transfectants secreted an antibody (HuVNP -IgE) with heavy chains comprising a HuVNP variable domain (i.e. a "humanised" mouse variable region) and human ε constant domains, and lambda 1 light chains from the J558L myeloma cells.

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2. US Patent 5656272 titled "Methods of treating TNF-α-mediated Crohn's disease using chimeric anti-TNF antibodies" teaches **a process for preparing chimeric anti-TNF antibodies**.

The process comprises the following steps:

i) injecting (subcutaneously and intraperitoneally) 40µg of purified *E. coli*-derived recombinant human TNF (rhTNF) emulsified with an equal volume of complete Freund's adjuvant in 0.4 ml to 10 week old female BALB/c mice.
One week later, an injection of 5 µg of rhTNF in incomplete Freund's adjuvant is given i.p. followed by four consecutive i.p. injections of 10µg of TNF without adjuvant. Eight weeks after the last injection, the mouse is boosted i.p. with 10 µg of TNF.

ii) after 4 days the mouse is sacrificed, the spleen is obtained and a spleen cell suspension is prepared. Spleen cells are fused with cells of the nonsecreting hybridoma, Sp2/0, at a 4:1 ratio of spleen cells to Sp2/0 cells, in the presence of 0.3 ml of 30% polyethylene glycol, PEG 1450. The cells are incubated at 37° C for 6 hours.

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After incubation, the fused cells are distributed in 0.2 ml aliquots into 96-well plates at concentrations of 2×10^4 SP2/0 cells per well. Feeder cells, in the form of 5×10^4 normal BALB/c spleen cells, are added to each well.

iii) The growth medium used consisted of RPMl-1640 medium, 10% heat-inactivated fetal bovine serum (FBS), 0.1 mM minimum essential medium (MEM) nonessential amino acids, 1 mM sodium pyruvate, 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and, for selection, hypoxanthine-aminopterin-thymidine.
A solid-phase radioimmunoassay (RIA) is employed for screening supernatants for the presence of mAbs specific for rhTNFα. The background binding in this assay is about 500 cpm. A supernatant is considered positive if it yielded binding of 2000 cpm or higher.

iv) Positive wells are subcloned at limiting dilution on mouse feeder cells. Upon further analysis of the supernatants in neutralization assays, the positive clone showing potent inhibiting and/or neutralizing activity is selected and the line is maintained in RPMl-1640 medium with 10% FBS, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin.

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3. US Patent 6054297 titled "Humanized antibodies and methods for making them" teaches a method for **making a humanized antibody comprising non-human, import Complementarity Determining Region (CDR) amino acid residues and human Framework Region (FR) amino acid residues.**

The process comprises the following steps:

i) obtaining the amino acid sequences of at least a portion of an import antibody variable domain and of a consensus variable domain;

ii) identifying Complementarity Determining Region (CDR) amino acid sequences in the import and the human variable domain sequences;

iii) substituting an import CDR amino acid sequence for the corresponding human CDR amino acid sequence;

iv) aligning the amino acid sequences of a Framework Region (FR) of the import antibody and the corresponding FR of the consensus antibody;

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v) identifying import antibody FR residues in the aligned FR sequences that are non-homologous to the corresponding consensus antibody residues;

vi) determining if the non-homologous import amino acid residue is reasonably expected to have at least one of the following effects:
1. non-covalently binds antigen directly,
2. interacts with a CDR; or
3. participates in the V_L -V_H interface; and

vii) for any non-homologous import antibody amino acid residue which is reasonably expected to have at least one of these effects, substituting that residue for the corresponding amino acid residue in the consensus antibody FR sequence.

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4. US Patent 6090382 titled "Human antibodies that bind human TNFα" teaches a **process for preparing human antibodies that bind to human TNFα.**

A series of single alanine mutations are introduced by standard methods along the CDR3 domain of the D2E7 VL and the D2E7 VH regions.

The kinetics of rhTNFα interaction with an antibody composed of wild-type D2E7 VI, and VH is compared to that of antibodies composed of 1) a wild-type D2E7 VL paired with an alanine-substituted D2E7 VH; 2) a wild-type D2E7 VH paired with an alanine-substituted D2E7 VL; or 3) an alanine-substituted D2E7 VL paired with an alanine-substituted D2E7 VH. All antibodies are tested as full-length, IgG4 molecules.

Kinetics of interaction of antibodies with rhTNFα is determined by surface plasmon resonance.

