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SCAFFOLD PROTEINS

ABSTRACT

The invention relates to a polypeptide, such as an Affimer polypeptide, comprising an amino acid sequence having at least 80% identity to amino acid residues 1 to 11, 13 to 15, 17 to 19, 21 to 25, 27 to 28, 35 to 37, 39, 41, 43 to 44, 46 to 47, 49 to 50, 52 to 53, 55 to 58, 63 to 64, 66, 68 to 82, 84 to 85, and 87 to 98 of SEQ ID NO: 1;

characterised in that said polypeptide comprises one or more mutations relative to SEQ ID NO: 1 selected from the group consisting of:

T51L, T51V, M65V, N32G, A59I, L38A, V20I, A40I, L38V, A12I, A12V, I16L, V20L, Q26E, E29M, T31K, N32D, N32H, T34V, T34R, T34D, T34P, A40V, Q42D, T45I, T45V, V48E, V48G, V48A, T51F, T51A, A59L, L67I, (V20I, L38A), (V20L, L38A), (V20I, L38V), (V20L, L38V), (E29K, K30E, E33K), (Y54D, T83D, Q86E), (A59L, G60N, D61G, N62K), (A59V, D61N, N62K), (G60N, D61G, N62K), (G60N, ΔD61, N62G), ΔD61, (A59L, G60N, ΔD61, N62G), (A59V, G60N, D61G, N62K), (A59I, G60N, D61G, N62K), (A59I, G60N, ΔD61, N62G), (A59V, G60N, ΔD61, N62G), (A59V, ΔD61), (G60P, ΔD61, N62P), (G60P, D61P, N62K), (G60P, ΔD61, N62G), (G60P, D61G, N62K), (D61N, N62K) and (T83D, Q86E).

The invention also relates to various methods and nucleic acids.

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Background to the Invention

WO 2009/136182 discloses the mutation of prior art scaffold STM using D48L and G50S, which are disclosed as leading to increased expression in the bacterial system (page 22, lines 12 to 13).

WO 2009/136182 as well as WO 2006/131749 have each previously disclosed mutation of the V48 site of wild-type Stefin A, in particular to V48D which is useful for the abolition of domain-swap dimerisation (WO 2006/131749 page 35, line 25).

Simultaneous mutation of E78A and L80R has been disclosed for scaffold SQT in WO 2009/136182, as well as disclosing simultaneous mutation of L82R and T83S in the SQM scaffold (paragraph bridging pages 22 to 23 of WO 2009/136182). These pairs of simultaneous mutations have been disclosed as exhibiting high expression in *E. coli*.

WO 2009/136182 discloses modified Stefin A scaffold proteins. In particular, the disclosures in this document focus on the "position 4 mutation", which corresponds to the G4 site of Stefin A or the W4 site of STM. In particular, this document teaches the special advantages of a G4R mutant. This document makes no teachings whatsoever regarding alteration of thermal stability of the scaffold protein. Thermal stability is mentioned in only one paragraph in this document, merely as a one of several properties which might be measured in order to assess a scaffold protein's resistance to being deformed by a target peptide (WO 2009/136182 page 13, first paragraph).

WO 2006/131749 discloses the use of Stefin A as a scaffold protein, and discloses several mutations useful for ensuring biological neutrality of the scaffold. This document discloses a thermostability assay (page 32, first paragraph) and shows data on the heat stability of STM (page 35, lines 9 to 12). Thermostability in this document is solely disclosed as a property which can be measured in order to assess the scaffold protein's resistance to being deformed by the target peptide (see paragraph bridging pages 6 to 7 of WO 2006/131749). There is no teaching whatsoever regarding the variation or modulation of the thermal stability in this document.

WO 2014/125290 discloses scaffold proteins derived from plant cystatins. In particular, this document discloses three preferred synthetic proteins as SEQ ID No. 1, SEQ ID No. 2 and SEQ ID No. 3 of WO 2014/125290. This document discloses measurement of the thermal stability of their preferred scaffold by differential scanning calorimetry (page 48, lines 31 to 32 and Figure 7a). There is no disclosure anywhere in this document of mutations which might be made to affect the thermal stability. Indeed, the inventors do not comment on any way of affecting the thermal stability of their scaffold proteins. They assert that the thermal stability of their preferred scaffold is "high", but attribute this to the compact nature of their scaffold:

"The compact nature of the scaffold, which is more pronounced than seen in other structures of stefins or cystatins seems likely to contribute to its high thermal stability." (WO 2014/125290 page 53, lines 17 to 19).

There is no teaching in this document as to how thermal stability might be modulated or altered. Moreover, the only discussion of mutations in this document is in connection with prior art mutations, or with the need to use trimer insertions or deletions so as not to produce frame shift mutations. The scientific focus of this document is concentrated on designing a consensus sequence derived from plant cystatin proteins – there is no disclosure of manipulating a sequence to influence thermal stability.

Summary of the Invention

The inventors studied the Stefin A scaffold protein with aims of improving its properties such as its thermal stability. Building on their insights into the protein structure and function, they designed a range of changes and substitutions to improve those properties. This led to various surprising results including unpredicted anomalies where for example it might have been expected for the protein to behave in a certain manner, but for which the objective findings were at odds with predictions (for example the Q42D/E anomaly discussed in detail below). The result of this substantial research and intellectual effort is a comprehensive teaching of how to alter the thermal stability of the Stefin A protein and how those changes in thermal stability deliver benefits to its use as a scaffold protein.

Thus the invention relates to a polypeptide, such as an Affimer polypeptide, comprising an amino acid sequence having at least 80% identity to amino acid residues 1 to 11, 13 to 15, 17 to 19, 21 to 25, 27 to 28, 35 to 37, 39, 41, 43 to 44, 46 to 47, 49 to 50, 52 to 53, 55 to 58, 63 to 64, 66, 68 to 82, 84 to 85, and 87 to 98 of SEQ ID NO: 1;

characterised in that said polypeptide comprises one or more mutations relative to SEQ ID NO: 1 selected from the group consisting of:

T51L, T51V, M65V, N32G, A59I, L38A, V20I, A40I, L38V, A12I, A12V, I16L, V20L, Q26E, E29M, T31K, N32D, N32H, T34V, T34R, T34D, T34P, A40V, Q42D, T45I, T45V, V48E, V48G, V48A, T51F, T51A, A59L, L67I, (V20I, L38A), (V20L, L38A), (V20I, L38V), (V20L, L38V), (E29K, K30E, E33K), (Y54D, T83D, Q86E), (A59L, G60N, D61G, N62K), (A59V, D61N, N62K), (G60N, D61G, N62K), (G60N, ΔD61, N62G), ΔD61, (A59L, G60N, ΔD61, N62G), (A59V, G60N, D61G, N62K), (A59I, G60N, D61G, N62K), (A59I, G60N, ΔD61, N62G), (A59V, G60N, ΔD61, N62G), (A59V, ΔD61), (G60P, ΔD61, N62P), (G60P, D61P, N62K), (G60P, ΔD61, N62G), (G60P, D61G, N62K), (D61N, N62K) and (T83D, Q86E).

The invention relates to a polypeptide, such as an Affimer polypeptide, comprising an amino acid sequence having at least 80% identity to SEQ ID NO: 1;

characterised in that said polypeptide comprises one or more mutations relative to SEQ ID NO: 1 selected from the group consisting of:

T51L, T51V, M65V, N32G, A59I, L38A, V20I, A40I, L38V, A12I, A12V, I16L, V20L, Q26E, E29M, T31K, N32D, N32H, T34V, T34R, T34D, T34P, A40V, Q42D, T45I, T45V, V48E, V48G, V48A, T51F, T51A, A59L, L67I, (V20I, L38A), (V20L, L38A), (V20I, L38V), (V20L, L38V), (E29K, K30E, E33K), (Y54D, T83D, Q86E), (A59L, G60N, D61G, N62K), (A59V, D61N, N62K), (G60N, D61G, N62K), (G60N, ΔD61, N62G), ΔD61, (A59L, G60N, ΔD61, N62G), (A59V, G60N, D61G, N62K), (A59I, G60N, D61G, N62K), (A59I, G60N, ΔD61, N62G), (A59V, G60N, ΔD61, N62G), (A59V, ΔD61), (G60P, ΔD61, N62P), (G60P, D61P, N62K), (G60P, ΔD61, N62G), (G60P, D61G, N62K), (D61N, N62K) and (T83D, Q86E).

The invention relates to a polypeptide, such as an Affimer polypeptide, comprising an amino acid sequence having at least 80% identity to amino acid residues 1 to 98 of SEQ ID NO: 1;

characterised in that said polypeptide comprises one or more mutations relative to SEQ ID NO: 1 selected from the group consisting of:

T51L, T51V, M65V, N32G, A59I, L38A, V20I, A40I, L38V, A12I, A12V, I16L, V20L, Q26E, E29M, T31K, N32D, N32H, T34V, T34R, T34D, T34P, A40V, Q42D, T45I, T45V, V48E, V48G, V48A, T51F, T51A, A59L, L67I, (V20I, L38A), (V20L, L38A), (V20I, L38V), (V20L, L38V), (E29K, K30E, E33K), (Y54D, T83D, Q86E), (A59L, G60N, D61G, N62K), (A59V, D61N, N62K), (G60N, D61G, N62K), (G60N, ΔD61, N62G), ΔD61, (A59L, G60N, ΔD61, N62G), (A59V, G60N, D61G, N62K), (A59I, G60N, D61G, N62K), (A59I, G60N, ΔD61, N62G), (A59V, G60N, ΔD61, N62G), (A59V, ΔD61), (G60P, ΔD61, N62P), (G60P, D61P, N62K), (G60P, ΔD61, N62G), (G60P, D61G, N62K), (D61N, N62K) and (T83D, Q86E).

Suitably said one or more mutations relative to SEQ ID NO: 1 is or are selected from the group consisting of:

T51L, T51V, M65V, N32G, A59I, E29M, T34V, T34R, T45I, T45V, T51F, A59L, L67I, (E29K, K30E, E33K), (A59L, G60N, D61G, N62K), (A59V, D61N, N62K), (G60N, D61G, N62K), (G60N, ΔD61, N62G), ΔD61, (A59L, G60N, ΔD61, N62G), (A59V, G60N, D61G, N62K), (A59I, G60N, D61G, N62K), (A59I, G60N, ΔD61, N62G), (A59V, G60N, ΔD61, N62G), and (A59V, ΔD61);

preferably wherein said polypeptide has a Tm higher than the Tm of SEQ ID NO: 1.

Suitably said one or more mutations relative to SEQ ID NO: 1 is or are selected from the group consisting of:

L38A, V20I, A40I, L38V, A12I, A12V, I16L, V20L, Q26E, T31K, N32D, N32H, T34D, T34P, A40V, Q42D, V48E, V48G, V48A, T51A, (V20I, L38A), (V20L, L38A), (V20I, L38V), (V20L, L38V), (Y54D, T83D, Q86E), (G60P, ΔD61, N62P), (G60P, D61P, N62K), (G60P, ΔD61, N62G), (G60P, D61G, N62K), (D61N, N62K) and (T83D, Q86E);

preferably wherein said polypeptide has a Tm lower than the Tm of SEQ ID NO: 1.

Another aspect of the invention provides a polypeptide, such as an Affimer polypeptide, comprising an amino acid sequence represented in the general formula:

MIP-Xaa1-GLSEAKPA^{12}TPEI^{16}QEIV^{20}DKVKPQ^{26}LEE^{29}K^{30}T^{31}N^{32}E^{33}T^{34}YGKL^{38}EA^{40}VQ^{42}YKT^{45}QVV^{48}A-(Xaa)n-Xaa2-T^{51}NYY^{54}IKVRA^{59}G^{60}D^{61}N^{62}KYM^{65}HL^{67}KVF-Xaa3-Xaa4-Xaa5-(Xaa)m-Xaa6-D-Xaa7-VLT^{83}GYQ^{86}VDKNKDDELTGF

wherein

Xaa, individually for each occurrence, is an amino acid residue;
n and m are each, independently, an integer from 3 to 20;

Xaa1 is Gly, Ala, Val, Arg, Lys, Asp, or Glu;
Xaa2 is Gly, Ala, Val, Ser or Thr;
Xaa3 is Arg, Lys, Asn, Gln, Ser, Thr;
Xaa4 is Gly, Ala, Val, Ser or Thr;
Xaa5 is Ala, Val, Ile, Leu, Gly or Pro;
Xaa6 is Gly, Ala, Val, Asp or Glu; and
Xaa7 is Ala, Val, Ile, Leu, Arg or Lys;

and wherein at least one of the following amino acid positions is selected from a recited alternative amino residue

A^{12} is Ala, or an alternative amino acid residue selected from Val, Ile or Leu, and preferably from Ile or Val;
I^{16} is Ile or an alternative amino acid residue selected from Val or Leu, preferably Leu;
V^{20} is Val or an alternative amino acid residue selected from Ala, Ile or Leu, preferably Ile or Leu;
Q^{26} is Gln or an alternative amino acid residue selected from Asp or Glu, preferably Glu;
E^{29} is Glu, or an alternative amino acid residue selected from Asp or Met, preferably Met;
K^{30} is Lys, or an alternative amino acid residue selected from Arg, His, Glu or Asp, preferably Glu;
T^{31} is Thr, or an alternative amino acid residue selected from Ser, Arg or Lys, preferably Lys;
N^{32} is Asn, or an alternative amino acid residue selected from Gly, Asp, Glu or His, preferably Gly, Asp or His;
E^{33} is Glu, or an alternative amino acid residue selected from Arg, His, Lys or Asp, preferably Lys;

T^{34} is Thr, or an alternative amino acid residue selected from Ser, Ala, Val, Ile, Leu, Arg, Lys, Asp, Glu or Pro, preferably Val, Arg, Asp or Pro;
L^{38} is Leu, or an alternative amino acid residue selected from Gly, Ala or Val, preferably Ala or Val;
A^{40} is Ala, or an alternative amino acid residue selected from Gly, Val, Leu or Ile, preferably Ile or Val;
Q^{42} is Gln, or an alternative amino acid residue selected from Asp, Glu, or Asn, preferably Asp;
T^{45} is Thr, or an alternative amino acid residue selected from Ser, Ala, Val, Ile or Leu, preferably Ile or Val;
V^{48} is Val, or an alternative amino acid residue selected from Gly, Ala, Ile, Leu, Glu or Asp, preferably Gly, Ala or Glu;
T^{51} is Thr, or an alternative amino acid residue selected from Gly, Ala, Val, Ile, Leu, Ser or Phe, preferably Phe, Ala, Val or Leu;
Y^{54} is Tyr, or an alternative amino acid residue selected from Asp or Glu, preferably Asp;
A^{59} is Ala, or an alternative amino acid residue selected from Gly, Val, Ile or Leu, preferably Ile, Leu or Val;
G^{60} is Gly, or an alternative amino acid residue selected from Asn, Gln, Gly or Pro, preferably Asn or Pro;
D^{61} is Asp, or an alternative amino acid residue selected from ΔD61 (absent), Gly, Pro, Glu, Asn or Gln, preferably ΔD61 (absent), Gly, Pro or Asn;
N^{62} is Asn, or an alternative amino acid residue selected from Gln, Lys, Arg, His, Gly or Pro, preferably Lys, Pro or Gly;
M^{65} is Met, or an alternative amino acid residue selected from Ala, Val, Ile, Leu, preferably Val;
L^{67} is Leu, or an alternative amino acid residue selected from Ala, Val or Ile, preferably Ile;
T^{83} is Thr, or an alternative amino acid residue selected from Ser, Asp or Glu, preferably Asp; and
Q^{86} is Gln, or an alternative amino acid residue selected from Asn, Glu or Asp, preferably Glu.

