CLAIMS

1. An *in vitro* method for production of a heterodimeric protein comprising the following steps:

a) incubating a first homodimeric protein with a second homodimeric protein under reducing conditions sufficient to allow reduction of the inter-chain disulfide bonds in the hinge region, and

wherein said first homodimeric protein comprises an Fc region of an immunoglobulin, said Fc region comprising a first CH3 region, and said second homodimeric protein comprises an Fc region of an immunoglobulin, said Fc region comprising a second CH3 region, wherein said first homodimeric protein has an amino acid substitution in the CH3 region at a position selected from the group consisting of: 366, 368, 370, 399, 405, 407 and 409, and said second homodimeric protein has an amino acid substitution in the CH3 region at a position selected from the group consisting of: 366, 368, 370, 399, 405, 407 and 409, and wherein said first homodimeric protein and said second homodimeric protein is not substituted in the same positions so that the sequences of said first and second CH3 regions are different and are such that the heterodimeric interaction between said first and second CH3 regions is stronger than each of the homodimeric interactions of said first and second CH3 regions, ,

b) subjecting at least 10 mL of the composition obtained from step a) to oxidizing conditions sufficient to allow oxidation of cysteines in the heterodimeric protein to inter-chain disulfide bonds,

c) obtaining the heterodimeric protein.

2. An *in vitro* method according to any of the preceding claims, wherein the reducing conditions in step a) comprise adding a reducing agent.

3. An *in vitro* method according to claim 2, wherein the reducing agent is selected from the group consisting of: 2-mercaptoethylamine, a chemical derivative of 2-mercaptoethylamine, L-cysteine, and D-cysteine.

4. An *in vitro* method according to any of the preceding claims, wherein step a) comprises adding a metal chelating agent.

5. An *in vitro* method according to claim 4, wherein the metal chelating agent is EDTA, EGTA or citric acid.

6. An *in vitro* method according to any one of the preceding claims, wherein step a) is performed under reducing conditions with a redox potential between -150 and -600 mV, such as between -350 and -450 mV.

7. An *in vitro* method according to any one of the preceding claims, wherein step a) comprises incubation for at least 30 min at a temperature of at least 20°C in the presence of at least 25 mM of a reducing agent selected from the group consisting of 2-mercaptoethylamine, L-cysteine and D-cysteine.

8. An *in vitro* method according to any of the preceding claims, wherein the first and second homodimeric proteins are in a buffer.

9. An *in vitro* method according to claim 8, wherein the buffer comprises in the range of 1-100 mM phosphate, such as 1-50 mM phoshate or in the range of 1-25 mM, or in the range of 5-20 mM.

10. An *in vitro* method according to claim 9, wherein the buffer has pH in the range of 4.5-8.5, such as in the range of 6.5 to 7.5.

11. An *in vitro* method according to claims 9 - 10, wherein the buffer is selected from the group consisting of a) 8.1 mM sodium phosphate (Na_2HPO_4-$7H_2O$), 1.5 mM potassium phosphate (KH_2PO_4), 138 mM sodium chloride (NaCl), 2.7 mM potassium chloride (KCl) pH 5.0; b) 8.1 mM sodium phosphate (Na_2HPO_4-$7H_2O$), 1.5 mM potassium phosphate (KH_2PO_4), 138 mM sodium chloride (NaCl), 2.7 mM potassium chloride (KCl) pH 7.0; 3) 20 mM Tris-HCl, pH 7.8

12. An *in vitro* method according to any of the preceding claims, wherein step b) comprises a pH in the range of 6-8.5.

13. An *in vitro* method according to any of the preceding claims, wherein step b) comprises a redox potential of at least -300 mV.

14. An *in vitro* method according to any of the preceding claims, wherein the oxidizing conditions in step b) comprise the presence of at least 0.05 mM oxygen.

15. An *in vitro* method according to any of the preceding claims, wherein the oxidizing conditions in step b) comprise adding oxygen.

16. An *in vitro* method according to claim 15, wherein adding oxygen is performed mechanically, e.g. by agitating, stirring, increasing pressure or creating flow.