A series of antibodies related in sequence to D2E7 are analyzed for their binding to rhTNFα, as compared to D2E7, by surface plasmon resonance.

The functional activity of D2E7 is examined by measuring the ability of the antibody to inhibit hTNFα activity, either in vitro or in vivo.

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The functional activity of D2E7 is examined by measuring the ability of the antibody to inhibit hTNFα activity, either in vitro or in vivo.

The binding specificity of D2E7 is examined by measuring its ability to neutralize tumor necrosis factors from various primate species and from mouse, using theL929 cytotoxicity assay.

A 96-well microtiter plate containing 100 µl of anti-hTNFα Abs is serially diluted 1/3 down the plate in duplicates using RPMI medium containing 10% fetal bovine serum (FBS).

Fifty microliters of rhTNFα is added for a final concentration of 500 pg/ml in each sample well.
The plates are then incubated for 30 minutes at room temperature.

Then, 50 µl of TNFα-sensitive L929 mouse fibroblasts cells are added for a final concentration of 5×10-4 cells per well, including 1 µg/ml Actinomycin-D.

Controls included medium plus cells and rhTNFα plus cells. These controls, and a TNFα standard curve, ranging from 2 ng/ml to 8.2 pg/ml, are used to determine the quality of the assay and provide a window of neutralization.

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The plates are then incubated overnight (18-24 hours) at 37° C. in 5% CO2.

One hundred microliters of medium is removed from each well and 50 µl of 5 mg/ml 3,(4,4-dimethylthiazol-2-yl)2,5-diphenyl-tetrazolium bromide (MTT) in PBS is added.

The plates are then incubated for 4 hours at 37° C.

Fifty microliters of 20% sodium dodecyl sulfate (SDS) is then added to each well and the plates are incubated overnight at 37° C.

The optical density at 570/630 nm is measured, curves are plotted for each sample and IC50 s are determined by standard methods.

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5. US Patent 6217866 titled "Monoclonal antibodies specific to human epidermal growth factor receptor and therapeutic methods employing same" teaches **a method producing a therapeutic composition for inhibiting growth of human tumor cells that express human EGF receptors and are mitogenically stimulated by EGF.**

Monoclonal antibodies are prepared by a process comprising the following steps:

i) Balb/c mice are immunized by intraperitoneal injections of CH 71 [Chinese hamster ovary cells which have been transfected with a plasmid bearing a truncated form (deletion of most of the intracellular domain of the EGF-R) of the EGF-R cDNA] cells or CH 71 cell membrane preparation. The mice are immunized three times on day 0, 13, and 32.

The two best responding mice are each boosted by three intraperitoneal injections of CH 71 cells three consecutive days before the fusion. On day 65, the spleen cells of the mice are then fused with NS1 myeloma cells (ratio 5/1) according to the general procedure of Kohler and Milstein, using PEG 4000 as the fusing agent.

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ii) The fusion product is diluted in hypoxanthineazaserine (HA) selection medium and distributed in 96 well plates.
The presence of specific antibodies in the medium of the wells of the growing hybridoma cells is first assayed by radioimmunoassay.
Cells expressing or not expressing the EGF receptor are plated in 96 well plates. At confluency, they are washed once with binding medium (DMEM, 20 mM Hepes, 0.2 BSA) and incubated for 90 minutes at room temperature with 100 µl of culture supernatant from the different growing hybridomas. Cells were then washed 3 times with binding medium and incubated for a further 60 minutes at room temperature with 100 µl of a solution of iodinated goat antimouse immunoglobulins (250,000 cpm/100 µl.).
After 3 washes with PBS (pH 7.5) the cells are scraped from the wells and the radioactivity which is associated with their surface is counted using a gamma counter.
The positive hybridomas are cloned by limiting dilution and further tested by measuring their ability to immunoprecipitate 35S methionine or 32P labeled EGF-R from lysates of cell lines of different species (human, mouse, chicken).

The monoclonal antibodies are found to be specific for the human EGF-R. These antibodies are also tested for their ability to inhibit the binding of iodinated EGF to the surface of cells expressing EGF-R – the antibodies are found to inhibit the binding of iodinated EGF to the surface of cells expressing EGF-R.