In one aspect, the invention relates to a polypeptide as described above,

wherein said polypeptide further comprises at least one heterologous peptide insertion,

wherein said heterologous peptide insertion comprises a heterologous peptide inserted at one of the following positions relative to SEQ ID NO: 1:

a) 47-<heterologous peptide>-55

b) 46-<heterologous peptide>-54

c) 46-<heterologous peptide>-50

d) 48-<heterologous peptide>-50

e) 49-<heterologous peptide>-51

f) 50-<heterologous peptide>-52

g) 66-<heterologous peptide>-85

h) 67-<heterologous peptide>-84

i) 70-<heterologous peptide>-74

j) 72-<heterologous peptide>-74

k) 71-<heterologous peptide>-73

l) 72-<heterologous peptide>-81

m) 73-<heterologous peptide>-80

n) 79-<heterologous peptide>-81

o) 80-<heterologous peptide>-81

p) 82-<heterologous peptide>-83

q) 72-<heterologous peptide>-77

r) 73-<heterologous peptide>-78

s) 74-<heterologous peptide>-79

t) 4-<heterologous peptide>-5

Suitably said polypeptide comprises two heterologous peptide insertions, a first heterologous peptide insertion at any of positions (a) to (f), and a second heterologous peptide insertion at any of positions (g) to (s).

Suitably said polypeptide comprises two heterologous peptide insertions, a first heterologous peptide insertion at any of positions (a) to (f), and a second heterologous peptide insertion at position (t).

Suitably said polypeptide comprises two heterologous peptide insertions, a first heterologous peptide insertion at any of positions (g) to (s), and a second heterologous peptide insertion at position (t).

Suitably said polypeptide comprises three heterologous peptide insertions, a first heterologous peptide insertion at any of positions (a) to (f), and a second heterologous peptide insertion at any of positions (g) to (s), and a third heterologous peptide insertion at position (t).

In one aspect, the invention relates to a polypeptide, such as an Affimer polypeptide, comprising an amino acid sequence having at least 80% identity to amino acid residues 1 to 11, 13 to 15, 17 to 19, 21 to 25, 27 to 28, 35 to 37, 39, 41, 43 to 44, 46 to 47, 49 to 50, 52 to 53, 55 to 58, 63 to 64, 66, 68 to 82, 84 to 85, and 87 to 98 of SEQ ID NO: 1;

wherein said polypeptide comprises at least one heterologous peptide insertion;

characterised in that said polypeptide comprises one or more mutations relative to SEQ ID NO: 1 selected from the group consisting of:

M65I, T51I, T51L, T51V, M65V, A59V, N32G, A59I, L38A, V20A, V20I, A40I, L38V, G50S, L38F, A12I, A12V, I16L, V20L, Q26E, E29M, T31K, N32D, N32H, T34V, T34R, T34K, T34D, T34P, A40V, Q42E, Q42D, T45I, T45V, V48E, V48D, V48G, V48A, V48L, T51F, T51A, A59L, K63R, L67I, N90T, (V20I, L38A), (V20L, L38A), (V20I, L38V), (V20L, L38V), (E29K, K30E, E33K), (Y54D, T83D, Q86E), (A59L, G60N, D61G, N62K), (A59V, D61N, N62K), (G60N, D61G, N62K), (G60N, ΔD61, N62G), ΔD61, (A59L, G60N, ΔD61, N62G), (A59V, G60N, D61G, N62K), (A59I, G60N, D61G, N62K), (A59I, G60N, ΔD61, N62G), (A59V, G60N, ΔD61, N62G), (A59V, ΔD61), (G60P, ΔD61, N62P), (G60P, D61P, N62K), (G60P, ΔD61, N62G), (G60P, D61G, N62K), (D61N, N62K), and (T83D, Q86E);

wherein said heterologous peptide insertion comprises a heterologous peptide inserted at one of the following positions relative to SEQ ID NO: 1:

d) 48-<heterologous peptide>-50,

e) 49-<heterologous peptide>-51,

f) 50-<heterologous peptide>-52,

q) 72-<heterologous peptide>-77,

r) 73-<heterologous peptide>-78; or

s) 74-<heterologous peptide>-79.

In one aspect, the invention relates to a polypeptide, such as an Affimer polypeptide, comprising an amino acid sequence having at least 80% identity to SEQ ID NO: 1;

wherein said polypeptide comprises at least one heterologous peptide insertion;

characterised in that said polypeptide comprises one or more mutations relative to SEQ ID NO: 1 selected from the group consisting of:

M65I, T51I, T51L, T51V, M65V, A59V, N32G, A59I, L38A, V20A, V20I, A40I, L38V, G50S, L38F, A12I, A12V, I16L, V20L, Q26E, E29M, T31K, N32D, N32H, T34V, T34R, T34K, T34D, T34P, A40V, Q42E, Q42D, T45I, T45V, V48E, V48D, V48G, V48A, V48L, T51F, T51A, A59L, K63R, L67I, N90T, (V20I, L38A), (V20L, L38A), (V20I, L38V), (V20L, L38V), (E29K, K30E, E33K), (Y54D, T83D, Q86E), (A59L, G60N, D61G, N62K), (A59V, D61N, N62K), (G60N, D61G, N62K), (G60N, ΔD61, N62G), ΔD61, (A59L, G60N, ΔD61, N62G), (A59V, G60N, D61G, N62K), (A59I, G60N, D61G, N62K), (A59I, G60N, ΔD61, N62G), (A59V, G60N, ΔD61, N62G), (A59V, ΔD61), (G60P, ΔD61, N62P), (G60P, D61P, N62K), (G60P, ΔD61, N62G), (G60P, D61G, N62K), (D61N, N62K), and (T83D, Q86E);

wherein said heterologous peptide insertion comprises a heterologous peptide inserted at one of the following positions relative to SEQ ID NO: 1:

d) 48-<heterologous peptide>-50,

e) 49-<heterologous peptide>-51,

f) 50-<heterologous peptide>-52,

q) 72-<heterologous peptide>-77,

r) 73-<heterologous peptide>-78; or

s) 74-<heterologous peptide>-79.

In one aspect, the invention relates to a polypeptide, such as an Affimer polypeptide, comprising an amino acid sequence having at least 80% identity to amino acid residues 1 to 98 of SEQ ID NO: 1;

wherein said polypeptide comprises at least one heterologous peptide insertion;

characterised in that said polypeptide comprises one or more mutations relative to SEQ ID NO: 1 selected from the group consisting of:

M65I, T51I, T51L, T51V, M65V, A59V, N32G, A59I, L38A, V20A, V20I, A40I, L38V, G50S, L38F, A12I, A12V, I16L, V20L, Q26E, E29M, T31K, N32D, N32H, T34V, T34R, T34K, T34D, T34P, A40V, Q42E, Q42D, T45I, T45V, V48E, V48D, V48G, V48A, V48L, T51F, T51A, A59L, K63R, L67I, N90T, (V20I, L38A), (V20L, L38A), (V20I, L38V), (V20L, L38V), (E29K, K30E, E33K), (Y54D, T83D, Q86E), (A59L, G60N, D61G, N62K), (A59V, D61N, N62K), (G60N, D61G, N62K), (G60N, ΔD61, N62G), ΔD61, (A59L, G60N, ΔD61, N62G), (A59V, G60N, D61G, N62K), (A59I, G60N, D61G, N62K), (A59I, G60N, ΔD61, N62G), (A59V, G60N, ΔD61, N62G), (A59V, ΔD61), (G60P, ΔD61, N62P), (G60P, D61P, N62K), (G60P, ΔD61, N62G), (G60P, D61G, N62K), (D61N, N62K), and (T83D, Q86E);

wherein said heterologous peptide insertion comprises a heterologous peptide inserted at one of the following positions relative to SEQ ID NO: 1:

d) 48-<heterologous peptide>-50,

e) 49-<heterologous peptide>-51,

f) 50-<heterologous peptide>-52,

q) 72-<heterologous peptide>-77,

r) 73-<heterologous peptide>-78; or

s) 74-<heterologous peptide>-79.

Suitably said polypeptide comprises two heterologous peptide insertions, a first heterologous peptide insertion at any of positions (d) to (f), and a second heterologous peptide insertion at any of positions (q) to (s).

Suitably said one or more mutations relative to SEQ ID NO: 1 is or are selected from the group consisting of:

M65I, T51I, T51L, T51V, M65V, A59V, N32G, A59I, E29M, T34V, T34R, T34K, Q42E, T45I, T45V, T51F, A59L, K63R, L67I, N90T, (E29K, K30E, E33K), (A59L, G60N, D61G, N62K), (A59V, D61N, N62K), (G60N, D61G, N62K), (G60N, ΔD61, N62G), ΔD61, (A59L, G60N, ΔD61, N62G), (A59V, G60N, D61G, N62K), (A59I, G60N, D61G, N62K), (A59I, G60N, ΔD61, N62G), (A59V, G60N, ΔD61, N62G), and (A59V, ΔD61);

preferably wherein said polypeptide has a Tm higher than the Tm of SEQ ID NO: 1.

Suitably said one or more mutations relative to SEQ ID NO: 1 is or are selected from the group consisting of:

L38A, V20A, V20I, A40I, L38V, G50S, L38F, A12I, A12V, I16L, V20L, Q26E, T31K, N32D, N32H, T34D, T34P, A40V, Q42D, V48E, V48D, V48G, V48A, V48L, T51A, (V20I, L38A), (V20L, L38A), (V20I, L38V), (V20L, L38V), (Y54D, T83D, Q86E), (G60P, ΔD61, N62P), (G60P, D61P, N62K), (G60P, ΔD61, N62G), (G60P, D61G, N62K), (D61N, N62K) and (T83D, Q86E);

preferably wherein said polypeptide has a Tm lower than the Tm of SEQ ID NO: 1.

Suitably said one or more mutations relative to SEQ ID NO: 1 is or are selected from the group consisting of:

T51L, T51V, M65V, N32G, A59I, L38A, V20I, A40I, L38V, A12I, A12V, I16L, V20L, Q26E, E29M, T31K, N32D, N32H, T34V, T34R, T34D, T34P, A40V, Q42D, T45I, T45V, V48E, V48G, V48A, T51F, T51A, A59L, L67I, (V20I, L38A), (V20L, L38A), (V20I, L38V), (V20L, L38V), (E29K, K30E, E33K), (Y54D, T83D, Q86E), (A59L, G60N, D61G, N62K), (A59V, D61N, N62K), (G60N, D61G, N62K), (G60N, ΔD61, N62G), ΔD61, (A59L, G60N, ΔD61, N62G), (A59V, G60N, D61G, N62K), (A59I, G60N, D61G, N62K), (A59I, G60N, ΔD61, N62G), (A59V, G60N, ΔD61, N62G), (A59V, ΔD61), (G60P, ΔD61, N62P), (G60P, D61P, N62K), (G60P, ΔD61, N62G), (G60P, D61G, N62K), (D61N, N62K) and (T83D, Q86E).

Suitably said one or more mutations relative to SEQ ID NO: 1 is or are selected from the group consisting of:

T51L, T51V, M65V, N32G, A59I, E29M, T34V, T34R, T45I, T45V, T51F, A59L, L67I, (E29K, K30E, E33K), (A59L, G60N, D61G, N62K), (A59V, D61N, N62K), (G60N, D61G, N62K), (G60N, ΔD61, N62G),

ΔD61, (A59L, G60N, ΔD61, N62G), (A59V, G60N, D61G, N62K), (A59I, G60N, D61G, N62K), (A59I, G60N, ΔD61, N62G), (A59V, G60N, ΔD61, N62G), and (A59V, ΔD61);

preferably wherein said polypeptide has a Tm higher than the Tm of SEQ ID NO: 1.

Suitably said one or more mutations relative to SEQ ID NO: 1 is or are selected from the group consisting of:

L38A, V20I, A40I, L38V, A12I, A12V, I16L, V20L, Q26E, T31K, N32D, N32H, T34D, T34P, A40V, Q42D, V48E, V48G, V48A, T51A, (V20I, L38A), (V20L, L38A), (V20I, L38V), (V20L, L38V), (Y54D, T83D, Q86E), (G60P, ΔD61, N62P), (G60P, D61P, N62K), (G60P, ΔD61, N62G), (G60P, D61G, N62K), (D61N, N62K) and (T83D, Q86E);

preferably wherein said polypeptide has a Tm lower than the Tm of SEQ ID NO: 1.

In one aspect, the invention relates to a polypeptide as described above wherein said polypeptide has a Tm higher than the Tm of SEQ ID NO: 1.

In one aspect, the invention relates to a polypeptide as described above wherein said polypeptide has a Tm lower than the Tm of SEQ ID NO: 1.

Suitably said polypeptide further comprises one or more mutations relative to SEQ ID NO: 1 selected from the group consisting of: G4R, G4W, V48D, V48E, G50 S, Y35W, Y43W, Y53W, Y54W, Y64W, F70W, Y85W, F98W, (K71N S72G L73P), or (E78A L80R).

Suitably the polypeptide as described above comprises five or fewer mutations relative to SEQ ID NO: 1.

Suitably said five or fewer mutations are selected from the group consisting of Y35W, N32G, V48D, M65I, Q42E and T51L.

In one aspect, the invention relates to a polypeptide as described above wherein said five or fewer mutations are selected from the group consisting of N32G, V48D, M65I, Q42E and T51L.

In one embodiment, the invention relates to a polypeptide as described above having each of the mutations in one of the following groups:

i) N32G V48D

ii) N32G V48D M65I

iii) N32G V48D M65I T51L

iv) N32G V48D M65I Q42E

v) N32G V48D M65I Q42E T51L.

In one embodiment, the invention relates to a polypeptide as described above having each of the mutations in one of the following groups:

i) N32G V48D

ii) N32G V48D M65I

iii) N32G V48D M65I T51L

iv) N32G V48D M65I Q42E

v) N32G V48D M65I Q42E T51L, and having no further mutations relative to SEQ ID NO: 1.

In one embodiment, the invention relates to a polypeptide as described above having each of the mutations iv) N32G, V48D, M65I and Q42E.

In one embodiment, the invention relates to a polypeptide as described above having each of the mutations iv) N32G, V48D, M65I and Q42E, and having no further mutations relative to SEQ ID NO: 1.

In one embodiment, the invention relates to a polypeptide as described above having each of the mutations in one of the following groups:

a) Y35W N32G V48D M65I Q42E T51L (A59V ΔD61) (E29K K30E E33K)

b) Y35W N32G V48D M65I Q42E T51L (A59V G60N ΔD61 N62G) (E29K K30E E33K).

In one embodiment, the invention relates to a polypeptide as described above having each of the mutations in one of the following groups:

a) Y35W N32G V48D M65I Q42E T51L (A59V ΔD61) (E29K K30E E33K)

b) Y35W N32G V48D M65I Q42E T51L (A59V G60N ΔD61 N62G) (E29K K30E E33K), and having no further mutations relative to SEQ ID NO: 1.

In one embodiment, the invention relates to a polypeptide as described above having each of the mutations b) Y35W N32G V48D M65I Q42E T51L (A59V G60N ΔD61 N62G) (E29K K30E E33K).

In one embodiment, the invention relates to a polypeptide as described above having each of the mutations b) Y35W N32G V48D M65I Q42E T51L (A59V G60N ΔD61 N62G) (E29K K30E E33K), and having no further mutations relative to SEQ ID NO: 1.

Suitably said heterologous peptide is 6 to 36 amino acids in length.

In one aspect, the invention relates to a fusion protein comprising:

- a polypeptide as described above; and

- one or more additional amino acid sequences selected from the group consisting of: secretion signal sequences, peptide linker sequences, affinity tags, transmembrane domains, cell surface retention sequence, substrate recognition sequences for post-translational modifications, multimerization domains to create multimeric structures of the protein aggregating through protein-protein interactions, half-life extending polypeptide moieties, polypeptide sequences for altering tissue localization and antigen binding site of an antibody, one or more additional polypeptides as described above binding to the same or different targets, and one or more additional Affimer polypeptide sequences binding to the same or different targets.

Suitably said fusion protein comprises one or more half-life extending polypeptide moieties selected from the group consisting of an Fc domain or portion thereof, an albumin protein or portion thereof, an albumin-binding polypeptide moiety, transferrin or portion thereof, a transferrin-binding polypeptide moiety, fibronectin or portion thereof, or a fibronectin-binding polypeptide moiety.

Suitably the Fc domain or a portion thereof retains FcN binding.

Suitably the Fc domain or a portion thereof is from IgA, IgD, IgE, IgG, and IgM or a subclass (isotype) thereof such as IgG1, IgG2, IgG3, IgG4, IgA1 or IgA2.

Suitably the Fc domain or a portion thereof retains effector function selected from C1q binding, complement dependent cytotoxicity (CDC), antibody-dependent cell-mediated cytotoxicity (ADCC); phagocytosis; down regulation of B cell receptor, or a combination thereof.

Suitably the half-life extending polypeptide moiety increases the serum half-life of the protein by at least 5-fold relative to its absence from the protein.