17. An *in vitro* method according to any of claims 15-16, wherein adding oxygen is performed by sparging with oxygen or air or increasing pressure.

18. An *in vitro* method according to any of the preceding steps, wherein the oxidizing conditions in step b) comprise an oxidizing agent.

19. An *in vitro* method according to claim 18, wherein the oxidizing agent is dehydroascorbic acid (dhAA).

20. An *in vitro* method according to any of the preceding claims, wherein step b) comprises separating the heterodimeric protein and the reducing agent.

21. An *in vitro* method according to any of the preceeding claims , wherein step b) comprises subjecting the composition obtained from step a) to chromatography or filtration.

22. An *in vitro* method according to claim 21, wherein the chromatography is column chromatography.

23. An *in vitro* method according to claim 21, wherein the filtration is diafiltration.

24. A method according to claim 23, wherein the diafiltration is tangential flow filtration (TFF) or normal flow filtration (NFF).

25. An *in vitro* method according to claim 24, wherein the diafiltration is TFF.

26. An *in vitro* method according to claim 23, wherein the diafiltration is performed by circulating the composition through a hollow fiber cartridge comprising a cut-off value in the range of 10-50 kDa, and with a surface area in the range of 0.05-1 m^2, and with a cartridge inlet pressure in the range of 70-280 kPa, until one to seven volumes of buffer exchange have taken place.

27. An *in vitro* method according to any of claims 23-26, wherein separating the heterodimeric protein and the reducing agent comprises exchanging the buffer or solution of the composition obtained from step a) with a buffer or solution without said reducing agent.

28. An *in vitro* method according to claim 27, comprising in the range of 3-12 volumes of buffer or solution exchanges, such as in the range of 4-10 volumes.

29. An *in vitro* method according to any of claims 21-27, wherein separating the heterodimeric protein and the reducing agent is a continous process.

30. An *in vitro* method according to any of claims 21-27, wherein separating the heterodimeric protein and the reducing agent is a batch process.

31. An *in vitro* method according to any of the preceding claims, wherein the oxidizing conditions in step b) comprise the steps of:

I) diafiltration of the composition obtained from step a)
II) incubation of the retentate obtained from step I)
III) diafiltration of the composition obtained from step II).

32. An *in vitro* method according to claim 31, wherein the diafiltration in steps I) and/or III) comprises in the range of 3-12 volumes of buffer exchange.

33. An *in vitro* method according to any of claims 31-32, wherein step II) comprises incubation at a temperature in the range of 15-35°C for a period of 12-48 hours.

34. An *in vitro* method according to any of the preceding claims, wherein the concentration of heterodimeric protein in the composition obtained from step a) is in the range of 1-100 g/L.

35. An *in vitro* method according to any of the preceding claims, wherein the oxidizing conditions in step b) comprise a metal ion.

36. An *in vitro* method according to claim 35, wherein the oxidizing conditions in step b) comprise adding a metal ion.

37. An *in vitro* method according to any of claims 35-36, wherein the concentration of the metal ion is in the range of 0.1 to 100 μM.

38. An *in vitro* method according to any of claims 35-37, wherein the metal ion is selected from the group consisting of: Copper, Manganese, Magnesium, Iron, Nickel and Cobalt.

39. An *in vitro* method according to any of the preceding claims, wherein the ratio of first to second homodimeric protein in step a) is in the range of 1:1.01 to 1:2.

40. An *in vitro* method according to any of the preceding claims, wherein either the first or second homodimeric protein is not able to bind to Protein A and/or Protein G.

41. An *in vitro* method according to any of the preceding claims, wherein the first and second homodimeric protein comprise different light chains.

42. An *in vitro* method according to claim 41, wherein step c) comprises separation of the heterodimeric protein from residual first and/or second homodimeric protein by passing it over a material to which only one of the light chains bind.

43. An *in vitro* method according to any of the preceding claims, wherein the Fc region of the first and second homodimeric protein are of different allotypes.

44. An *in vitro* method according to claim 41, wherein step c) comprises separation of the heterodimeric protein from residual first and/or second homodimeric protein by passing it over a material to which only one of the allotypes bind.