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6. US Patent 6331415 titled "Methods of producing immunoglobulins, vectors and transformed host cells for use therein" teaches **a process for producing an immunoglobulin molecule or an immunologically functional immunoglobulin fragment comprising at least the variable domains of the immunoglobulin heavy and light chains, in a single host cell.**

Construction of Expression Vectors for Murine anti-CEA Antibody Chains and Peptide Synthesis:

i) Total RNA from CEA.66-E3 cells is extracted and cells are pelleted by centrifugation and approximately 1 g portions of pellet is resuspended in 10 ml of 10 mM NaCl, 10 mM Tris HCl (pH 7.4), 1.5 mM MgCl2. The resuspended cells are lysed by addition of non-ionic detergent NP-40 to a final concentration of 1 percent, and nuclei removed by centrifugation. After addition of SDS (pH 7.4) to 1 percent final concentration, the supernatant is extracted twice with 3 ml portions of phenol (redistilled)/chloroform: isoamyl alcohol 25:1 at 4° C. The aqueous phase is made 0.2 M in NaCl and total RNA is precipitated by addition of two volumes of 100 percent ethanol and overnight storage at −20° C. After centrifugation, polyA mRNA is purified from total RNA by oligo-dT cellulose chromatography, 142 µg of polyA mRNA is obtained from 1 g of cells.

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ii) 5 µg of the unfractionated polyA mRNA prepared is used as template for oligo-dT primed preparation of double-stranded (ds) cDNA by standard procedures. The cDNA is size fractionated by 6 percent polyacrylamide gel electrophoresis and 124 ng of ds cDNA greater than 600 base pairs in length is recovered by electroelution. A 20 ng portion of ds cDNA is extended with deoxy C residues using terminal deoxynucleotidyl transferase, and annealed with 200 ng of the plasmid pBR322 which has been cleaved with Pst I and tailed with deoxy G. Each annealed mixture is then transformed into *E. coli* K12 strain 294 (ATCC No. 31446). Approximately 8500 ampicillin sensitive, tetracycline resistant transformants are obtained.

iii) The 14mer, 5′ GGTGGGAAGATGGA 3′ complementary to the coding sequence of constant region for mouse MOPC21 kappa chain which begins 25 basepairs 3′ of the variable region DNA sequence is used as kappa chain probe. A 15 mer, 5′ GACCAGGCATCCCAG 3′, complementary to a coding sequence located 72 basepairs 3′ of the variable region DNA sequence for mouse MOPC21 gamma chain is used to probe gamma chain gene.

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Both probes are synthesized by the phosphotriester method and made radioactive by kinasing as follows: 250 ng of deoxyoligonucleotide are combined in 25 µl of 60 mM Tris HCl (pH 8), 10 mM MgCl2, 15 mM beta-mercaptoethanol, and 100 µCi (γ-32P) ATP. 5 units of T4 polynucleotide kinase are added and the reduction is allowed to proceed at 37° C for 30 minutes and terminated by addition of EDTA to 20 mM.

iv) 2000 colonies prepared as described in step 2 are individually inoculated into wells of microtitre dishes containing LB +5 µg/ml tetracycline and stored at −20° C, after addition of DMSO to 7 percent. Individual colonies from this library are transferred to duplicate sets of Schleicher and Schuell BA85/20 nitrocellulose filters and grown on agar plates containing LB+5 µg/ml tetracycline. After 10 hours growth at 37° C the colony filters are transferred to agar plates containing LB+5 µg/ml tetracycline and 12.5 µg/ml chloramphenicol and reincubated overnight at 37° C. The DNA from each colony is then denatured and fixed to the filter by a modification of the Grunstein-Hogness procedure. Each filter is floated for 3 minutes on 0.5 N NaOH, 1.5 M NaCl to lyse the colonies and denature the DNA, then neutralized by floating for 15 minutes on 3 M NaCl, 0.5 M Tris HCl (pH 7.5).

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The filters are then floated for an additional 15 minutes on 2XSSC, and subsequently baked for 2 hours in an 80° C vacuum oven. The filters are prehybridized for 2 hours at room temperature in 0.9 M NaCl, 1X Denhardts, 100 mM Tris HCl (pH 7.5), 5 mM Na-EDTA, 1 mM ATP, 1 M sodium phosphate (dibasic), 1 mM sodium pyrophosphate, 0.5 percent NP-40, and 200 µg/ml E. coli t-RNA, and hybridized in the same solution overnight, using 40×10^6 cpm of either the kinased kappa or gamma probe.