Suitably the polypeptide is an affimer or an affimer polypeptide. Affimers/ affimer polypeptides are described in more detail below.

In one aspect, the invention relates to a nucleic acid comprising nucleotide sequence encoding a polypeptide as described above.

In one aspect, the invention relates to a vector comprising a nucleic acid as described above.

In one aspect, the invention relates to a library comprising a population of polypeptides as described above, wherein at least two individual polypeptides within said population comprise different heterologous peptide insertions.

In one aspect, the invention relates to a library comprising a population of nucleic acids, said nucleic acids comprising nucleotide sequences encoding a population of polypeptides as described above. Suitably each nucleic acid encodes a single polypeptide as described above.

In one aspect, the invention relates to a host cell comprising a polypeptide as described above, a nucleic acid as described above, or a library as described above.

In one aspect, the invention relates to a polypeptide as described above for use in medicine.

In one aspect, the invention relates to a method for identifying a peptide capable of binding a structure of interest, said method comprising:

(i) providing a polypeptide as described above comprising a heterologous peptide insertion;

(ii) contacting said polypeptide with said structure of interest; and

(iii) monitoring the association between the polypeptide and the structure of interest;

wherein association of the polypeptide with the structure of interest identifies the peptide as a candidate peptide capable of binding said structure.

In one aspect, the invention relates to use of a polypeptide as described above as a scaffold protein.

More suitably the invention relates to a polypeptide as described above wherein said heterologous peptide insertion comprises a heterologous peptide inserted at one of the following positions relative to SEQ ID NO: 1:

d) 48-<heterologous peptide>-50

e) 49-<heterologous peptide>-51

f) 50-<heterologous peptide>-52

q) 72-<heterologous peptide>-77

r) 73-<heterologous peptide>-78

s) 74-<heterologous peptide>-79

t) 4-<heterologous peptide>-5.

More suitably said polypeptide comprises two heterologous peptide insertions, a first heterologous peptide insertion at any of positions (d) to (f), and a second heterologous peptide insertion at any of positions (q) to (s).

More suitably said polypeptide comprises two heterologous peptide insertions, a first heterologous peptide insertion at any of positions (d) to (f), and a second heterologous peptide insertion at position (t).

More suitably said polypeptide comprises two heterologous peptide insertions, a first heterologous peptide insertion at any of positions (q) to (s), and a second heterologous peptide insertion at position (t).

More suitably said polypeptide comprises three heterologous peptide insertions, a first heterologous peptide insertion at any of positions (d) to (f), and a second heterologous

peptide insertion at any of positions (q) to (s), and a third heterologous peptide insertion at position (t).

Suitably the polypeptide of the invention comprises amino acid sequence corresponding to amino acids 1 to 98 of SEQ ID NO: 1, comprising mutation(s) as described. A small number of mutations described are deletions e.g. the amino acid corresponding to amino acid 61 of SEQ ID NO: 1 may be mutated by substitution or by deletion. If mutated by deletion, the final resulting polypeptide may comprise only 97 amino acids corresponding to SEQ ID NO: 1 - in other words when D61 is deleted then ΔD61 corresponds to D61 of SEQ ID NO: 1 - the skilled person will interpret such feature accordingly. Most suitably mutations are substitutions.

In a broad aspect, also disclosed is a polypeptide, such as an Affimer polypeptide, comprising an amino acid sequence corresponding to amino acids 1 to 98 of SEQ ID NO: 1, said polypeptide comprising a mutation relative to SEQ ID NO: 1 selected from the group consisting of

M65I, T51I, T51L, T51V, M65V, A59V, N32G, A59I, L38A, V20A, V20I, A40I, L38V, G50S, L38F, A12I, A12V, I16L, V20L, Q26E, E29M, T31K, N32D, N32H, T34V, T34R, T34K, T34D, T34P, A40V, Q42E, Q42D, T45I, T45V, V48E, V48D, V48G, V48A, V48L, T51F, T51A, A59L, K63R, L67I, N90T, (V20I, L38A), (V20L, L38A), (V20I, L38V), (V20L, L38V), (E29K, K30E, E33K), (Y54D, T83D, Q86E), (A59L, G60N, D61G, N62K), (A59V, D61N, N62K), (G60N, D61G, N62K), (G60N, ΔD61, N62G), ΔD61, (A59L, G60N, ΔD61, N62G), (A59V, G60N, D61G, N62K), (A59I, G60N, D61G, N62K), (A59I, G60N, ΔD61, N62G), (A59V, G60N, ΔD61, N62G), (A59V, ΔD61), (G60P, ΔD61, N62P), (G60P, D61P, N62K), (G60P, ΔD61, N62G), (G60P, D61G, N62K), (D61N, N62K), and (T83D, Q86E);

and further comprising a heterologous peptide insertion. Suitably said heterologous peptide insertion comprises a heterologous peptide inserted at one of the following positions relative to SEQ ID NO: 1:

d) 48-<heterologous peptide>-50,

e) 49-<heterologous peptide>-51,

f) 50-<heterologous peptide>-52,

q) 72-<heterologous peptide>-77,

r) 73-<heterologous peptide>-78, or

s) 74-<heterologous peptide>-79.

An Affimer® is an engineered non-antibody binding protein (i.e. a polypeptide affinity reagent). Affimer (AFFIMER) is a registered trade mark.

As is known in the art, aptamers and Affimer® reagents are not the same. Affimer technology has been engineered to overcome many of the problems associated with aptamers or with antibodies and possesses a number of benefits. For example, sensitivity to the assay environment has been improved because Affimer® scaffolds are resistant to a wide pH range, making them suitable for a wide range of assay conditions. Affimer® molecules are also not sensitive to EDTA (a problem for aptamers that require Mg++ for folding and function). An aptamer is not constrained, whereas an Affimer® protein constrains the displayed heterologous peptide.

Suitably the Affimer® reagents such as Affimer® scaffolds/polypeptides described herein are based on or derived from the human Stefin A protein. This is described in more detail below.

Suitably the polypeptide is a Stefin A polypeptide.

In one aspect the invention relates to a Stefin A polypeptide having a combination of one or more mutations from a set of defined mutations which all share the common technical feature of each being demonstrated to affect the stability such as thermal stability (Tm) of the polypeptide AND a heterologous peptide insertion. To the best of the inventors' knowledge and belief at the filing date these combinations are novel.

A small number of the amino acids at particular positions taught by the inventors may have occurred in the art before (e.g. as a naturally occurring residue in a distantly related Stefin A homologue i.e. without a heterologous peptide insertion and therefore not occurring in the above described novel combination); or in combination with a heterologous peptide insertion e.g. in prior art scaffold STM. Examples of such mutations (substitutions) which may have occurred in the art before include: V20A,T34K,L38F,Q42E,V48D,G50S,T51I,A59V,K63R,M65I,and N90T. In one embodiment suitably the polypeptide of the invention does not comprise a mutation selected from this group. Suitably when the polypeptide of the invention has a mutation selected from this group, it also has at least one heterologous peptide insertion, most suitably at one or more of the following positions relative to SEQ ID NO: 1:

d) 48-<heterologous peptide>-50,

e) 49-<heterologous peptide>-51,

f) 50-<heterologous peptide>-52,

q) 72-<heterologous peptide>-77,

r) 73-<heterologous peptide>-78, or

s) 74-<heterologous peptide>-79.

Suitably when the polypeptide of the invention has a mutation selected from this group, it also has at least one further mutation selected from Table A.

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Table A - novel mutations for modulating thermal stability of Stefin A polypeptides

A12I,A12V,I16L,V20I,V20L,Q26E,E29M,T31K

N32G,N32D,N32H,T34V,T34R,T34D,T34P,L38A

L38V,A40I,A40V,Q42D,T45I,T45V,V48E,V48G

V48A,T51F,T51V,T51L,T51A,A59L,A59I,M65V

L67I

(V20I,L38A),(V20L,L38A),(V20I,L38V),(V20L,L38V)

(E29K,K30E,E33K),(Y54D,T83D,Q86E)

(A59L,G60N,D61G,N62K),(A59V,D61N,N62K)

(G60N,D61G,N62K),(G60N,ΔD61,N62G)

ΔD61,(A59L,G60N,ΔD61,N62G)

(A59V,G60N,D61G,N62K),(A59I,G60N,D61G,N62K)

(A59I,G60N,ΔD61,N62G),(A59V,G60N,ΔD61,N62G)

(A59V,ΔD61),(G60P,ΔD61,N62P)

(G60P,D61P,N62K),(G60P,ΔD61,N62G)

(G60P,D61G,N62K),(D61N,N62K) and

(T83D,Q86E)

With reference to increasing the Tm of the Stefin A polypeptide, examples of such mutations (substitutions) which may have occurred in the art before include: T34K,Q42E,G50S,T51I,A59V,K63R,M65I,and N90T. In one embodiment suitably the polypeptide of the invention does not comprise a mutation selected from this group. Suitably when the polypeptide of the invention has a mutation selected from this group, it also has at least one heterologous peptide insertion, most suitably at one or more of the following positions relative to SEQ ID NO: 1:

d) 48-<heterologous peptide>-50,

e) 49-<heterologous peptide>-51,

f) 50-<heterologous peptide>-52,

q) 72-<heterologous peptide>-77,

r) 73-<heterologous peptide>-78, or

s) 74-<heterologous peptide>-79.

Suitably when the polypeptide of the invention has a mutation selected from this group, it also has at least one further mutation selected from Table B.

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Table B - novel mutations for increasing thermal stability of Stefin A polypeptides

E29M,N32G,T34V,T34R,T45I,T45V,T51F,T51V

T51L,A59L,A59I,M65V,L67I

(E29K,K30E,E33K),(A59L,G60N,D61G,N62K)

(A59V,D61N,N62K),(G60N,D61G,N62K)

(G60N,ΔD61,N62G),ΔD61

(A59L,G60N,ΔD61,N62G),(A59V,G60N,D61G,N62K)

(A59I,G60N,D61G,N62K),(A59I,G60N,ΔD61,N62G)

(A59V,G60N,ΔD61,N62G), and (A59V,ΔD61)

With reference to decreasing the Tm of the Stefin A polypeptide, examples of such mutations (substitutions) which may have occurred in the art before include: V20A, L38F, and V48D. In one embodiment suitably the polypeptide of the invention does not comprise a mutation selected from this group. Suitably when the polypeptide of the invention has a mutation selected from this group, it also has at least one heterologous peptide insertion, most suitably at one or more of the following positions relative to SEQ ID NO: 1:

d) 48-<heterologous peptide>-50,

e) 49-<heterologous peptide>-51,

f) 50-<heterologous peptide>-52,

q) 72-<heterologous peptide>-77,

r) 73-<heterologous peptide>-78, or

s) 74-<heterologous peptide>-79.

Suitably when the polypeptide of the invention has a mutation selected from this group, it also has at least one further mutation selected from Table C.

Table C - novel mutations for decreasing thermal stability of Stefin A polypeptides

A12I,A12V,I16L,V20I,V20L,Q26E,T31K,N32D

N32H,T34D,T34P,L38A,L38V,A40I,A40V,Q42D

V48E,V48G,V48A,T51A

(V20I,L38A),(V20L,L38A),(V20I,L38V),(V20L,L38V)

(Y54D,T83D,Q86E),(G60P,ΔD61,N62P)

(G60P,D61P,N62K),(G60P,ΔD61,N62G)

(G60P,D61G,N62K),(D61N,N62K) and

(T83D,Q86E)

Suitably the polypeptide comprises amino acid sequence corresponding to amino acids 1 to 98 of SEQ ID NO: 1, comprising mutation(s) as described above.

Thus in one aspect the invention relates to a polypeptide, such as an Affimer polypeptide, comprising an amino acid sequence corresponding to amino acids 1 to 98 of SEQ ID NO: 1,

wherein said amino acid sequence has at least 80% identity to amino acid residues 1 to 11, 13 to 15, 17 to 19, 21 to 25, 27 to 28, 35 to 37, 39, 41, 43 to 44, 46 to 47, 49 to 50, 52 to 53, 55 to 58, 63 to 64, 66, 68 to 82, 84 to 85, and 87 to 98 of SEQ ID NO: 1;

characterised in that said polypeptide comprises one or more mutations relative to SEQ ID NO: 1 selected from a group disclosed above.

Suitably the polypeptide comprises at least 80 amino acids; suitably at least 81 amino acids, suitably at least 82 amino acids, suitably at least 83 amino acids, suitably at least 84 amino acids, suitably at least 85 amino acids, suitably at least 86 amino acids, suitably at least 87 amino acids, suitably at least 88 amino acids, suitably at least 89 amino acids, suitably at least 90 amino acids, suitably at least 91 amino acids, suitably at least 92 amino acids, suitably at least 93 amino acids, suitably at least 94 amino acids, suitably at least 95 amino acids, suitably at least 96 amino acids; more suitably at least 97 amino acids; most suitably 98 amino acids (excluding any heterologous peptide insertions). Suitably the polypeptide comprises amino acid sequence corresponding to full length hSteA. Suitably full length hSteA means 98aa, most suitably corresponding to those shown in SEQ ID NO: 1.

Scaffold

As is well known in the art, the term 'scaffold' refers to a protein which can present target peptide(s) to solvent without its own structure being deformed by the target peptide.

Regarding the presentation of peptide to solvent, this can be tested using immunoprecipitation experiments. For example, an indication that a peptide is being presented to solvent may be obtained by its availability to an antibody capable of recognising it. Thus, in order to test the ability of a scaffold protein to present a peptide to solvent, the scaffold comprising the peptide would be expressed and an antibody recognising the peptide would be used to try to immunoprecipitate the scaffold-peptide fusion. If this protein can be immunoprecipitated or captured on the antibody, this shows that the peptide was presented to solvent as is required by a scaffold protein. Another, or an alternative, indication that a peptide is being presented to solvent may be obtained by phosphorylation studies. By incorporating a phosphate acceptor site into the target peptide, and then contacting the scaffold-peptide fusion with the cognate kinase in conditions permissive of phosphorylation, then the presentation of the peptide to solvent can be verified. Phosphorylation of the peptide indicates correct presentation to solvent.

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A scaffold protein must be able to accept a peptide insert i.e. a heterologous peptide insertion ('target' peptide) as taught herein. Preferably the peptide insert is 36 amino acids or fewer, preferably 20 amino acids or fewer. Preferably the peptide insert is 12 amino acids or fewer.

Concerning a scaffold protein's resistance to being deformed by the target peptide which it bears, this can be tested using techniques such as circular dichroism. Specifically, a circular dichroism analysis of a scaffold protein without target peptide inserted into it should be substantially the same as the circular dichroism characteristics of the same scaffold protein when bearing a target peptide. This provides a demonstration that the presence of the target peptide in the scaffold protein has not compromised or deformed the structure of the scaffold protein bearing it.

For example, the near UV CD spectra report on the environment of the aromatic side chains. Peaks, positive or negative, in a near UV CD spectrum indicate that the environment around the aromatic residues is fixed. This is interpreted as a protein having a fixed tertiary structure. Incorporating heterologous peptides into insertion site(s) as taught herein suitably results in near UV CD spectra with the same shape as the "empty" scaffolds, indicating that the addition of heterologous peptides has not disrupted the tertiary structure of the scaffold proteins. As will be realised by the person skilled in the art, the spectra may have different magnitudes, e.g. the peaks may be higher and/or the troughs may be lower, but retention of the same shaped spectra is the important factor.

For example, the far UV CD spectra report on gross secondary structure. The alpha-helix, beta-strand and random coil all have distinctly different far UV CD spectra. Incorporating heterologous peptides into insertion site(s) as taught herein suitably results in far UV CD spectra with the same shape as the "empty" scaffolds, indicating that the addition of heterologous peptides has not disrupted the gross secondary structure of the scaffold proteins. As will be realised by the person skilled in the art, the spectra may have different magnitudes, e.g. the peaks may be higher and/or the troughs may be lower, but retention of the same shaped spectra is the important factor.

Suitably a scaffold protein constrains the target peptide. The presence of a constraint effect in a scaffold protein can be demonstrated by comparing the affinity of an entity binding the target peptide when the target peptide is in the scaffold protein with the affinity when the peptide is not in the scaffold protein. A difference in these two affinities indicates that the scaffold protein is constraining the peptide to assume a particular three dimensional conformation. Suitably a scaffold protein constrains a peptide so that it demonstrates an increased binding affinity when present in the context of the scaffold protein. In other words, suitably the scaffold protein decreases the entropic cost of binding and so increases the measured affinity when compared with binding of a free peptide.