45. An *in vitro* method according to any of the preceding claims, wherein step c) comprises subjecting the composition obtained from step b) to a purification method.

46. An *in vitro* method according to claim 45, wherein the purification method is selected from the group consisting of protein A or protein G chromatography, affinity chromatography based on antigen-binding, affinity chromatography based on anti-idiotypic antibodies, ion exchange, hydrophobic interaction chromatography, Mixed Mode Chromatography (such as hydroxyapatite), Immobilized Metal Affinity Chromatography and Thiophilic Adsorption Chromatography.

47. An *in vitro* method according to any of the preceding claims, wherein step a) comprises the steps of:

i) providing a first homodimeric protein comprising an Fc region of an immunoglobulin, said Fc region comprising a first CH3 region,

ii) providing a second homodimeric protein comprising an Fc region of an immunoglobulin, said Fc region comprising a second CH3 region,

wherein the sequences of said first and second CH3 regions are different and are such that the heterodimeric interaction between said first and second CH3 regions is stronger than each of the homodimeric interactions of said first and second CH3 regions,

iii) incubating said first protein together with said second protein under reducing conditions sufficient to allow reduction of the inter-chain disulfide bonds in the hinge region.

48. An *in vitro* method according to any of the preceding claims, wherein step b) comprises subjecting at least 30 mL of the composition obtained from step a) to oxidizing conditions sufficient to allow oxidation of cysteines in the heterodimeric protein to inter-chain disulfide bonds.

49. An *in vitro* method according to any of the preceding claims, wherein the total concentration of first homodimeric and second homodimeric protein in step a) is at least 0.25 mg/mL.

50. An *in vitro* method according to any of the preceding claims, wherein said first homodimeric protein has no more than one amino acid substitution in the CH3 region, and the second homodimeric protein has no more than one amino acid substitution in the CH3 region relative to the wild-type CH3 regions.

51. An *in vitro* method according to any of the preceding claims, wherein said first homodimeric protein has an amino acid other than Lys, Leu or Met at position 409 and said second homodimeric protein has an amino acid substitution at a position selected from the group consisting of: 366, 368, 370, 399, 405 and 407.

52. An *in vitro* method according to any of the preceding claims, wherein said first homodimeric protein has an amino acid other than Lys, Leu or Met at position 409 and said second homodimeric protein has an amino acid other than Phe at position 405.

53. An *in vitro* method according to any of the preceding claims, wherein said first homodimeric protein has an amino acid other than Lys, Leu or Met at position 409 and said second homodimeric protein has an amino acid other than Phe, Arg or Gly at position 405.

54. An *in vitro* method according to any of the preceding claims, wherein said first homodimeric protein has an amino acid selected from the group consisting of Ala,

Asp, Glu, Phe, Gly, His, Ile, Asn, Gln, Arg, Ser, Thr, Val, Trp and Tyr at position 409 and said second homodimeric protein has an amino acid selected from the group consisting of Ala, Asp, Glu, Gly, His, Ile, Lys, Leu, Met, Asn, Gln, Arg, Ser, Thr, Val , Trp and Tyr at position 405.

55. An *in vitro* method according to claim 54, wherein said first homodimeric protein has an amino acid selected from the group consisting of Arg, Gly, His, Val and Ile at position 409, and said second homodimeric protein has Leu at position 405.

56. An *in vitro* method according to any of claims 51-55, wherein said first homodimeric protein has Arg at position 409.

57. An *in vitro* method according to any of claims 51-56, wherein said second homodimeric protein has Leu at position 405.

58. An *in vitro* method according to claim 57, wherein said first homodimeric protein has Arg at position 409 and said second homodimeric protein has Leu at position 405.

59. An *in vitro* method according to any of the preceding claims, wherein the first and/or second homodimeric protein do not contain the c-terminal lysine.

60. An *in vitro* method according to claim 59, wherein the first and/or second homodimeric proteins are genetically modified to lack the c-terminal lysine in the heavy chain.

61. An *in vitro* method according to claim 59, wherein said method comprises a step of removing the c-terminal lysine from the heavy chain.