After extensive washing at 37° C in 6X SSC, 0.1 percent SDS, the filters are exposed to Kodak XR-5 X-ray film with DuPont Lightning-Plus intensifying screens for 16-24 hours at –80° C. Approximately 20 colonies which hybridized with kappa chain probe and 20 which hybridized with gamma chain probe are characterized.

v) Plasmid DNAs isolated from several different transformants which hybridized to kappa chain probe are cleaved with Pst I and fractionated by polyacrylamide gel electrophoresis (PAGE). This analysis demonstrated that a number of plasmid DNAs contained cDNA inserts large enough to encode full length kappa chain.
The complete nucleotide sequence of the cDNA insert of one of these plasmids is determined by the dideoxynucleotide chain termination method after subcloning restriction endonuclease cleavage fragments into M13 vectors.

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The entire coding region of mouse anti-CEA kappa chain is isolated on this one large DNA fragment. The amino acid sequence of kappa chain, deduced from the nucleotide sequence of the pK17G4 cDNA insert, corresponds perfectly with the first 23 N-terminal amino acids of mature mouse anti-CEA kappa chain as determined by amino acid sequence analysis of purified mouse anti-CEA kappa chain. The coding region of pK17G4 contains 27 basepairs or 9 amino acids of the presequence and 642 basepairs or 214 amino acids of the mature protein. The mature unglycosylated protein (MW 24,553) has a variable region of 119 amino acids, including the J1 joining region of 12 amino acids, and a constant region of 107 amino acids. After the stop codon behind amino acids 215 begins 212 basepairs of 3′ untranslated sequence up to the polyA addition. The kappa chain probe used to identify pK17G4 hybridizes to nucleotides.

vi) Plasmid DNA isolated from several transformants positive for hybridization with the heavy chain gamma 1 probe is subjected to Pst I restriction endonuclease analysis. Plasmid DNAs demonstrating the largest cDNA insert fragments are selected for further study. Nucleotide sequence coding for mouse heavy (gamma-1) chain, shows an NcoI restriction endonuclease cleavage site near the junction between variable and constant region.

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Selected plasmid DNAs are digested with both PstI and NcoI and sized on polyacrylamide. This analysis allowed identification of a number of plasmid DNAs that contain NcoI restriction endonuclease sites, although none that demonstrate cDNA insert fragments large enough to encode the entire coding region of mouse anti-CEA heavy chain.

vii) Construction of a Plasmid for Direct Expression of Mouse Mature Anti-CEA Kappa Chain Gene, pKCEAtrp207-1*: First, an intermediate plasmid pHGH207-1*, having a single trp promoter, is prepared - plasmid pHGH 207 has a double lac promoter followed by the trp promoter, flanked by EcoR I sites and is used to prepare pHGH207-1. pHGH207 is digested with BamH 1, followed by partial digestion with EcoR I. The largest fragment, which contains the entire trp promoter, is isolated and ligated to the largest EcoR I- BamH I fragment from pBR322, and the ligation mixture is used to transform E. coli 294. TetR AmpR colonies are isolated, and most of them contained pHGH207-1. pHGH207-1* which lacks the EcoR1 site between the ampR gene and the trp promoter, is obtained by partial digestion of pHGH207-1 with EcoR I, filling in the ends with Klenow and dNTPs, and religation. 5 µg of pHGH207-1* is digested with EcoRI, and the ends extended to blunt ends using 12 units of DNA Polymerase I in a 50 µl reaction containing 60 mM NaCl, 7 mM MgCl2, 7 mM Tris HCl (pH 7.4) and 1 mM in each dNTP at 37° C for 1 hour,

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followed by extraction with phenol/CHCl3 and precipitation with ethanol. The precipitated DNA was digested with BamH I, and the large vector fragment (fragment 1) purified using 5 percent polyacrylamide gel electrophoresis, electroelution, phenol/CHCl3 extraction and ethanol precipitation.

The DNA is resuspended in 50 µl of 10 mM Tris pH 8, 1 mM EDTA and treated with 500 units Bacterial Alkaline Phosphatase (BAP) for 30′ at 65° followed by phenol/CHCl3 extraction and ethanol precipitation.

A DNA fragment containing part of the light chain sequence is prepared as follows: 7 µg of pH17G4 DNA is digested with Pst I and the kappa chain containing cDNA insert is isolated by 6 percent gel electrophoresis, and electroelution. After phenol/CHCl3 extraction, ethanol precipitation and resuspension in water, this fragment is digested with Ava II. The 333 bp Pst I-Ava II DNA fragment is isolated and purified from a 6 percent polyacrylamide gel.