A scaffold protein is suitably biologically neutral. By 'biologically neutral' it is meant that known interactions with other proteins have been abolished. Furthermore, any signalling abilities possessed by the protein are preferably removed. Mutations for biological neutrality of Stefin A based scaffolds are known, and preferred examples are discussed herein.

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Combination of such mutations for biological neutrality with the mutations taught herein for modulation of thermal stability is expressly contemplated.

Suitably the polypeptide of the invention is a scaffold protein.

In another aspect, the invention relates to use of a polypeptide as disclosed herein as a scaffold protein.

Thermal Stability

A key part of the invention is the modulation of thermal stability. Without knowledge of the invention, in principle mutating any residue in the polypeptide of the invention might lead to a change in thermal stability. However, the inventors studied the protein in detail at all levels of its structure and designed particular changes in the protein in order to influence thermal stability. To their surprise, not all of the mutations which the inventors designed produced the expected effects. Thus, there is a large degree of unpredictability associated with making mutations to influence thermal stability in a scaffold protein. As a result of significant intellectual effort and experimentation, the inventors have arrived at a very detailed teaching presented herein regarding which residues may be used to influence thermal stability.

Also included for comparison purposes are various data surprisingly showing residues with no effect or neutral effect on stability such as Tm - those data further illustrate how the invention could not have been arrived at by prediction from the art.

Thermal stability may be assessed by any suitable means known in the art. Most suitably thermal stability is suitably read out or assessed by measuring the Tm. Thus, suitably references to 'thermal stability' may be understood as references to 'Tm'. Unless otherwise apparent from the context, 'improved' thermal stability/Tm means increased thermal stability/Tm. 'Increased' is a relative term - unless otherwise apparent from the context, 'increased' means 'increased relative to wild type Stefin A'. In other words, relative to Stefin A having the wild type amino acid residue at the stated position; most suitably relative to wild type Stefin A having the amino acid sequence of SEQ ID NO: 1.

It should be noted that the majority of the measurements presented herein are relative to hSteA Y35W; the same relative difference to hSteA is a scientific assumption.

In addition to providing an assessment of thermal stability, the Tm is a good proxy for assessing how easy the scaffold protein is to make – scaffolds with higher Tm's are advantageously easier to make. Moreover, an increase in the Tm can show that the protein is "stiffer" and so better suited to accepting of heterologous peptide insertions. Moreover, the Tm is a good indication of stability, for example the shelf life of a protein may be advantageously increased when it is more stable i.e. having a higher Tm. Moreover, increased Tm may provide further advantage(s) such as improved performance in downstream formatting, long-term stability, and/or flexibility in assay design.

Tm of proteins is also known as the denaturation midpoint, and is defined as the temperature at which both the folded and unfolded states are present in equal amounts or populations at equilibrium. Tm is determined in this manner assuming a two state protein folding.

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Tm values given herein refer to the 'empty' polypeptide of the invention / scaffold protein i.e. scaffold protein without heterologous peptide insertions (unless otherwise indicated). Insertion of heterologous sequence(s) can, and often does, reduce the thermal stability such as Tm and therefore the most meaningful value is obtained when empty scaffold proteins are compared.

Melting temperature is a particularly useful indicator of protein stability. As used herein, melting temperature means the apparent mid-point of a thermal unfolding transition. The relative proportions of folded and unfolded proteins can be determined by any technique known to the skilled person, including differential scanning calorimetry, UV difference spectroscopy, fluorescence, circular dichroism (CD), or NMR (for example see Pace, C. Nick, and J. Martin Scholtz. "Measuring the conformational stability of a protein." Protein structure: A practical approach 2 (1997): 299-321.).

Measurement Techniques

It is possible to use alternative measurement techniques to assess the same property such as Tm. Preferred techniques for measuring Tm include Optim (most suitably Optim 2) and/or differential scanning calorimetry (DSC). Most suitably assessment of thermal stability such as Tm is carried out by differential scanning calorimetry (DSC) using any commercially available instrument, and/or by using an 'Optim 2' High Throughput Protein Stability Instrument (also known as UNit) available from Avacta Analytical (Unit 20, Ash Way Thorp Arch Estate, Wetherby LS23 7FA, UK) and/or from Unchained Labs (6870 Koll Center Parkway, Pleasanton, CA 94566, USA).

In more detail, it will be appreciated by any scientist/person skilled in the art that it is not likely to produce the same absolute value when a property is measured by two different techniques. Having said that, Tm measured by Optim and measured by DSC produces very similar, but sometimes not precisely identical, values. Tm's measured by DSC and Tm's measured by Optim produce the same indication of changes. Therefore, if the Tm of a polypeptide is measured by Optim and the Tm of the same polypeptide is measured by DSC, they may not give precisely the same absolute values, but they will both give the same indication of change e.g. +10°C. Moreover, the ranking of individual polypeptides is the same using either technique. Thus, it must be borne in mind that, in accordance with normal scientific practice, the absolute values quoted may vary slightly depending on the technique used to make the measurement. Unless otherwise stated, values herein are suitably determined by Optim. Advantages of Optim include that it is quick, and that it is reliable using very little input material. One limitation of Optim is that measurements are most reliable when the Tm is below 95 °C, suitably below 92 °C, most suitably below 90 °C. An advantage of DSC is that it works very well at higher temperatures, and may be slightly better for avoiding interference effects. Thus, if it is desired to attempt to determine absolute Tm's, then DSC would be the measurement technique of choice. However, as a practical matter when measuring "deltas" or differences between individual polypeptides then Optim is a very convenient and very reliable and robust technique to use. Unless otherwise stated, values provided herein are

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determined using Optim. In all circumstances, it is good scientific practice to compare measurements between individual polypeptides which were determined using the same measurement technique. Suitably the Tm is Tm where determined by DSC or Optim. Most suitably, Tm is Tm when determined by Optim.

Most suitably the stability such as Tm is assessed for wild type Stefin A in the same manner as stability such as Tm is assessed for the polypeptides / scaffold proteins bearing mutations as disclosed herein. For reference purposes, the Tm of wild type Stefin A when assessed by DSC (r/t) is 89.0 ºC.

For comparison purposes, the prior art SQT scaffold protein has a Tm of 64.6 ºC when assessed by the same techniques.

Measurement techniques are discussed in more detail below.

Similarly, human SteA is the preferred starting point for the scaffolds before mutating and/or inserting heterologous peptides as described. Advantageously the Y35W mutation is included to give a signal that can be measured to easily monitor thermal stability. Thus many or most of the measurements provided have been made in scaffolds including Y35W. For example, when we show data for N32G increasing the stability by 3 degrees, the measurement was that hSteA Y35W N32G increases the stability by 3 degrees over hSteA Y35W.

It is a reasonable and robust scientific expectation that the effect of a mutation as measured on hSteA Y35W has the same effect on hSteA. This has been carefully validated and we present data to support this: We carried out a DSC experiment on several mutations in both hSteA and hSteA Y35W. We include these data below, giving the difference in stability from hSteA / hSteA Y35W and showing this expectation is scientifically acceptable:

Difference in stability from hSteA / hSteA Y35W:	
N23G	3.3 / 2.1
M65I	7.6 / 6.6
Q42E	10 / 9.6
T51L	14.4 / 12.4
A59V dD61	13.6 / 11.6
A59V dD61 G60N N62G	17.1 / 13.6

Suitably the polypeptide of the invention / scaffold protein has a melting temperature (Tm) of at least 90 °C, more preferably at least 91 °C, more preferably at least 92 °C, more preferably at least 94 °C, more preferably at least 95 °C, more preferably at least 98 °C, and most preferably at least 100 °C.

In one aspect, the invention relates to increasing thermal stability of a Stefin A polypeptide such as a Stefin A scaffold protein by use of mutations as taught herein.

In another aspect, the invention relates to reduction of thermal stability of a Stefin A polypeptide such as a Stefin A scaffold protein by use of mutations as taught herein.

Thus in one aspect the invention relates to a method of increasing the thermal stability of a Stefin A polypeptide such as a Stefin A scaffold protein by making one or more mutation(s) in said Stefin A polypeptide such as a Stefin A scaffold protein, which mutation(s) are selected from the group consisting of:

E29M, N32G, T34V, T34R, T34K, Q42E, T45I, T45V, T51F, T51V, T51L, T51I, A59L, A59I, A59V, K63R, M65V, M65I, L67I, N90T, (E29K, K30E, E33K), (A59L, G60N, D61G, N62K), (A59V, D61N, N62K), (G60N, D61G, N62K), (G60N, ΔD61, N62G), ΔD61, (A59L, G60N, ΔD61, N62G), (A59V, G60N, D61G, N62K), (A59I, G60N, D61G, N62K), (A59I, G60N, ΔD61, N62G), (A59V, G60N, ΔD61, N62G), and (A59V, ΔD61);

more suitably selected from the group consisting of:

E29M, N32G, T34V, T34R, T45I, T45V, T51F, T51V, T51L, A59L, A59I, M65V, L67I, (E29K, K30E, E33K), (A59L, G60N, D61G, N62K), (A59V, D61N, N62K), (G60N, D61G, N62K), (G60N, ΔD61, N62G), ΔD61, (A59L, G60N, ΔD61, N62G), (A59V, G60N, D61G, N62K), (A59I, G60N, D61G, N62K), (A59I, G60N, ΔD61, N62G), (A59V, G60N, ΔD61, N62G), and (A59V, ΔD61).

Thus in one aspect the invention relates to a method of decreasing the thermal stability of a Stefin A polypeptide such as a Stefin A scaffold protein by making one or more mutation(s) in said Stefin A polypeptide such as a Stefin A scaffold protein, which mutation(s) are selected from the group consisting of:

A12I, A12V, I16L, V20A, V20I, V20L, Q26E, T31K, N32D, N32H, T34D, T34P, L38A, L38V, L38F, A40I, A40V, Q42D, V48E, V48D, V48G, V48A, V48L, T51A, (V20I, L38A), (V20L, L38A), (V20I, L38V), (V20L, L38V), (Y54D, T83D, Q86E), (G60P, ΔD61, N62P), (G60P, D61P, N62K), (G60P, ΔD61, N62G), (G60P, D61G, N62K), (D61N, N62K) and (T83D, Q86E);

more suitably selected from the group consisting of:

A12I, A12V, I16L, V20I, V20L, Q26E, T31K, N32D, N32H, T34D, T34P, L38A, L38V, A40I, A40V, Q42D, V48E, V48G, V48A, T51A, (V20I, L38A), (V20L, L38A), (V20I, L38V), (V20L, L38V), (Y54D, T83D, Q86E), (G60P, ΔD61, N62P), (G60P, D61P, N62K), (G60P, ΔD61, N62G), (G60P, D61G, N62K), (D61N, N62K) and (T83D, Q86E).

The invention is remarkable in that the mutations which are taught are not interdependent (unless otherwise stated in the text). In other words, the mutations (or groups of mutations if otherwise mentioned in the text) taught herein to influence thermal stability are independent

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may be combined in different permutations to achieve the same technical benefit of the invention i.e. to increase (or decrease) thermal stability. Thus, if a particular subset of mutations is desired to be used in a polypeptide according to the invention, then the subset may be chosen from the overall collection of mutations disclosed herein, since advantageously those mutations are not reliant on other parts of the protein in order to deliver their advantageous effects. Thus, if a large change in thermal stability is required, then a person skilled in the art will select a larger number of mutations as disclosed. If an increase in thermal stability is required, then the person skilled in the art may select mutations disclosed herein which increase thermal stability, or may select any subset of those mutations combined in order to deliver the desired level of increase in thermal stability.

Equally, if a reduction of thermal stability is required, then the person skilled in the art may select mutations disclosed herein which reduce thermal stability, or may select any subset of those mutations combined in order to deliver the desired level of reduction of thermal stability.

Each of the mutations disclosed herein as useful in the manipulation or modulation of the thermal stability (such as Tm) of the polypeptide of the invention may be used independently. As explained, in some embodiments the skilled operator may seek to choose each of the mutations useful for increasing the Tm in order to provide a polypeptide with a higher thermal stability (unless otherwise stated, higher/increased or lower/decreased are relative to the thermal stability of the wild type Stefin A, the sequence of which is already provided herein). In another set of embodiments, the skilled operator may choose each of the mutations disclosed to decrease or reduce the thermal stability (e.g. Tm) of the polypeptide such as scaffold protein relative to wild type Stefin A. In another set of embodiments, the skilled operator may select mutations from those which increase thermal stability and from those which decrease thermal stability – such "mixed" embodiments may advantageously allow the skilled operator to arrive at a more precise preselected thermal stability (such as Tm) for the polypeptide / scaffold protein produced in this manner. For example, if a particular thermal stability (such as Tm) is desired, then in selecting which particular mutants to use the skilled person would carry out arithmetic in order to select mutations which might (for example) increase the thermal stability towards the desired value. However, since each mutation contributes a slightly different value to the increase of thermal stability (such as Tm), then it may be necessary to also choose one or mutations from those which decrease thermal stability in order to "balance" the overall desired selected thermal stability value. For example, if the desired thermal stability is 6° higher than wild type Stefin A, it may be possible to select two different mutations which each increase thermal stability by 4°, and one further mutation which decreases thermal stability by 2°, thereby arriving at an overall effect of thermal stability increased relative to wild type Stefin A by 6° (+4, +4, -2). Any such combination of mutations may be used by the skilled operator, expressly including combinations of mutations which are selected from both those which increase Tm and those which decrease Tm relative to wild type Stefin A. This may be important when designing scaffolds incorporating further mutations, for example those mutations useful in achieving biological neutrality. In particular,

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when it is desired to use a V48 mutation such as V48D, this inhibits domain swap dimerisation and so is extremely useful in proving biological neutrality. However, this mutation also has a destabilising (decreasing thermal stability such as Tm) effect on the polypeptide / scaffold protein. Therefore, in order to affect the thermal stability and arrive at the desired value, it will be important to select mutations in order to "balance" the effect of selecting the V48D biological neutrality mutation. An exemplary polypeptide / scaffold protein designed in this manner might have the following mutations: V48D, N32G, M65I (referred to herein as 3 T2).

Thus the exemplary 3t series scaffolds are examples of "mixed" embodiments. These have the destabilising V48D mutation, plus different numbers of stabilising mutations to 'restore' (i.e. move back towards wild type Tm by increasing Tm from the destabilised lower Tm V48D mutant) and then further increase the Tm. Data demonstrating the Tm of different examples of such mixed embodiments as follows:

hSteA N32G V48D = 3t1 = 85.6 °C

hSteA N32G V48D M65I = 3t2 = 89.2 °C

hSteA N32G V48D M65I T51L = 3t3 = 91.6 °C

hSteA N32G V48D M65I Q42E = 3t4 = 92.4 °C

hSteA N32G V48D Q42E T51L M65I = 3t5 = 94.7 °C

Clearly it will be possible to produce polypeptides / scaffold proteins according to the invention with combined mutations which result in a modest final change in thermal stability e.g. Tm relative to wild type Stefin A. For example, one such embodiment may comprise a polypeptide / scaffold protein having a V48D mutation, which lowers Tm, and also having one or more mutations according to the present invention which raise Tm, so that the final Tm of that polypeptide / scaffold protein might be higher or lower than the Tm of wild type Stefin A depending on the mutations selected. The change in Tm relative to wild type Stefin A is therefore suitably not determinative whether or not a scaffold protein is a scaffold protein of the invention - that will be as defined in the claims - but it is important that a scaffold protein of the invention will comprise at least one mutation as taught herein for affecting Tm such as increasing it or reducing it relative to Stefin A. If those mutation(s) are used in a polypeptide / scaffold protein in combination with other mutations such as the known V48D mutation then the resulting polypeptide / scaffold protein is still regarded as a part of the invention by virtue of including the mutation(s) disclosed herein for influencing thermal stability such as Tm, whether or not the final product has a Tm higher or lower than wild type Stefin A.

The assessment of whether the mutation raises or lowers Tm relative to wild type Stefin A is suitably determined for the mutation (or group of interdependent mutations as appropriate as apparent from the context) in isolation i.e. when made singly (or as a single group of interdependent mutations as appropriate as apparent from the context) and when the polypeptide / scaffold protein so mutated is compared to wild type Stefin A.