CLAIMS

1. An *in vitro* method for production of a heterodimeric protein comprising the following steps:

a) incubating a first homodimeric protein with a second homodimeric protein under reducing conditions sufficient to allow reduction of the inter-chain disulfide bonds in the hinge region, and

wherein said first homodimeric protein comprises an Fc region of an immunoglobulin, said Fc region comprising a first CH3 region, and said second homodimeric protein comprises an Fc region of an immunoglobulin, said Fc region comprising a second CH3 region, wherein said first homodimeric protein has an amino acid substitution in the CH3 region at a position selected from the group consisting of: 366, 368, 370, 399, 405, 407 and 409, and said second homodimeric protein has an amino acid substitution in the CH3 region at a position selected from the group consisting of: 366, 368, 370, 399, 405, 407 and 409, and wherein said first homodimeric protein and said second homodimeric protein is not substituted in the same positions so that the sequences of said first and second CH3 regions are different and are such that the heterodimeric interaction between said first and second CH3 regions is stronger than each of the homodimeric interactions of said first and second CH3 regions, wherein the amino acid positions are numbered according to the EU-index,

b) subjecting at least 10 mL of the composition obtained from step a) to oxidizing conditions sufficient to allow oxidation of cysteines in the heterodimeric protein to inter-chain disulfide bonds,

c) obtaining the heterodimeric protein, wherein the method yields a heterodimeric protein wherein more than 80% of the total product is the desired heterodimeric protein.

2. An *in vitro* method according to any of the preceding claims, wherein the reducing conditions in step a) comprise adding a reducing agent.

3. An *in vitro* method according to claim 2, wherein the reducing agent is selected from the group consisting of: 2-mercaptoethylamine, a chemical derivative of 2-mercaptoethylamine, L-cysteine, and D-cysteine.

4. An *in vitro* method according to any of the preceding claims, wherein step a) comprises adding a metal chelating agent.

5. An *in vitro* method according to claim 4, wherein the metal chelating agent is EDTA, EGTA or citric acid.

6. An *in vitro* method according to any one of the preceding claims, wherein step a) is performed under reducing conditions with a redox potential between -150 and -600 mV, such as between -350 and -450 mV.

7. An *in vitro* method according to any one of the preceding claims, wherein step a) comprises incubation for at least 30 min at a temperature of at least 20°C in the presence of at least 25 mM of a reducing agent selected from the group consisting of 2-mercaptoethylamine, L-cysteine and D-cysteine.

8. An *in vitro* method according to any of the preceding claims, wherein the first and second homodimeric proteins are in a buffer.

9. An *in vitro* method according to claim 8, wherein the buffer comprises in the range of 1-100 mM phosphate, such as 1-50 mM phoshate or in the range of 1-25 mM, or in the range of 5-20 mM.

10. An *in vitro* method according to claim 9, wherein the buffer has pH in the range of 4.5-8.5, such as in the range of 6.5 to 7.5.

11. An *in vitro* method according to claims 9 - 10, wherein the buffer is selected from the group consisting of a) 8.1 mM sodium phosphate (Na_2HPO_4-$7H_2O$), 1.5 mM potassium phosphate (KH_2PO_4), 138 mM sodium chloride (NaCl), 2.7 mM potassium chloride (KCl) pH 5.0; b) 8.1 mM sodium phosphate (Na_2HPO_4-$7H_2O$), 1.5 mM potassium phosphate (KH_2PO_4), 138 mM sodium chloride (NaCl), 2.7 mM potassium chloride (KCl) pH 7.0; 3) 20 mM Tris-HCl, pH 7.8

12. An *in vitro* method according to any of the preceding claims, wherein step b) comprises a pH in the range of 6-8.5.

13. An *in vitro* method according to any of the preceding claims, wherein step b) comprises a redox potential of at least -300 mV.

14. An *in vitro* method according to any of the preceding claims, wherein the oxidizing conditions in step b) comprise the presence of at least 0.05 mM oxygen.

15. An *in vitro* method according to any of the preceding claims, wherein the oxidizing conditions in step b) comprise adding oxygen.

16. An *in vitro* method according to claim 15, wherein adding oxygen is performed mechanically, e.g. by agitating, stirring, increasing pressure or creating flow.