A 15 nucleotide DNA primer is synthesized by the phosphotriester method. 500 ng of this primer is phosphorylated at the 5′ end with 10 units T4 DNA kinase in 20 µl reaction containing 0.5 mM ATP. 200 ng of the Pst I-Ava II DNA fragment is mixed with the 20 µl of the phosphorylated primer, heated to 95° C for 3 minutes and quick frozen in a dry-ice ethanol bath. The denatured DNA solution is made 60 mM NaCl, 7 mM MgCl2, 7 mM Tris HCl (pH 7.4), 12 mM in each dNTP and 12 units DNA

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Polymerase I-Large Fragment is added. After 2 hours incubation at 37° C this primer repair reaction is phenol/CHCl3 extracted, ethanol precipitated, and digested to completion with Sau 3A. The reaction mixture is then electrophoresed on a 6 percent polyacrylamide gel and 50 ng of the 182 basepair amino-terminal blunt-end to Sau 3A fragment (fragment 2) is obtained after electroelution.

100 ng of fragment 1 and 50 ng of fragment 2 are combined in 20 µl of 20 mM Tris HCl (pH 7.5), 10 mM MgCl2, 10 mM DTT, 2.5 mM ATP and 1 unit of T4 DNA ligase. After overnight ligation at 14° C the reaction is transformed into *E. coli* K12 strain 294. Restriction endonuclease digestion of plasmid DNA from a number of ampicillin resistant transformants indicated the proper construction and DNA sequence analysis proved the desired nucleotide sequence through the initiation codon of this new plasmid, pKCEAInt1.

The remainder of the coding sequence of the kappa light chain gene is synthesized: The Pst I cDNA insert fragment from 7 µg of K17G4 DNA is partially digested with Ava II and the Ava II cohesive ends are extended to blunt ends in a DNA Polymerase I large fragment reaction.

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After 6 percent polyacrylamide gel electrophoresis, the 686 basepair Pst I to blunt ended Ava II DNA fragment is isolated, purified and subjected to Hpa II restriction endonuclease digestion. The 497 basepair Hpa II to blunt ended Ava II DNA fragment (fragment 3) is isolated and purified after gel electrophoresis.

10 µg of pKCEAInt1 DNA is digested with Ava I, extended with DNA polymerase I large fragment, and digested with Xba I. Both the large blunt ended Ava I to Xba I vector fragment and the small blunt ended Ava I to Xba I fragment are isolated and purified from a 6 percent polyacrylamide gel after electrophoresis. The large vector fragment (fragment 4) is treated with Bacterial Alkaline Phosphatase (BAP), and the small fragment is digested with Hpa II, electrophoresed on a 6 percent polyacrylamide and the 169 basepair Xba I-Hpa II DNA fragment (fragment 5) is purified.

75 ng of fragment 4, 50 ng of fragment 3 and 50 ng of fragment 5 are combined in a T4 DNA ligase reaction and incubated overnight at 14°C, and the reaction mixture transformed into *E. coli* K12 strain 294. Plasmid DNA from six ampicillin resistant transformants are analyzed by restriction endonuclease digestion. The plasmid DNA that demonstrated the proper construction is designated pKCEAInt2.

Final construction is effected by ligating the K-CEA fragment, including the trp promoter from pKCEAInt2 into pBR322(XAP). (pBR322(XAP) is prepared from pBR322 by deletion of the AvaI-PvuII fragment followed by ligation.)

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The K-CEA fragment is prepared by treating pKCEAInt2 with Ava I, blunt ending with DNA polymerase I (Klenow fragment) in the presence of DNTPs, digestion with Pst I and isolation of the desired fragment by gel electrophoresis and electroelution.

The large vector fragment from pBR322(XAP) is prepared by successive treatment with EcoR I, blunt ending with polymerase, and redigestion with Pst I, followed by isolation of the large vector fragment by electrophoresis and electroelution.

The K-CEA and large vector fragments prepared are ligated with T4 DNA ligase, and the ligation mixture transformed into *E. coli* . Plasmid DNA from several ampicillin resistant transformants are selected for analysis, and the plasmid DNA that demonstrated the proper construction, is designated pKCEAtrp207-I*.

viii) Plasmid Vector for Direct Expression of Mouse Mature Anti-CEA Heavy (Gamma 1) Chain Gene, pγCEAtrp207-1* is also constructed.