Nevertheless, in preferred embodiments of the invention the polypeptide / scaffold protein has a thermal stability increased relative to wild type Stefin A. Thus suitably when the

polypeptide / scaffold protein comprises mutations which lower its thermal stability such as Tm relative to wild type Stefin A (e.g. the V48D mutation), suitably said polypeptide / scaffold protein further comprises mutations as disclosed herein sufficient to compensate for the decrease in thermal stability such as Tm caused by that mutation or mutations (e.g. the V48D mutation) and thereby still result in a polypeptide / scaffold protein with increased thermal stability relative to wild type Stefin A.

Thus, suitably the polypeptide / scaffold protein has a Tm increased by at least +1 ºC relative to wild type Stefin A, suitably increased by at least +2 ºC, suitably increased by at least +3 ºC, suitably increased by at least +4 ºC, suitably increased by at least +5 ºC, suitably increased by at least +6 ºC, suitably increased by at least +7 ºC, suitably increased by at least +10 ºC, suitably increased by at least +13 ºC, suitably increased by at least +14 ºC, suitably increased by at least +17 ºC, relative to wild type Stefin A.

Examples of such values for particular mutations or combinations of mutations include:

hSteA N32G M65I +7.6 ºC

hSteA N32G M65I Q42E +10.0 ºC

hSteA N32G M65I Q42E T51L +14.4 ºC

hSteA N32G M65I Q42E T51L A59V dD61 +13.6 ºC

hSteA N32G M65I Q42E T51L A59V G60N dD61 N62G +17.1 ºC

Examples of mixed embodiments (i.e. sequences including a destabilising mutation but with compensating stabilising mutations) may be made. As examples, scaffolds which include V48D are made and their Tm determined - values shown below:

hSteA N32G V48D M65I = 3t2; Tm 89.2 °C

hSteA N32G V48D M65I T51L = 3t3; Tm 91.6 °C

hSteA N32G V48D M65I Q42E = 3t4; Tm 92.4 °C

hSteA N32G V48D Q42E T51L M65I = 3t5; Tm 94.7 °C

hSteA Y35W V48D N32G Q42E T51L M65I (A59V ΔD61) (E29K K30E E33K) = 3r1; Tm 95.1 °C

hSteA Y35W V48D N32G Q42E T51L M65I (A59V G60N ΔD61 N62G) (E29K K30E E33K) = 3r2; Tm >98.3 °C

Exemplary scaffolds such as 3t1 may be slightly less stable than wild type hSteA and 3t2 is approximately as stable as hSteA (slightly more stable than hSteA) but such scaffolds are still useful. For example 3t1 has very few mutations compared to wild type (only 3 mutations) and so represents an excellent compromise between maintaining sequence close to wild type whilst still designing a useful scaffold with good stability. In 3t1, the destabilising V48D is strongly compensated for by the two additional stabilising mutations, resulting in a very stable scaffold yet still bearing the useful V48D mutation for biological neutrality.

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Suitably the polypeptide of the invention comprises D at position 48, such as V48D.

Decreasing Thermal Stability

Equally, in certain peripheral embodiments of the invention the polypeptide / scaffold protein may have a thermal stability decreased relative to wild type Stefin A. Thus suitably when the polypeptide / scaffold protein comprises mutations which raise its thermal stability such as Tm relative to wild type Stefin A (e.g. the N32G mutation), suitably said polypeptide / scaffold protein further comprises mutations as disclosed herein sufficient to compensate for the increase in thermal stability such as Tm caused by that mutation or mutations (e.g. the N32G mutation) and thereby still result in a polypeptide / scaffold protein with decreased thermal stability relative to wild type Stefin A.

For reference, wild type human Stefin A has a Tm of 89.0ºC.

Thus in some embodiments, suitably the polypeptide / scaffold protein has a Tm reduced by at least -1 ºC relative to wild type Stefin A, suitably reduced by at least -2 ºC, suitably reduced by at least -3 ºC, suitably reduced by at least -4 ºC, suitably reduced by at least -5 ºC, suitably reduced by at least -6 ºC, suitably reduced by at least -7 ºC, suitably reduced by at least -8 ºC, suitably reduced by at least -9 ºC, suitably reduced by at least -10 ºC, suitably reduced by at least -11 ºC, suitably reduced by at least -12 ºC, suitably reduced by at least -13 ºC, suitably reduced by at least -15 ºC, suitably reduced by at least -16 ºC, suitably reduced by at least -21 ºC, relative to wild type Stefin A.

Examples of such values for particular mutations or combinations of mutations include:

hSteA Y35W V20I L38A -16.7 ºC

hSteA Y35W V20L L38A -15.3 ºC

hSteA Y35W Y54D T83D Q86E -21.1 ºC

More suitable examples are:

hSteA N32D V48D Y54D T83D Q86E (Tm approx. 58 °C)

hSteA V48D Y35W V20A L38A (Tm approx. 57 °C)

hSteA V48D Y35W A40I T31K A12I V20I (Tm approx. 50 °C)

The person skilled in the art will be aware that destabilising mutations should be chosen whilst maintaining a scaffold protein which folds correctly for example at room temperature e.g. 18-24 °C. Clearly if all the destabilising mutations taught herein are simultaneously introduced into a SteA polypeptide, that polypeptide might have a Tm lower than room temperature and would be of limited practical use. Thus in this embodiment suitably mutations as taught herein are used in combinations resulting in a polypeptide with a Tm no lower than 25°C, suitably no lower than 30°C, suitably no lower than 40°C, suitably no lower than 50°C. Thus in

this embodiment suitably mutations as taught herein are used in combinations resulting in a polypeptide with a Tm of 25°C or more, suitably 30°C or more, suitably 40°C or more, suitably 50°C or more. Thus in this embodiment suitably mutations as taught herein are used in combinations resulting in a polypeptide with a Tm of 25-88°C or more, suitably 30-88°C or more, suitably 40-88°C or more, suitably 50-88°C or more.

Assessing the correct folding (e.g. structure or conformation) is taught herein, for example using near and far UV CD spectra. Thus if there is any doubt, the skilled worker needs only to make the protein with the mutation combination to be checked, and then assess the correct folding (e.g. structure or conformation), and/or measure the Tm as taught herein.

Most preferred embodiments are those with increased thermal stability relative to wild type Stefin A.

P25 Site

P25 may be mutated. P25 mutants such as P25S can be useful in improving the polypeptide / scaffold protein, for example by making it stiffer. The P25 site is located at a kink in the helix of the Stefin A based scaffold protein. Mutating P25 to another residue (such as P25S) retains the kink in the helix. P25 mutants such as P25S show no significant effect, or no effect on thermal stability.

V48 Site

V48 is important for domain swap dimerisation. Suitably V48 is mutated to prevent or inhibit domain swap dimerisation. Most suitably V48 is V48D.

V48 is also important for Cathepsin binding. Therefore, for superior biological neutrality suitably V48 is mutated. Most suitably V48 is mutated to V48D.

V48G or V48D mutations may be used, most suitably V48D.

N32 Site

Advantageously the N32 site is mutated. For example, it may be mutated to N32G. This has an advantageous effect in increasing thermal stability of the polypeptide / scaffold protein. In addition, this has the advantageous property of removing a glycosylation site from the polypeptide / scaffold protein, thereby helping to ensure biological neutrality.

Other mutations at the N32 site may be used. For example, the N32G mutation prevents glycosylation by removing the residue that would be glycosylated. However, the N is only glycosylated when it is recognised in an NXS/T motif where X is any residue except P. In hSteA the sequence at this site is NET. We can prevent glycosylation by either mutating the N to anything else, or the T to anything but an S. N32G has the advantage of extra thermostability. Suitably T34 is substituted for any amino acid except S. T34E has the property that it does not

change the thermostability. Either N32 or T34 mutations will stop glycosylation at N32 by removing the required motif.

N32G has a further benefit in helix-capping. G is found more than twice as often than average at this position. Next most favoured is histidine, closely followed by N. All other mutations are less favoured than N for helix capping. Suitably histidine at 32 (N32H) is not used, as this amino acid residue adds a titratable side chain which may not be desirable.

An additional advantage of the N32G mutation is that this optimises the cap at the end of the helix in this area of the polypeptide / scaffold protein. Thus, although other N32 mutants may be used, N32G delivers particular advantages as noted above.

As a matter of convention, if certain mutations are bracketed together, for example as shown in some of the figures or tables herein, this indicates that those mutations are suitably made together i.e. in combination. For example "(T83D,Q86E)" means "substitution of T83D AND substitution of Q86E" in the same polypeptide. In other words, mutations which are bracketed together are suitably made as a group in a polypeptide / scaffold protein according to the invention. This can be important, for example when a pair of mutations are made in a "charge-swap" arrangement – in this case it is important to mutate a first position to swap the charge, and to make the corresponding opposite charge-swap mutation at the second site thereby preserving the charge-charge interaction between those two residues in the final polypeptide / scaffold protein. For example, the E29K K30E E33K mutations are surface charge mutations and are suitably mutated as a group and so are described as a single option bracketed together: "(E29 K30 E33)" i.e. suitably all three mutations E29K K30E E33K or none of the three mutations are made in an individual polypeptide / scaffold protein according to the invention. An advantage of the (E29K K30E E33K) set of mutations is the formation of favourable Coulombic interactions on the surface of the scaffold.

Reference Sequence

Suitably all sequences herein are discussed with reference to human wild-type Stefin A (Cystatin A) having the sequence Uniprot P01040 (most suitably Uniprot P01040-1). For the avoidance of doubt, this sequence is presented below:

SEQ ID NO: 1 - Uniprot P01040 - wild type human Cystatin A

```
         10          20          30          40          50
MIPGGLSEAK PATPEIQEIV DKVKPQLEEK TNETYGKLEA VQYKTQVVAG
         60          70          80          90
TNYYIKVRAG DNKYMHLKVF KSLPGQNEDL VLTGYQVDKN KDDELTGF
```

When particular amino acid residues are referred to herein using numeric addresses, the numbering is taken with reference to the wild type Stefin A (Cystatin A) amino acid sequence

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(or to the polynucleotide sequence encoding same if referring to nucleic acid). An exemplary nucleic acid encoding wild type Stefin A (Cystatin A) is:

SEQ ID NO: 2 (DNA sequence; artificial (not natural); the amino acid sequence SEQ ID NO: 1 was used to generate a codon-optimised DNA sequence suitable for expression in *E. coli*):

ATGATTCCTGGTGGTTTGTCGGAAGCCAAACCGGCTACTCCGGAAATCCAGGAGATTGTGGACAAAGT
CAAACCGCAACTGGAGGAAAAGACCAATGAAACCTATGGCAAACTCGAAGCGGTACAGTACAAAACCC
AAGTCGTTGCGGGTACGAACTACTACATCAAAGTACGCGCAGGAGATAACAAGTATATGCATCTGAAA
GTGTTCAAAAGCTTACCAGGGCAGAATGAGGATCTGGTTCTTACGGGCTATCAGGTGGATAAGAACAA
AGACGATGAACTGACAGGCTTT

Suitably the current version of sequence database(s) are relied upon. Alternatively, the release in force at the date of filing is relied upon. For the avoidance of doubt, UniProt release 2017_02 is relied upon. In more detail, the UniProt consortium European Bioinformatics Institute (EBI), SIB Swiss Institute of Bioinformatics and Protein Information Resource (PIR)'s UniProt Knowledgebase (UniProtKB) Release 2017_02 published 15 February 2017 is relied upon. UniProt (Universal Protein Resource) is a comprehensive catalogue of information on proteins ("UniProt: the universal protein knowledgebase" Nucleic Acids Res. 45: D158-D169 (2017)).

This is to be used as is well understood in the art to locate the residue of interest. This is not always a strict counting exercise - attention must be paid to the context. For example, if the protein of interest is of a slightly different length, then location of the correct residue in that sequence may require the sequences to be aligned and the equivalent or corresponding residue picked. This is well within the ambit of the skilled reader.

Mutating has it normal meaning in the art and may refer to the substitution or truncation or deletion of one or more residues, motifs or domains. Mutation may be effected at the polypeptide level, for example, by synthesis of a polypeptide having the mutated sequence, or may be effected at the nucleotide level, for example, by making a polynucleotide encoding the mutated sequence, which polynucleotide may be subsequently translated to produce the mutated polypeptide. Suitably, the mutations to be used are as set out herein. Unless otherwise apparent from the context, mutations mentioned herein are substitutions. For example 'N32G' means that the residue corresponding to 'N32' in the wild type Stefin A (SEQ ID NO: 1) is substituted with G.

Sequence Variation

The polypeptides described herein may comprise sequence changes relative to the wild type sequence in addition to the key mutations described herein for modulating thermal stability such as Tm. Specifically the polypeptides described herein may comprise additional sequence changes at sites which do not significantly compromise the function or operation of the polypeptides described herein. The sequence changes may be at the polypeptide or the nucleotide level.

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Polypeptide function may be easily tested using the methods as set out in the examples section, for example in order to verify that the peptide structure or conformation has not been significantly altered. Thus, provided that the polypeptide retains its structure or conformation which can be easily tested as set out herein, sequence variations may be made in the polypeptide relative to the wild type reference sequence.

For example, a polypeptide may be tested to see if it retains its structure or conformation by assessing the near and far UV CD spectra; these are recorded in a compatible buffer (for example 50 mM sodium phosphate, pH 7.4) at 0.6 mg/mL and 0.2 mg/mL, respectively. Spectra are processed by subtracting a buffer spectrum from the sample spectrum and converting the units to molar ellipticity (for near UV CD spectra) or mean residue ellipticity (far UV CD spectra). Near UV CD spectra should have a maximum between 275 nm – 280 nm, and an addition minimum around 295 nm if a single tryptophan is present at position 35. This could be a maximum if a different position is chosen for the tryptophan. Far UV CD spectra should resemble that of a typical beta-sheet protein, having a minimum around 218 nm. If these conditions are met, the polypeptide has retained correct structure or conformation. If there is no near UV CD spectrum, or the far UV CD spectrum has minima at 208 nm and 222 nm, or a minimum at 198 nm, polypeptide has not retained correct structure or conformation and may have been significantly altered.

Polypeptides include variants produced by introducing any type of additional alterations (for example, insertions, deletions, or substitutions of amino acids; changes in glycosylation states; changes that affect refolding or isomerizations, three-dimensional structures, or self-association states), which can be deliberately engineered. The variant may have alterations which produce a silent change and result in a functionally equivalent polypeptide. Deliberate amino acid substitutions may be made on the basis of similarity in polarity, charge, solubility, hydrophobicity, hydrophilicity and the amphipathic nature of the residues as long as the structure or conformation of the polypeptide is retained. For example, negatively charged amino acids include aspartic acid and glutamic acid; positively charged amino acids include lysine and arginine; and amino acids with uncharged polar head groups having similar hydrophilicity values include leucine, isoleucine, valine, glycine, alanine, asparagine, glutamine, serine, threonine, phenylalanine, and tyrosine.

Conservative substitutions may be made, for example according to the table below. Amino acids in the same block in the second column and suitably in the same line in the third column may be substituted for each other:

ALIPHATIC	Non-polar	G A P
		I L V
	Polar - uncharged	C S T M
		N Q
	Polar - charged	D E
		K R

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AROMATIC		H F W Y

In considering what mutations, substitutions or other such changes might be made relative to the wild type sequence, retention of the structure or conformation of the polypeptide is important. Typically conservative amino acid substitutions would be less likely to adversely affect the function.

When engineering the polypeptide to modulate thermal stability as taught herein, one or more of amino acids

A12I, A12V, I16L, V20A, V20I, V20L, Q26E, E29M, T31K, N32G, N32D, N32H, T34V, T34R, T34K, T34D, T34P, L38A, L38V, L38F, A40I, A40V, Q42E, Q42D, T45I, T45V, V48E, V48D, V48G, V48A, V48L, G50S, T51F, T51V, T51L, T51I, T51A, A59L, A59I, A59V, K63R, M65V, M65I, L67I, N90T, (V20I, L38A), (V20L, L38A), (V20I, L38V), (V20L, L38V), (E29K, K30E, E33K), (Y54D, T83D, Q86E), (A59L, G60N, D61G, N62K), (A59V, D61N, N62K), (G60N, D61G, N62K), (G60N, ΔD61, N62G), ΔD61, (A59L, G60N, ΔD61, N62G), (A59V, G60N, D61G, N62K), (A59I, G60N, D61G, N62K), (A59I, G60N, ΔD61, N62G), (A59V, G60N, ΔD61, N62G), (A59V, ΔD61), (G60P, ΔD61, N62P), (G60P, D61P, N62K), (G60P, ΔD61, N62G), (G60P, D61G, N62K), (D61N, N62K), and (T83D, Q86E);

should be substituted as taught herein, or if not substituted should suitably correspond to SEQ ID NO: 1. Residues other than as shown in SEQ ID NO: 1 or substitutions other than as taught herein are suitably not used at these positions.