17. An *in vitro* method according to any of claims 15-16, wherein adding oxygen is performed by sparging with oxygen or air or increasing pressure.

18. An *in vitro* method according to any of the preceding steps, wherein the oxidizing conditions in step b) comprise an oxidizing agent.

19. An *in vitro* method according to claim 18, wherein the oxidizing agent is dehydroascorbic acid (dhAA).

20. An *in vitro* method according to any of the preceding claims, wherein step b) comprises separating the heterodimeric protein and the reducing agent.

21. An *in vitro* method according to any of the preceeding claims , wherein step b) comprises subjecting the composition obtained from step a) to chromatography or filtration.

22. An *in vitro* method according to claim 21, wherein the chromatography is column chromatography.

23. An *in vitro* method according to claim 21, wherein the filtration is diafiltration.

24. A method according to claim 23, wherein the diafiltration is tangential flow filtration (TFF) or normal flow filtration (NFF).

25. An *in vitro* method according to claim 24, wherein the diafiltration is TFF.

26. An *in vitro* method according to claim 23, wherein the diafiltration is performed by circulating the composition through a hollow fiber cartridge comprising a cut-off value in the range of 10-50 kDa, and with a surface area in the range of 0.05-1 m^2, and with a cartridge inlet pressure in the range of 70-280 kPa, until one to seven volumes of buffer exchange have taken place.

27. An *in vitro* method according to any of claims 23-26, wherein separating the heterodimeric protein and the reducing agent comprises exchanging the buffer or solution of the composition obtained from step a) with a buffer or solution without said reducing agent.

28. An *in vitro* method according to claim 27, comprising in the range of 3-12 volumes of buffer or solution exchanges, such as in the range of 4-10 volumes.

29. An *in vitro* method according to any of claims 21-27, wherein separating the heterodimeric protein and the reducing agent is a continous process.

30. An *in vitro* method according to any of claims 21-27, wherein separating the heterodimeric protein and the reducing agent is a batch process.

31. An *in vitro* method according to any of the preceding claims, wherein the oxidizing conditions in step b) comprise the steps of:

I) diafiltration of the composition obtained from step a)
II) incubation of the retentate obtained from step I)
III) diafiltration of the composition obtained from step II).

32. An *in vitro* method according to claim 31, wherein the diafiltration in steps I) and/or III) comprises in the range of 3-12 volumes of buffer exchange.

33. An *in vitro* method according to any of claims 31-32, wherein step II) comprises incubation at a temperature in the range of 15-35°C for a period of 12-48 hours.

34. An *in vitro* method according to any of the preceding claims, wherein the concentration of heterodimeric protein in the composition obtained from step a) is in the range of 1-100 g/L.

35. An *in vitro* method according to any of the preceding claims, wherein the oxidizing conditions in step b) comprise a metal ion.

36. An *in vitro* method according to claim 35, wherein the oxidizing conditions in step b) comprise adding a metal ion.

37. An *in vitro* method according to any of claims 35-36, wherein the concentration of the metal ion is in the range of 0.1 to 100 μM.

38. An *in vitro* method according to any of claims 35-37, wherein the metal ion is selected from the group consisting of: Copper, Manganese, Magnesium, Iron, Nickel and Cobalt.

39. An *in vitro* method according to any of the preceding claims, wherein the ratio of first to second homodimeric protein in step a) is in the range of 1:1.01 to 1:2.

40. An *in vitro* method according to any of the preceding claims, wherein either the first or second homodimeric protein is not able to bind to Protein A and/or Protein G.

41. An *in vitro* method according to any of the preceding claims, wherein the first and second homodimeric protein comprise different light chains.

42. An *in vitro* method according to claim 41, wherein step c) comprises separation of the heterodimeric protein from residual first and/or second

homodimeric protein by passing it over a material to which only one of the light chains bind.

43. An *in vitro* method according to any of the preceding claims, wherein the Fc region of the first and second homodimeric protein are of different allotypes.

44. An *in vitro* method according to claim 41, wherein step c) comprises separation of the heterodimeric protein from residual first and/or second homodimeric protein by passing it over a material to which only one of the allotypes bind.