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ix) *E. coli* strain W3110 are transformed with pγCEAtrp207-1* or pKCEAtrp207-1* using standard techniques.

x) In order to obtain heavy and light chain preparations for reconstitution, transformed cells are grown in larger batches, harvested and frozen.

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7. US Patent 8048854 titled "Amidated insulin glargine" teaches a **process for An insulin glargine modified by amidation, in particular Gly(A21), Arg(B31), Arg-amide (B32) human insulin (insulin glargine amide)**

A primer (completely covering the gene segment coding for amino acids A15-A21 of the insulin analog) is synthesized to prepare a sequence coding for a fusion protein comprising miniproinsulin (with Gly(A21), Arg(B31) human insulin being is later produced from the miniproinsulin).

The primer is combined with a primer for hirf1. Plasmid pADH2Hir_ins is used as a template for generating a DNA fragment.
A DNA fragment generated by PCR, is digested with restriction enzymes KpnI and NcoI and inserted into the open vector (pADH2Hir_ins_glyA21) to obtain a fusion protein.
The fusion protein is expressed using bakers yeast system.
The fusion protein is processed to Gly(A21)-miniproinsulin and by purified by cation exchange chromatography (pH 3.5) in the presence of 2-propanol.
DNA of the plasmid pADH2Hir_ins_glyA21 is used to prepare a vector for direct secretion of Gly(A21), Arg(B31) human insulin
A primer covering the sequence encoding C terminus of the alpha-factor, codons for Lys-Arg and of the N-terminus of the miniproinsulin sequence is synthesized. The primer hybridizes with the 3′ end of the insulin analog sequence cloned into the plasmid pADH2Hir_ins_glyA21.

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A DNA fragment generated by PCR, is digested with restriction enzymes KpnI and NcoI and inserted into a vector to obtain a fusion protein and used to transform Yeast strain Y79 cells. Gly(A21) , Arg(B31) miniproinsulin is purified using cation exchange chromatography in the presence of 30% 2-propanol and pH 3.5.
Gly(A21) , Arg(B31) miniproinsulin is concentrated and then cleaved by trypsin and again purified by cation exchange chromatography.

A primer ins_gly_rev2 is used for the expression of of Gly(A21) human insulin in the plasmid pPich_Hir_ins-GlyA21, which is cloned in the P. pastoris system and the obtained Gly(A21), Arg(B31)-miniproinsulin is purified by cation exchange chromatography.

A plasmid containing the DNA of the plasmid pPich_Hir_ins-GlyA21 and two primers pich_insgly_dirf and pich_insgly_dirrev is constructed.
The Gly(A21), Arg(B31)-miniproinsulin obtained is purified by cation exchange chromatography.

Preparation of Gly(A21), Arg(B31), Arg(B32)-NH2-Human Insulin from a Gly(A21), Arg(B31) Human Insulin Precursor is carried out by Coupling with Argininamide: 100mg of Gly(A21), Arg(B31) human insulin are dissolved in 0.95 ml of argininamide solution (446 g/L), and 0.13 mL of M Na acetate buffer (pH 5.8) and 2 ml of DMF is added. The reaction mixture is cooled to 12°C and 0.094 ml of trypsin is added.

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The reaction is stopped after 8 hours by adding Trifluoroacetic acid (pH =2.5) and HPLC analysis is carried out. The amidated insulin analog is purified by chromatographic techniques.

Two sets of primers covering the sequence of the C terminus of the alpha-factor, codons for Lys-Arg and of the N terminus of the miniproinsulin sequence are synthesized.

DNA of the plasmid pADH2Hir_ins serves as the template for the PCR reactions.
In the first reaction, primers a29_a30_k31f and insnco1rev are used
In the second reaction, the primers a29_a30_k31 rev and alpha_insf1 are used.
The PCR fragments are isolated and aliquots of the 2 reactions are combined, which serve as template for a third reaction with the primers insnco1rev and pich_insgly_dirrev.

The PCR fragment obtained is cloned and expressed resulting in Ala(B31), Ala(C1), Lys(C1)-miniproinsulin, which is converted with lysyl endopeptidase in to B(1-29)-A(1-21) split insulin and serves as intermediate for preparing the amidated insulin glargine.