When engineering the polypeptide to increase thermal stability as taught herein, one or more of amino acids

E29M, N32G, T34V, T34R, T34K, Q42E, T45I, T45V, T51F, T51V, T51L, T51I, A59L, A59I, A59V, K63R, M65V, M65I, L67I, N90T, (E29K, K30E, E33K), (A59L, G60N, D61G, N62K), (A59V, D61N, N62K), (G60N, D61G, N62K), (G60N, ΔD61, N62G), ΔD61, (A59L, G60N, ΔD61, N62G), (A59V, G60N, D61G, N62K), (A59I, G60N, D61G, N62K), (A59I, G60N, ΔD61, N62G), (A59V, G60N, ΔD61, N62G), and (A59V, ΔD61);

should be substituted as taught herein, or if not substituted should suitably correspond to SEQ ID NO: 1. Residues other than as shown in SEQ ID NO: 1 or substitutions other than as taught herein are suitably not used at these positions.

When engineering the polypeptide to decrease thermal stability as taught herein, one or more of amino acids

A12I, A12V, I16L, V20A, V20I, V20L, Q26E, T31K, N32D, N32H, T34D, T34P, L38A, L38V, L38F, A40I, A40V, Q42D, V48E, V48D, V48G, V48A, V48L, G50S, T51A, (V20I, L38A), (V20L, L38A), (V20I, L38V), (V20L, L38V), (Y54D, T83D, Q86E), (G60P, ΔD61, N62P), (G60P, D61P, N62K), (G60P, ΔD61, N62G), (G60P, D61G, N62K), (D61N, N62K) and (T83D, Q86E);

should be substituted as taught herein, or if not substituted should suitably correspond to SEQ ID NO: 1. Residues other than as shown in SEQ ID NO: 1 or substitutions other than as taught herein are suitably not used at these positions.

When engineering the polypeptide to provide aromatic residue(s) for fluorescence/absorbance as taught herein, one or more of amino acids Y35W, Y43W, Y53W, Y54W, Y64W, F70W or Y85W should be substituted as taught herein, or if not substituted should suitably correspond to SEQ ID NO: 1. Residues other than as shown in SEQ ID NO: 1 or substitutions other than as taught herein are suitably not used at these positions.

Suitably the polypeptide of the invention comprises W at position 35, e.g. Y35W.

It should be noted that Y85W displays a clear transition upon unfolding as judged by BCM of intrinsic fluorescence emission spectra and improved absorbance properties compared to hSteA, but no obvious improvement in amplitude of the raw fluorescence emission spectra. For the avoidance of doubt, Y85W may still be used for transitions in unfolding – small improvements are provided. Therefore, this mutation is still useful to observe transitions, but does not increase fluorescence very much, but still works in this setting.

When engineering the polypeptide for biological neutrality as taught herein, one or more amino acids from the following table should be substituted as taught herein, or if not substituted should suitably correspond to SEQ ID NO: 1. Residues other than as shown in SEQ ID NO: 1 or substitutions other than as taught herein are suitably not used at these positions:

SUBSTITUTION(S) FOR BIOLOGICAL NEUTRALITY			
Amino acid (in SEQ ID NO: 1 - wild type Stefin A)	**substitution(s)**	**advantage(s)**	**notes**
G4	R W	increases the accessibility of the recognition (target binding) surface	G4 is also part of the inhibitory sequence motif

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V48	D E	abolish domain swap dimerisation	V48 is also part of the inhibitory sequence motif
G50	S	increased expression in a bacterial system	G50 is also part of the inhibitory sequence motif
Y35	W	introduces a fluorophore	
Y43	W	introduces a fluorophore	
Y53	W	introduces a fluorophore	
Y54	W	introduces a fluorophore	
Y64	W	introduces a fluorophore	
F70	W	introduces a fluorophore	
Y85	W	introduces a fluorophore	
F98	W	introduces a fluorophore	
^{71}KSL	^{71}NGP	Blocks secondary structure from the loop 4 propagating into the scaffold	
^{78}**EDL**	^{78}**ADR**		most suitably for research scaffolds; present in prior art SQT scaffold
71**KSL**PGQN**EDL**	71**NGP**PGQN**ADR**		most suitably for research scaffolds; present in prior art SQT scaffold

Residues which are less conserved between proteins are more likely to tolerate mutation.

In total, as many as 15 substitutions relative to SEQ ID NO: 1 may be made to increase thermal stability.

In addition, as many as 2 substitutions relative to SEQ ID NO: 1 may be made for biological neutrality.

In addition, as many as 1 substitutions relative to SEQ ID NO: 1 may be made to provide aromatic residue(s) for fluorescence/absorbance.

Therefore, in one embodiment the invention relates to polypeptide(s) having up to 18 substitutions relative to SEQ ID NO: 1.

By way of example, in one embodiment the invention relates to a polypeptide having the following substitutions relative to SEQ ID NO: 1:

1 for fluorescence (e.g. Y35W); 2 for biological neutrality (e.g. N32G, V48D); 15 for Tm (T34, Q42, T45, T51, K63, M65, L67, N90 (these are all single mutations), E29K K30E E33K (charge interactions), A59 G60 D61 N62 (the b-turn).

Additional mutation, most suitably substitution, of one or more residues other than those expressly mentioned above may be made, provided that of the structure or conformation of the polypeptide is retained. Therefore, in one embodiment the invention relates to polypeptide(s) having up to 25 mutations most suitably substitutions relative to SEQ ID NO: 1.

For example (1 - novel and Tm increased or decreased):

MIPGGLSEAK P**X1**TPE**X2**QEI**X3** DKVKP**X4**LE**X5X6** **X7X8X9X10**YGK**X11**E**X12** V**X13**YK**X14**QV**X15**AG **X16**NY**X17**IKVR**X18X19** **X20X21**KY**X22**H**X23**KVF KSLPGQNEDL VL**X24**GY**X25**VDKN KDDELTGF

X1 where wild type is A and mutations are I, V

X2 where wild type is I and mutations are L

X3 where wild type is V and mutations are I, L

X4 where wild type is Q and mutations are E

X5 where wild type is E and mutations are M

X7 where wild type is T and mutations are K

X8 where wild type is N and mutations are G, D, H

X10 where wild type is T and mutations are V, R, D, P

X11 where wild type is L and mutations are A, V

X12 where wild type is A and mutations are I, V

X13 where wild type is Q and mutations are D

X14 where wild type is T and mutations are I, V

X15 where wild type is V and mutations are E, G, A

X16 where wild type is T and mutations are F, V, L, A

X18 where wild type is A and mutations are L, I

X20 where wild type is D and mutations are Δ

X22 where wild type is M and mutations are V

X23 where wild type is L and mutations are I

(X3, X11) where wild type is (V, L) and mutations are (I, A), (L, A), (I, V), (L, V)

(X5, X6, X9) where wild type is (E, K, E) and mutations are (K, E, K)

(X17, X24, X25) where wild type is (Y, T, Q) and mutations are (D, D, E)

(X18, X19, X20, X21) where wild type is (A, G, D, N) and mutations are (L, N, G, K), (L, N, Δ, G), (V, N, G, K), (I, N, G, K), (I, N, Δ, G), (V, N, Δ, G)

(X18, X20, X21) where wild type is (A, D, N) and mutations are (V, N, K)

(X18, X20) where wild type is (A, D) and mutations are (V, Δ)

(X19, X20, X21) where wild type is (G, D, N) and mutations are (N, G, K), (N, Δ, G), (P, Δ, P), (P, P, K), (P, Δ, G), (P, G, K)

(X24, X25) where wild type is (T, Q) and mutations are (D, E)

(X20, X21) where wild type is (D, N) and mutations are (N, K)

Therefore, in one embodiment the invention relates to polypeptide(s) having up to 13 mutations most suitably substitutions relative to SEQ ID NO: 1. For example (2 - novel and Tm increased):

MIPGGLSEAK PATPEIQEIV DKVKPQLE**X1X2** T**X3X4X5**YGKLEA VQYK**X6**QVVAG **X7**NYYIKVR**X8X9** **X10X11**KY**X12**H**X13**KVF KSLPGQNEDL VLTGYQVDKN KDDELTGF

X1 where wild type is E and mutations are M

X3 where wild type is N and mutations are G

X5 where wild type is T and mutations are V, R

X6 where wild type is T and mutations are I, V

X7 where wild type is T and mutations are F, V, L

X8 where wild type is A and mutations are L, I

X10 where wild type is D and mutations are Δ

X12 where wild type is M and mutations are V

X13 where wild type is L and mutations are I

(X1, X2, X4) where wild type is (E, K, E) and mutations are (K, E, K)

(X8, X9, X10, X11) where wild type is (A, G, D, N) and mutations are (L, N, G, K), (L, N, Δ, G), (V, N, G, K), (I, N, G, K), (I, N, Δ, G), (V, N, Δ, G)

(X8, X10, X11) where wild type is (A, D, N) and mutations are (V, N, K)

(X9, X10, X11) where wild type is (G, D, N) and mutations are (N, G, K), (N, Δ, G)

(X8, X10) where wild type is (A, D) and mutations are (V, Δ)

Therefore, in one embodiment the invention relates to polypeptide(s) having up to 18 mutations most suitably substitutions relative to SEQ ID NO: 1. For example (3 - novel and Tm decreased):

MIPGGLSEAK P**X1**TPE**X2**QEI**X3** DKVKP**X4**LEEK **X5X6**E**X7**YGK**X8**E**X9** V**X10**YKTQV**X11**AG **X12**NY**X13**IKVRA**X14** **X15X16**KYMHLKVF KSLPGQNEDL VL**X17**GY**X18**VDKN KDDELTGF

X1 where wild type is A and mutations are I, V

X2 where wild type is I and mutations are L

X3 where wild type is V and mutations are I, L

X4 where wild type is Q and mutations are E

X5 where wild type is T and mutations are K

X6 where wild type is N and mutations are D, H

X7 where wild type is T and mutations are D, P

X8 where wild type is L and mutations are A, V

X9 where wild type is A and mutations are I, V

X10 where wild type is Q and mutations are D

X11 where wild type is V and mutations are E, G, A

X12 where wild type is T and mutations are A

(X3, X8) where wild type is (V, L) and mutations are (I, A), (L, A), (I, V), (L, V)

(X13, X17, X18) where wild type is (Y, T, Q) and mutations are (D, D, E)

(X14, X15, X16) where wild type is (G, D, N) and mutations are (P, Δ, P), (P, P, K), (P, Δ, G), (P, G, K)

(X15, X16) where wild type is (D, N) and mutations are (N, K)

(X17, X18) where wild type is (T, Q) and mutations are (D, E)

Therefore, in one embodiment the invention relates to polypeptide(s) having up to 28 mutations most suitably substitutions relative to SEQ ID NO: 1, suitably together with heterologous peptide insertion wherein said heterologous peptide insertion comprises a heterologous peptide inserted at one of the following positions relative to SEQ ID NO: 1:

d) 48-<heterologous peptide>-50,

e) 49-<heterologous peptide>-51,

f) 50-<heterologous peptide>-52,

q) 72-<heterologous peptide>-77,

r) 73-<heterologous peptide>-78; or

s) 74-<heterologous peptide>-79. For example (9 - Tm increased or decreased):

MIPGGLSEAK P**X1**TPE**X2**QEI**X3** DKVKP**X4**LE**X5X6** **X7X8X9X10**YGK**X11**E**X12** V**X13**YK**X14**QV**X15**A**X16** **X17**NY**X18**IKVR**X19X20** **X21X22X23**Y**X24**H**X25**KVF KSLPGQNEDL VL**X26**GY**X27**VDK**X28** KDDELTGF

X1 where wild type is A and mutations are I, V

X2 where wild type is I and mutations are D

X3 where wild type is V and mutations are A, I, L

X4 where wild type is Q and mutations are E

X5 where wild type is E and mutations are M

X7 where wild type is T and mutations are K

X8 where wild type is N and mutations are G, D, H

X10 where wild type is T and mutations are V, R, K, D, P

X11 where wild type is L and mutations are A, V, F

X12 where wild type is A and mutations are I, V

X13 where wild type is Q and mutations are E, D

X14 where wild type is T and mutations are I, V

X15 where wild type is V and mutations are E, D, G, A, L

X16 where wild type is G and mutations are S

X17 where wild type is T and mutations are F, V, L, I, A

X19 where wild type is A and mutations are L, I, V

X21 where wild type is D and mutations are Δ

X23 where wild type is K and mutations are R

X24 where wild type is M and mutations are V, I

X25 where wild type is L and mutations are I

X28 where wild type is N and mutations are T

(X3, X11) where wild type is (V, L) and mutations are (I, A), (L, A), (I, V), (L, V)

(X5, X6, X9) where wild type is (E, K, E) and mutations are (K, E, K)

(X18, X26, X27) where wild type is (Y, T, Q) and mutations are (D, D, E)

(X19, X20, X21, X22) where wild type is (A, G, D, N) and mutations are (L, N, G, K), (L, N, Δ, G), (V, N, G, K), (I, N, G, K), (I, N, Δ, G), (V, N, Δ, G)

(X19, X21, X22) where wild type is (A, D, N) and mutations are (V, N, K)

(X20, X21, X22) where wild type is (G, D, N) and mutations are (N, G, K), (N, Δ, G), (P, Δ, P), (P, P, K), (P, Δ, G), (P, G, K)

(X19, X21) where wild type is (A, D) and mutations are (V, Δ)

(X21, X22) where wild type is (D, N) and mutations are (N, K)

(X26, X27) where wild type is (T, Q) and mutations are (D, E)

Suitably when considering sequence identity, the amino acids marked as 'X' are not considered; suitably sequence identity is assessed across the amino acid residues specified or defined as above (i.e. the non-X residues).

In case any further guidance is required, to assess sequence identity when the reference sequence has variable residues (e.g. 'X' s) in it, sequence identity is calculated using the Protein BLAST server from the NIH (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The Query Sequence is

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the amino acid reference sequence with all the X's (Xn) removed. The Subject Sequence is the sequence of interest to be compared with the Query Sequence with equivalent residues removed. The analysis is then run and the Ident value taken as the result.

For example:

The sequence of hSteA containing X1 – X25 (reference sequence) is lined up with the sequence of interest - in this example canine SteA (cSteA):

hSteA (SEQ ID NO: 1)
MIPGGLSEAKPX1TPEX2QEIX3DKVKPX4LEX5X6X7X8X9X10YGKX11EX12VX13YKX14QVX15AGX16NYX17IKVRX18X19X20X21KYX22HX23KVFKSLPGQNEDLVLX24GYX25VDKNKDDELTGF

cSteA (SEQ ID NO: 3)

MMPGGLTEAKPA TPEV QEIA NEVKPQ LEE K T N E T YQEF EA VE YKT QVV AGI NYY IKVRV G D N SYI HL KIFKGLPGQNPTLTLT GYQ TDKSKDDELTGF

All Xn are removed, as are the corresponding residues in the sequence of interest cSteA:

hSteA - Query Sequence
MIPGGLSEAKPTPEQEIDKVKPLEYGKEVYKQVAGNYIKVRKYHKVFKSLPGQNEDLVLGYVDKNKDDELTGF

cSteA - Subject Sequence
MMPGGLTEAKPTPEQEINEVKPLEYQEEVYKQVAGNYIKVRSYHKIFKGLPGQNPTLTLGYTDKSKDDELTGF

These Query Sequence and Subject Sequence are compared using Protein BLAST. The result is these sequences have 59 of 73 residues identical, giving 81% sequence identity.