45. An *in vitro* method according to any of the preceding claims, wherein step c) comprises subjecting the composition obtained from step b) to a purification method.

46. An *in vitro* method according to claim 45, wherein the purification method is selected from the group consisting of protein A or protein G chromatography, affinity chromatography based on antigen-binding, affinity chromatography based on anti-idiotypic antibodies, ion exchange, hydrophobic interaction chromatography, Mixed Mode Chromatography (such as hydroxyapatite), Immobilized Metal Affinity Chromatography and Thiophilic Adsorption Chromatography.

47. An *in vitro* method according to any of the preceding claims, wherein step a) comprises the steps of:

i) providing a first homodimeric protein comprising an Fc region of an immunoglobulin, said Fc region comprising a first CH3 region,

ii) providing a second homodimeric protein comprising an Fc region of an immunoglobulin, said Fc region comprising a second CH3 region,

wherein the sequences of said first and second CH3 regions are different and are such that the heterodimeric interaction between said first and second CH3

regions is stronger than each of the homodimeric interactions of said first and second CH3 regions,

iii) incubating said first protein together with said second protein under reducing conditions sufficient to allow reduction of the inter-chain disulfide bonds in the hinge region.

48. An *in vitro* method according to any of the preceding claims, wherein step b) comprises subjecting at least 30 mL of the composition obtained from step a) to oxidizing conditions sufficient to allow oxidation of cysteines in the heterodimeric protein to inter-chain disulfide bonds.

49. An *in vitro* method according to any of the preceding claims, wherein the total concentration of first homodimeric and second homodimeric protein in step a) is at least 0.25 mg/mL.

50. An *in vitro* method according to any of the preceding claims, wherein said first homodimeric protein has no more than one amino acid substitution in the CH3 region, and the second homodimeric protein has no more than one amino acid substitution in the CH3 region relative to the wild-type CH3 regions.

51. An *in vitro* method according to any of the preceding claims, wherein said first homodimeric protein has an amino acid other than Lys, Leu or Met at position 409 and said second homodimeric protein has an amino acid substitution at a position selected from the group consisting of: 366, 368, 370, 399, 405 and 407.

52. An *in vitro* method according to any of the preceding claims, wherein said first homodimeric protein has an amino acid other than Lys, Leu or Met at position 409 and said second homodimeric protein has an amino acid other than Phe at position 405.

53. An *in vitro* method according to any of the preceding claims, wherein said first homodimeric protein has an amino acid other than Lys, Leu or Met at position 409 and said second homodimeric protein has an amino acid other than Phe, Arg or Gly at position 405.

54. An *in vitro* method according to any of the preceding claims, wherein said first homodimeric protein has an amino acid selected from the group consisting of Ala, Asp, Glu, Phe, Gly, His, Ile, Asn, Gln, Arg, Ser, Thr, Val, Trp and Tyr at position 409 and said second homodimeric protein has an amino acid selected from the group consisting of Ala, Asp, Glu, Gly, His, Ile, Lys, Leu, Met, Asn, Gln, Arg, Ser, Thr, Val , Trp and Tyr at position 405.

55. An *in vitro* method according to claim 54, wherein said first homodimeric protein has an amino acid selected from the group consisting of Arg, Gly, His, Val and Ile at position 409, and said second homodimeric protein has Leu at position 405.

56. An *in vitro* method according to any of claims 51-55, wherein said first homodimeric protein has Arg at position 409.

57. An *in vitro* method according to any of claims 51-56, wherein said second homodimeric protein has Leu at position 405.

58. An *in vitro* method according to claim 657, wherein said first homodimeric protein has Arg at position 409 and said second homodimeric protein has Leu at position 405.

59. An *in vitro* method according to any of the preceding claims, wherein the first and/or second homodimeric protein do not contain the c-terminal lysine.

60. An *in vitro* method according to claim 59, wherein the first and/or second homodimeric proteins are genetically modified to lack the c-terminal lysine in the heavy chain.

61. An *in vitro* method according to claim 59, wherein said method comprises a step of removing the c-terminal lysine from the heavy chain.