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The insulin precursor obtained is reacted with lysyl endopeptidase (LEP) and trypsin. 10 mg of insulin precursor is dissolved in Tris buffer (pH 8.0), and LEP is added (0.01 ml of a 1 mg/ml conc. solution in water, and incubated at room temperature for 2 h, and purified by RP-HPLC.
The Gly(A21) B(1-29)-A(1-20) split insulin precursor is reacted with Thr-Arg(Boc)-Arg(Boc)-NH2. 10 mg of the desthr-insulin precursor is dissolved in 0.25 ml of 10 M acetic acid, and 0.7 ml of 1.5 M Thr-Arg(Boc)-Arg(Boc)-NH2 dissolved in DMSO/1,3-butanediol (1:1) is added. Then 0.15 ml of LEP (15 mg/ml, dissolved in water) is added. The mixture is incubated at room temperature for 2 h. The protein is then precipitated by adding 5 ml of methanol/methyl tert-butyl ether (v/v=1:4) and dried. The protective group is eliminated by adding 1 M HCl/acetic acid and incubating at 0°C.

The hypoglycemic effect is investigated on healthy male dogs of the Beagle breed. A dose of 0.3 IU/kg of body weight is administered subcutaneously. In a controlled group, dogs are treated with the same dose of insulin glargine, and a further group receives a placebo injection without added insulin. Blood is taken from the animals for blood glucose determination every half hour for the first 2 hours after injection, and then each hour up to the eighth hour. It emerges that insulin glargine amide as well as insulin glargine exhibit a delayed time/effect profile, but the profile of the amide is distinctly advantageous because, on onset of the reaction, the blood glucose level falls without a clear low point (nadir).

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8. PCT Application WO2011024024 titled "A process for recovering darbepoeitin alfa isoforms" teaches **a Fermentation process for large scale production of darbepoeitin from a cell culture comprising mammalian cells that contain a gene encoding darbepoeitin**

A bioreactor is assembled and sterilized at 121°C for 45 minutes before seeding. After sterilization, the reactor is charged with 7200ml of a commercially available animal component free, chemically defined media.

The bioreactor is kept under positive pressure with air at a flow rate of 0.2 Litre per minute and is aerated over night for 100% air saturation.
The dO2 electrode is calibrated after stabilization of dissolved oxygen value.

A sterile connection is created between the seed bottle and the seed port on the bioreactor head plate.
The seed is then aseptically transferred to the bioreactor using peristaltic pump. The bioreactor is seeded with the density of 0.4×10^6 cells/mL.

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After seeding, the bioreactor is allowed to run at following pre-set parameters: pH: 6.9 - 7.2, dO2: 30 - 60% of air saturation, temperature: 30 - 38°C, Stir speed: 70-80RPM. Samples are monitored every 24/48 hours quality control analysis.

Harvesting is carried out at a cell viability of 70 - 80%.
The bioreactor process is a fed - batch process with feeding of different nutrients at definite culture stages.

The harvested cell culture is clarified using a cellulose disposable filter with 650 - 1000 cm2 effective filtration area and with an operating pressure of not more than 30 psi. The clarified harvest is diafilitered and buffer exchanged with Acetate buffer pH 4.5 - 5.5 using a 30 kDa - TFF membrane at a Trans Membrane Pressure of 5 - 10 psi. 2 Molar NaCI is added to the diafiltered product.

Hydrophobic interaction chromatography is used in binding and elution mode with column of 100 mm diameter for capturing; with Acetate buffer pH 4.5 - 5.5 containing 2 Molar NaCI as equilibration buffer.

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After the sample is loaded on to the column, it is washed with equilibration buffer. The protein of interest is eluted with Acetate buffer pH 4.5 - 5.5.

The elution fraction is filtered for virus removal using viral removal filter having an effective filtration area of 0.001 m2 . The nano filtrate is buffer exchanged with Phosphate buffered saline (pH 6.2±0.2) to get the drug substance.

Finally the sample is filtered using 0.2µm filter. The drug substance is characterized as per the specifications. The Drug Substance (Active Pharmaceutical Ingredient) is formulated by adding Polysorbate 80 to a final concentration of 0.05 mg/ml of protein solution to get the drug product.

The drug product is characterized by Western Blot, Isoelectric Focusing, Reverse Phase-HPLC, Size Exclusion HPLC, MALDI-TOF MS analysis, Peptide Mapping by HPLC, Glycan profiling, Deglycosylation analysis, Relative potency analysis.

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