Similarly, when a sequence of interest has 'gaps' or truncations e.g. is shorter than the reference sequence such as SEQ ID NO: 1, such gaps or truncations are assessed by the sequence comparison and contribute to a correspondingly lower sequence identity value. In this regard, sequence identity should suitably be assessed across the whole length of SEQ ID NO: 1. By convention, a shorter sequence might be compared to the reference sequence (SEQ ID NO: 1) only across the length of the shorter sequence of interest. However, unless otherwise apparent from the context, discussion of / determination of sequence identity (and sequence identity values mentioned herein) suitably treat truncations as 'gaps'. To give a practical example, if a sequence of interest was only 49 amino acids long and was compared to SEQ ID NO: 1 (which is 98 amino acids long) then the maximum sequence identity score would be 50%, (and any substitutions relative to SEQ ID NO: 1 would further reduce the sequence identity score). Thus a sequence identity score for a polypeptide of interest compared to SEQ ID NO: 1 of 50% implies presence of at least 49 amino acid residues in the polypeptide of interest, 49 of which are identical to their corresponding residues in SEQ ID NO: 1.

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Suitably when a residue is not mutated as above, it is 'wild-type' as above. In other words, suitably when a residue is not mutated as above, it is as shown in hSteA (SEQ ID NO: 1).

Sequence Identity

For precision, sequence relationships have been discussed in terms of substitutions relative to SEQ ID NO: 1 (wild type human Stefin A). However it may be desired to consider sequence relationships in terms of sequence identity.

Sequence comparisons can be conducted by eye or, more usually, with the aid of readily available sequence comparison programs. These publicly and commercially available computer programs can calculate percent homology (such as percent identity) between two or more sequences.

Percent identity may be calculated over contiguous sequences, i.e., one sequence is aligned with the other sequence and each amino acid in one sequence is directly compared with the corresponding amino acid in the other sequence, one residue at a time. This is called an "ungapped" alignment. Typically, such ungapped alignments are performed only over a relatively short number of residues (for example less than 50 contiguous amino acids).

Although this is a very simple and consistent method, it fails to take into consideration that, for example in an otherwise identical pair of sequences, one insertion or deletion will cause the following amino acid residues to be put out of alignment, thus potentially resulting in a large reduction in percent homology (percent identity) when a global alignment (an alignment across the whole sequence) is performed. Consequently, most sequence comparison methods are designed to produce optimal alignments that take into consideration possible insertions and deletions without penalising unduly the overall homology (identity) score. This is achieved by inserting "gaps" in the sequence alignment to try to maximise local homology/identity.

These more complex methods assign "gap penalties" to each gap that occurs in the alignment so that, for the same number of identical amino acids, a sequence alignment with as few gaps as possible - reflecting higher relatedness between the two compared sequences - will achieve a higher score than one with many gaps. "Affine gap costs" are typically used that charge a relatively high cost for the existence of a gap and a smaller penalty for each subsequent residue in the gap. This is the most commonly used gap scoring system. High gap penalties will of course produce optimised alignments with fewer gaps. Most alignment programs allow the gap penalties to be modified. However, it is preferred to use the default values when using such software for sequence comparisons. For example when using the GCG Wisconsin Bestfit package (see below) the default gap penalty for amino acid sequences is -12 for a gap and -4 for each extension.

Calculation of maximum percent homology therefore firstly requires the production of an optimal alignment, taking into consideration gap penalties. A suitable computer program for carrying out such an alignment is the GCG Wisconsin Bestfit package (University of Wisconsin, U.S.A; Devereux et al., 1984, *Nucleic Acids Research* 12:387). Examples of other software than can perform sequence comparisons include, but are not limited to, the BLAST package, FASTA

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(Altschul et al., 1990, *J. Mol. Biol.* 215:403-410) and the GENEWORKS suite of comparison tools.

Although the final percent homology can be measured in terms of identity, the alignment process itself is typically not based on an all-or-nothing pair comparison. Instead, a scaled similarity score matrix is generally used that assigns scores to each pairwise comparison based on chemical similarity or evolutionary distance. An example of such a matrix commonly used is the BLOSUM62 matrix - the default matrix for the BLAST suite of programs. GCG Wisconsin programs generally use either the public default values or a custom symbol comparison table if supplied. It is preferred to use the public default values for the GCG package, or in the case of other software, the default matrix, such as BLOSUM62. Once the software has produced an optimal alignment, it is possible to calculate percent homology, preferably percent sequence identity. The software typically does this as part of the sequence comparison and generates a numerical result.

Suitably the polypeptide of the invention has at least 80% sequence identity to SEQ ID NO: 1, more suitably 85%, more suitably 88%, more suitably 90%, more suitably 92%, more suitably 94%, more suitably 95%, more suitably 96%, more suitably 97%, more suitably 98%, more suitably 99% identity to SEQ ID NO: 1.

In one embodiment more suitably the polypeptide of the invention has at least 80% sequence identity to those residues of SEQ ID NO: 1 which are other than those specifically recited as being substituted when defining the polypeptide of the invention under consideration (e.g. in the appended claim(s)), more suitably 85%, more suitably 88%, more suitably 90%, more suitably 92%, more suitably 94%, more suitably 95%, more suitably 96%, more suitably 97%, more suitably 98%, more suitably 99% identity. In other words, in this embodiment, more suitably percent sequence identity is assessed for the sequence of interest compared to SEQ ID NO: 1, whilst excluding residues which are specifically defined (e.g. substitutions) in the particular embodiment of the polypeptide under consideration.

Suitably any heterologous peptide insertions are excluded from percent identity calculations.

In one embodiment suitably the substitutions and any heterologous peptide insertions are as defined, and sequence identity is judged against the remaining 'background' or 'backbone' sequence of the polypeptide compared to the corresponding residues of SEQ ID NO: 1.

In all discussions of sequence identity, it will be noted that SEQ ID NO: 1 is 98 amino acids in length. Therefore each single substitution is equivalent to 1.020408% change in identity if all 98 amino acids are considered. The above values are given to nearest whole percentage point and should be understood accordingly given that it is not possible to substitute partial amino acids within a polypeptide sequence. Clearly when fewer than 98 amino acids are considered (for example when considering sequence identity to those residues of SEQ ID NO: 1 which are other than those specifically recited as being substituted when defining the polypeptide of the invention under consideration) then each single amino acid substitution may correspond to greater than 1.020408% change in identity; the skilled reader can interpret the values accordingly given that it is not possible to substitute partial amino acids within a polypeptide sequence.

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In case any further guidance is needed, the table below shows how the percent sequence identity value varies taking into account number of residues compared and number of amino acid substitutions made within that number of residues compared. Any further values outside the examples in the table can be easily calculated by the skilled worker.

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Percent Sequence Identity Values									
total amino acids compared	**number of substitutions**								
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
	%	%	%	%	%	%	%	%	%
98	98.98	97.96	96.94	95.92	94.90	93.88	92.86	91.84	90.82
97	98.97	97.94	96.91	95.88	94.85	93.81	92.78	91.75	90.72
96	98.96	97.92	96.88	95.83	94.79	93.75	92.71	91.67	90.63
95	98.95	97.89	96.84	95.79	94.74	93.68	92.63	91.58	90.53
94	98.94	97.87	96.81	95.74	94.68	93.62	92.55	91.49	90.43
93	98.92	97.85	96.77	95.70	94.62	93.55	92.47	91.40	90.32
92	98.91	97.83	96.74	95.65	94.57	93.48	92.39	91.30	90.22
91	98.90	97.80	96.70	95.60	94.51	93.41	92.31	91.21	90.11
90	98.89	97.78	96.67	95.56	94.44	93.33	92.22	91.11	90.00
89	98.88	97.75	96.63	95.51	94.38	93.26	92.13	91.01	89.89
88	98.86	97.73	96.59	95.45	94.32	93.18	92.05	90.91	89.77
87	98.85	97.70	96.55	95.40	94.25	93.10	91.95	90.80	89.66
86	98.84	97.67	96.51	95.35	94.19	93.02	91.86	90.70	89.53
85	98.82	97.65	96.47	95.29	94.12	92.94	91.76	90.59	89.41
84	98.81	97.62	96.43	95.24	94.05	92.86	91.67	90.48	89.29
83	98.80	97.59	96.39	95.18	93.98	92.77	91.57	90.36	89.16
82	98.78	97.56	96.34	95.12	93.90	92.68	91.46	90.24	89.02
81	98.77	97.53	96.30	95.06	93.83	92.59	91.36	90.12	88.89
80	98.75	97.50	96.25	95.00	93.75	92.50	91.25	90.00	88.75
79	98.73	97.47	96.20	94.94	93.67	92.41	91.14	89.87	88.61

78	98.72	97.44	96.15	94.87	93.59	92.31	91.03	89.74	88.46

Where an amino acid sequence identity value is given for a particular polypeptide, the skilled reader will be aware that this particular polypeptide might also comprise a heterologous peptide insertion, and this must be borne in mind when assessing the sequence identity. Suitably the sequence identity is assessed across the amino acid residues corresponding to those in the wild type Stefin A sequence of SEQ ID NO: 1. In other words, suitably assessment of sequence identity does not include heterologous peptide insertions (or lack of insertions) but is considered across the amino acids corresponding to those present in SEQ ID NO: 1.

In one embodiment more suitably sequence identity is considered across the amino acids corresponding to those present in SEQ ID NO: 1 but excluding any heterologous peptide insertions and excluding any substitutions already defined in the claim under consideration.

Most suitably the percent sequence identity is calculated for amino acid residues excluding those substituted for thermal stability reasons.

For some applications, for example for intended therapeutic applications, it is desirable to minimise the number of mutations in the polypeptide / scaffold protein used. Thus, in some embodiments suitably the polypeptide / scaffold protein comprises five or fewer mutations compared to wild-type human Stefin A, suitably four or fewer, suitably three or fewer, suitably two or fewer mutations. In this context "mutations" refers to point mutations, or insertion or deletion of a small number such as five or fewer amino acids. Insertion of heterologous peptides into the polypeptide / scaffold protein of the invention is discussed separately. Insertion of one or more heterologous peptides is not counted as a "mutation" in this context. Most suitably mutation(s) means substitution(s).

It should be noted that the standard in the art is the SQT scaffold protein as disclosed in WO 2009/136182. This has a low thermal stability/Tm of 64.6 ºC. Suitably the SQT scaffold is a scaffold having a sequence of SEQ ID NO:24 of WO 2009/136182 (SQT): MIPRGLSEAK PATPEIQEIV DKVKPQLEEK TNETYGKLEA VQYKTQVLAS TNYYIKVRAG DNKYMHLKVF NGPPGQNADR VLTGYQVDKN KDDELTGF.

Thus, embodiments of the invention show significant advantages compared to the prior art SQT scaffold. However, it should be noted that comparative values expressed herein are relative to wild type Stefin A unless otherwise apparent from the context. Thus, it is possible to have a polypeptide / scaffold protein as disclosed herein having a reduced (lower) Tm compared to wild type Stefin A, but which nevertheless offers the advantage of improved (higher) Tm relative to SQT. The values are all expressed relative to wild type Stefin A (unless otherwise apparent from the context) for ease of understanding and consistency, but the advantages of the invention can be obtained in modulating the Tm whether the resulting Tm is higher or lower than that of wild type Stefin A. The Tm desired by the operator of the invention may be higher or lower than that of wild type Stefin A, or of SQT. The key advantage is that the skilled reader is now enabled to alter/modulate the Tm of the polypeptide / scaffold protein as taught herein.

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Suitably the scaffold of the invention has a Tm higher than SQT i.e. higher than 64.6ºC. Most suitably the scaffold of the invention has a Tm higher than wild type Stefin A i.e. higher than 89.0ºC.

It is an advantage of the invention that a polypeptide / scaffold protein which is biologically neutral is provided.

It is an advantage of the invention that a polypeptide / scaffold protein lacking glycosylation sites is provided.

It is an advantage of the invention that polypeptides / scaffold proteins with reduced or ameliorated propensity to dimerise are provided. This is advantageous because dimerisation can change the pharmacokinetics when the scaffold is a drug. This is also advantageous because dimerisation can affect affinity and therefore can affect interaction with the target. This is also advantageous because dimerisation can affect immunogenicity. Dimerisation can also change the effect of certain molecules from agonist to antagonist (or vice versa). In addition, in some cases dimerisation can promote higher-order structure formations and instability over time. Moreover, there are certain advantages in having monomers (i.e. reducing dimerisation), for example monomers are small (e.g. improving tumour penetration).

Clearly if the skilled operator desires dimers, then dimerisation residues can be treated accordingly e.g. retained as wild-type such as V48 to allow/promote domain-swap dimerisation if this is deemed advantageous for particular applications.

Heterologous Peptide Insertions

Suitably the heterologous peptide comprises 36 amino acids or fewer, more suitably 20 amino acids or fewer, more suitably 12 amino acids or fewer.

Suitably the heterologous peptide comprises 3 amino acids or more, more suitably 12 amino acids or more, more suitably 20 amino acids or more, more suitably up to 36 amino acids.

Suitably the heterologous peptide comprises 3 to 36 amino acids, more suitably 3 to 20 amino acids, more suitably 3 to 12 amino acids, more suitably 3 to 9 amino acids, more suitably 6 to 36 amino acids, more suitably 6 to 20 amino acids, more suitably 6 to 12 amino acids, more suitably 6 to 9 amino acids, most suitably 9 amino acids.

It is an advantage of the invention that a small increase in thermal stability is observed when heterologous peptide insertions are included in the polypeptide of the invention as taught herein.

Location of Heterologous Peptide Insertions

A heterologous peptide sequence may be inserted between amino acids 46 to 54 inclusive, (for example as described in WO 2006/131749 (describing scaffolds such as 'STM') or for example WO 2009/136182 (describing scaffolds such as 'SQT')), more suitably between amino acids 46 to 50 inclusive, more suitably between amino acids 48 to 50 inclusive. In this context

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'between...inclusive' means that the noted amino acids may be deleted in favour of the heterologous peptide insertion i.e. that any or all of the amino acid(s) from (e.g.) 46-54 may be deleted and the heterologous peptide insertion added in their place. Therefore an insertion which deletes amino acids 46-54 and replaces them is embraced by the phrase 'between amino acids 46 to 54'. There is no requirement for amino acid 46 and amino acid 54 to be retained - the peptide is inserted between those positions with reference to SEQ ID NO:1 rather than requiring the actual presence of amino acid 46 and amino acid 54 in order to be 'between' them. When all of amino acids 46-54 are deleted his may be represented as "45-<heterologous peptide>-55".

A heterologous peptide sequence may be inserted between amino acids 67 to 84 inclusive. A heterologous peptide sequence may be inserted between amino acids 71 to 73 inclusive. A heterologous peptide sequence may be inserted between amino acids 82 to 83 inclusive. The 'Leu 73' site is an insertion site for heterologous peptides (sometimes referred to as 'target peptides'). This represents a solvent exposed loop of the Stefin A protein, and is therefore amenable to the display of target peptides in a solvent accessible manner. The term 'Leu73 insertion' is used herein to describe insertion of a heterologous peptide close to or suitably at (e.g. within) the L73-L80 loop of human SteA. The term may refer to addition(s) to or insertion(s) at, or replacement of, Leu 80 of human stefin A. More suitably the term refers to addition(s) or insertion(s) at, or replacement of, Leu 73 of human stefin A. In one embodiment, the Leu73 mutation may comprise replacement of the whole loop between L73 and L80 with a heterologous peptide sequence. A heterologous peptide sequence may be inserted between amino acids 73 to 80 inclusive.

For example, insertions may be made like this (using the SQT sequence as an example):

MIPRGLSEAK PATPEIQEIV DKVKPQLEEK TNETYGKLEA VQYKTQVLA[heterologous peptide sequence in Loop2]S TNYYIKVRAG DNKYMHLKVF NGP[heterologous peptide sequence in Loop4][ΔPGQN]ADR VLTGYQVDKN KDDELTGF

Thus suitably, Loop 2 has a heterologous peptide sequence inserted between 49 and 50 (49-<heterologous peptide>-50) and Loop 4 has a heterologous peptide sequence inserted between 73 and 78 as residues 74-77 are replaced (73-<heterologous peptide>-78).

A heterologous peptide sequence may be inserted at the G4 site. A heterologous peptide sequence may be inserted at position 4. A heterologous peptide sequence may be inserted proximal to position 4 i.e. between position 4 and the centre of the polypeptide such as between amino acids 4 to 45 inclusive, or between amino acids 4 to 44 inclusive. Suitably the G4 residue is retained. Most suitably the G4 residue is mutated to G4R. Thus suitably a heterologous peptide sequence may be inserted between amino acids 5 to 45 inclusive, or between amino acids 5 to 44 inclusive. More suitably a heterologous peptide sequence may be inserted between amino acids 4 and 5 inclusive, most suitably immediately after amino acid 4, e.g. insertion at position 5.

Heterologous peptide(s) may be inserted in at least one of the following positions in the protein (see for example WO2014/125290 (describing scaffolds such as 'plant adhirons')): the

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loop between a first and second strand of β-sheet (sometimes known as LOOP1); and/or the loop between a third and fourth strand of β-sheet (sometimes known as LOOP2). In this context, first, second, third and fourth mean relative to the protein sequence, i.e. from the N- to C-terminus of the protein. Suitably heterologous peptides are inserted at both of these positions, i.e. at LOOP1 and LOOP2.

Loop Nomenclature

The secondary structure of the polypeptide / scaffold protein may be modelled using open source software, most suitably using "PPopen" available from the Technical University of Munich, Germany. The numbering of structures such as loops in the polypeptide / scaffold protein of the invention adheres to the systematic naming given using the PPopen software.

It should be noted that prior art documents have used an informal nomenclature. Therefore, disclosures in prior art documents such as WO 2006/131749 and/or WO 2009/136182 and/or WO2014/125290 might mention the informal name "loop 1", which would actually relate to "loop 2" using the systematic naming via PPopen software. The table below sets out the nomenclature of the different structural parts of the protein, and this will be adhered to throughout the text unless otherwise indicated.

Unless otherwise apparent from the context, the following loop nomenclature is adhered to. Amino acid residue numbers are as in, or corresponding to, SEQ ID NO: 1:

Name	Amino Acid address (SEQ ID NO: 1)
N-terminus	1 – 13
Helix 1	14 – 30
Loop 1	31 – 37
Strand 1	38 – 48
Loop 2	49 – 51
Strand 2	52 – 59
Loop 3	60 – 62
Strand 3	63 – 70
Loop 4	71 – 78
Strand 4	79 – 85
C-terminus	86 – 98 (not inc. GH6 tag)

As used here, the word "loop" means "connecting regular secondary structure" (i.e. alpha/beta).

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Occasionally informal nomenclature is used to relate to particular embodiments of the invention. For example, a preferred scaffold may be referred to as "3 T" or "3 R". The particular makeup of preferred embodiments of the invention is always disclosed with specific reference to mutated residues as set out in the text.

Suitably a population of scaffold polypeptides is created, each with a different heterologous peptide sequence (most suitably one per scaffold polypeptide) ie. a library.

'Heterologous' has its natural meaning i.e. the inserted polypeptide has an amino acid sequence which is heterologous to the stefin A sequence used e.g. the sequence derived from or corresponding to SEQ ID NO: 1. Therefore heterologous may mean from another species, and/or may mean from a polypeptide other than human wild type stefin A (SEQ ID NO: 1). Most suitably the heterologous polypeptide insertion comprises artificial amino acid sequence, most suitably artificial amino acid sequence designed by the operator.

Test heterologous peptide insertions may be used, for example GGS repeats such a nonameric peptide comprising GGSGGSGGS may be used. This is a particularly demanding peptide because it is entropically very difficult to close. Therefore, by inserting this as a heterologous peptide into a polypeptide / scaffold protein of the invention it is demonstrated that the scaffold performs very well in displaying such a soluble and entropically difficult to close loop. The capacity to display such a loop is a very strong indicator of the stability of the polypeptide / scaffold protein of the invention.

Especially Suitable Sites for Heterologous Peptide Insertion

It should be noted that the most preferred sites for heterologous peptide insertion in the polypeptides / scaffold proteins of the invention are themselves different to the sites disclosed for insertion in prior art disclosures of Stefin A based polypeptides / scaffold proteins.

Single insertions - Loop 2

48-<heterologous peptide>-50

49-<heterologous peptide>-51

50-<heterologous peptide>-52

Single insertions - Loop 4

72-<heterologous peptide>-77

73-<heterologous peptide>-78

74-<heterologous peptide>-79

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For the avoidance of doubt, "48-<heterologous peptide>-50" means between the amino acid corresponding to position 48 of SEQ ID NO: 1 and the amino acid corresponding to position 50 of SEQ ID NO: 1, and so on. Similarly "72-<heterologous peptide>-77" means between the amino acid corresponding to position 72 of SEQ ID NO: 1 and the amino acid corresponding to position 77 of SEQ ID NO: 1 (with the intervening amino acids being deleted (i.e. replaced by the heterologous peptide insertion), and so on.

Combinations - double insertions

Combinations of two insertions, one into Loop 2 and one onto Loop 4, may be made as disclosed in Figure 1.

Further Combinations

In addition to the specific example insertions mentioned above, single insertions or double insertions may be combined with an additional insertion at the N-terminus of the protein e.g. at or proximal to the G4 site as disclosed above - thereby making double insertions (G4 site + loop 2 or G4 site + loop 4) or triple insertions (G4 site + loop 2 + loop 4) as desired.

Most suitably a heterologous peptide is inserted between residues D48 and G50, deleting residue A49 (referred to as "48-<heterologous peptide>-50").

Most suitably a heterologous peptide is inserted between residues L73 and E78, deleting residues P74, G75, Q76 and N77 (referred to as "73-<heterologous peptide>-78").

Most suitably a double insertion is made by inserting a first heterologous peptide at 48-<first heterologous peptide>-50 and a second heterologous peptide at 73-<second heterologous peptide>-78.

First and second heterologous peptides are suitably different from each other.

First and second and third heterologous peptides are suitably different from each other.

Exemplary sequences showing exemplary insertions are below (X=any amino acid; in these examples the heterologous peptide insertions are 9 amino acids long):

exemplary scaffold 3r2

MIPGGLSEAKPATPEIQEIVDKVKPQLEKETGKTWGKLEAVEYKTQVD48XXXXXXXXXG50LNYYIKVRVN-GKYIHLKVFKSL73XXXXXXXXXE78DLVLTGYQVDKNKDDELTGF

exemplary scaffold 3t4

MIPGGLSEAKPATPEIQEIVDKVKPQLEEKTGETYGKLEAVEYKTQVD48XXXXXXXXXG50TNYYIKVRAGD NKYIHLKVFKSL73XXXXXXXXXE78DLVLTGYQVDKNKDDELTGF

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The specific preferred insertion points taught herein deliver certain additional advantages. These advantages are attributable to the choice of insertion site, and therefore could be advantageously employed on polypeptides / scaffold proteins having mutations different from those disclosed herein. In other words, the disclosure of the specific insertion sites made in this document is a discrete disclosure of an advantageous property provided by a technical feature. Therefore, in one aspect the invention may relate to a polypeptide / scaffold protein such as a Stefin A based polypeptide / scaffold protein having a heterologous peptide (or heterologous peptides) inserted at the specific position (or positions) as disclosed herein. For example, Single insertions - Loop 2: 48-<heterologous peptide>-50; 49-<heterologous peptide>-51; 50-<heterologous peptide>-52; Single insertions - Loop 4 - 72-<heterologous peptide>-77; 73-<heterologous peptide>-78; 74-<heterologous peptide>-79; Combinations - double insertions - combinations of two insertions, one into Loop 2 and one onto Loop 4, may be made as disclosed in Figure 1.

Affinity Maturation

Affinity maturation may be carried out by any suitable method known in the art. For example, a "mini-library" may be constructed keeping one heterologous peptide from a binder of interest constant whilst only varying the second or further heterologous peptide insertion(s).

Another approach is to alanine scan the heterologous peptide insertions in order to identify the amino acid(s) which are most important for interaction with the target.

An alternative approach is to carry out "slippy PCR" and to rescreen the resulting variants of the heterologous peptide insertion(s) against the target.

In the wild-type Stefin A, it is understood that one of its binding partners (Cathepsin) binds via all three loops on the relevant surface of the Stefin A molecule. Therefore, another approach to affinity maturation is to introduce heterologous peptide into the third position (the region comprising the G4 residue of Stefin A) at the N-terminal end of the protein and to rescreen the scaffold proteins carrying that set of heterologous peptides against the target.

As described herein, 'd' means 'deletion of' - for example 'dD61' means 'deletion of D61' - this is usually shown as 'ΔD61' (i.e. delta D61), as is common in the art. The residue numbering always follows human wild type SteA i.e. SEQ ID NO: 1.

In other words, when a deletion mutation is made in a polypeptide / scaffold protein according to the invention, then the numbering according to the human wild-type Stefin A protein is still adhered to. For example, if amino acid 61 is deleted, the numbering of that particular embodiment of a polypeptide / scaffold protein of the invention would go from amino acid 60 directly to amino acid 62 – in other words, the amino acid numbering is retained compared to the human wild-type Stefin A reference sequence rather than adjusting to consecutively number each of the amino acids present in the particular preferred embodiment of the polypeptide / scaffold protein of the invention. This is conventional and easily understood by the person skilled in the art as is normal in the field of molecular biology.

It should be noted that multiple insertion sites for heterologous peptides are disclosed. Thus, the polypeptide / scaffold protein may comprise a single heterologous peptide insertion, may comprise two heterologous peptide insertions at two different insertion sites (i.e. a single insertion at a first and a second site totalling two heterologous peptide insertions in total), or may comprise three heterologous peptide insertions at three different insertion sites (i.e. a single insertion at a first, second and third site totalling three heterologous peptide insertions in total). Libraries comprising polypeptides / scaffold proteins may comprise a single heterologous peptide insertion per polypeptide / scaffold protein, may comprise two heterologous peptide insertions per polypeptide / scaffold protein, or may comprise three heterologous peptide insertions per polypeptide / scaffold protein.

Embodiments of the invention resulting in a decreased thermostability find application in settings where it is desired to remove functionality of the polypeptide / scaffold protein by thermal treatment. One example is reducing the Tm of an Affimer® protein designed to inhibit an enzyme such that allows the enzyme to function by heating it to a set temperature (e.g. a hot-start polymerase). For example, Affimer® reagents such as a polypeptide / scaffold protein comprising a heterologous peptide insertion as described herein may be used to inhibit thermostable nucleic acid polymerase. These find application in PCR reaction mixtures. For example, it may be useful to produce an Affimer® reagent e.g. a polypeptide / scaffold protein according to the invention which inhibits the exonuclease function of thermostable nucleic acid polymerase. This is especially useful when primers are premixed into a PCR reaction mixture and then stored until used. If the exonuclease function of the thermostable nucleic acid polymerase is not inhibited, then that exonuclease function can degrade the primers in the reaction mixture. However, by including a polypeptide / scaffold protein according to the invention capable of inhibiting the exonuclease function of that polymerase into the reaction mixture, this drawback in prior art reaction mixtures can be avoided. In this scenario, it is important that the polypeptide / scaffold protein can be de-functionalised (such as denatured) in order to allow the PCR reaction to proceed.

Thus, suitably the polypeptide / scaffold protein used in this application has a decreased thermal stability so that it is degraded (such as denatured) during a first thermal cycle of the PCR reaction and thereby the inhibition of the exonuclease function of the thermostable polymerase is removed allowing the reaction to proceed.

Another example is an affinity ligand that could be co-eluted with the target protein but then removed (or its function removed) by a heating step where the Affimer® protein is designed to have a lower Tm than the target protein.

Fluorescence

In can be useful to exchange aromatic amino acids in the protein such as phenylalanine for alternative aromatic amino acids displaying fluorescence and/or absorbance at 280 nm properties. For example, phenylalanine may be mutated to tyrosine (which has weak fluorescence properties) or may be mutated to tryptophan (which has strong fluorescence properties). There are eight possible sites for these mutations within wild-type Stefin A. These

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have been tested by the inventors. The mutations and their usefulness in these applications are indicated in the table below.

Aromatic residue variants of human stefin A

Variant	Improved absorbance	Improved transition in intrinsic fluorescence properties upon unfolding[1]	Stability (Tm, °C)[2]
Y35W	Yes	Best	75
Y43W	Yes	Best	68
Y53W	Yes	Best	66
Y54W	Yes	Good	71
Y64W	Yes	Good	74
F70W	Yes	Good	73
Y85W	Yes	Good	75
F98W	Yes	No	N/A
F70Y	Marginal	No	N/A
F98Y	Marginal	No	N/A

[1] As judged by Optim thermal ramps.

[2] Measured using intrinsic fluorescence during Optim thermal ramp.

From this it can be concluded that certain of the above mutations are useful for absorbance at 280 nm, for example in determining protein concentrations. Moreover, certain mutations may be useful to introduce fluorescence which is helpful in monitoring changes in conformation of the protein such as protein unfolding.

More suitably the polypeptide of the invention comprises a substitution relative to SEQ ID NO: 1 selected from the group consisting of Y35W, Y43W, Y53W, Y54W, Y64W, F70W or Y85W. These all have good fluorescence properties.

Suitably the polypeptide of the invention comprises a substitution relative to SEQ ID NO: 1 selected from the group consisting of Y35W, Y43W and Y53W. These have the best fluorescence properties.

According to the above table, the most preferred mutation in a polypeptide / scaffold protein according to the invention would be Y35W. This has the advantages of being the least destabilising of the mutations and also offering the best spectra for analysis.

It should be noted that each tryptophan introduced into the polypeptide / scaffold protein of the invention is to some degree destabilising. This would be advantageous when considering embodiments of the invention resulting in reduced thermal stability. Alternatively, the reduced stability effect of these mutations may be ameliorated by making other mutations to increase the thermal stability as is taught throughout this application in detail. Therefore, in some embodiments the polypeptide / scaffold protein of the invention may comprise both stabilising and destabilising mutations depending on the aims or priorities for that particular polypeptide / scaffold protein.

It is an advantage of the N32G mutation that a particularly strong increase in thermal stability is delivered, for example an approximately 3°c increase.

It is an advantage of the preferred M65I mutation that an especially strong increase in thermal stability is delivered of +6 °C.

It is noted that the description of each mutation described herein as increasing the thermal stability of the polypeptide / scaffold protein of the invention is itself novel.

It should be noted that Cystatin C is only distantly related to the sequence of Cystatin A (Stefin A). Cystatin C is naturally a dimer, is a longer protein and has a low sequence identity level compared to Stefin A. The Cystatin fold is similar between Cystatin A and Cystatin C, but these proteins share only one region of homology corresponding to amino acids 49 to 95 of Cystatin C which correspond to amino acid positions 16 to 60 of Cystatin A. Over this region of relatedness, only 13/47 amino acids are identical corresponding to a sequence identity of 28%.

Subsets of Mutations

Mutation(s) from one group may be chosen to increase packing of the hydrophobic core.

Mutation(s) from one group may be chosen to increase charge interactions on the surface of the polypeptide / scaffold protein.

Mutation(s) from one group may be chosen to increase the geometry of a particular turn in the polypeptide structure. By "increase" we mean to make the turn energetically more favourable, for example energetically more stable. An example of one such mutation is deletion of D61 (ΔD61).

Mutation(s) from one group may be chosen to increase stability whilst still operating within the constraints of the three-dimensional structure for example at the end of β turns. More specifically, mutations may be carefully chosen to preserve the I-I+4 hydrogen bonding arrangements at the end of certain β turns.

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Mutation(s) from one group may be chosen to improve stability whilst advantageously changing the type of turn in the three-dimensional structure of the protein. For example, mutations may be made to change from a Type-1 to a Type-2 β turn if desired.

It should be noted that there is a comprehensive teaching in this application of the mutations which are useful in providing the disclosed technical features and advantages. For example, with reference to mutation at position Q42, mutating from E to D is widely regarded as a conservative substitution since this effectively drops only a single methylene group from the side chain of the amino acid. However, as can be seen from the data included in this application, a Q42E mutation provides an increase in thermal stability of +3°c, whereas a Q42D mutation provides a decrease in thermal stability of -3°c. Therefore, a seemingly conservative substitution providing a seemingly tiny change to the structure of the amino acid residue at this position can provide a dramatic difference of 6°c between the Tm's of the two polypeptide / scaffold protein variants. Therefore, the data in this application support unity of invention between each of the different mutations disclosed since they have been carefully chosen, designed and tested as well as demonstrated to each provide the same single special technical effect. Moreover, the claims of this application are limited to only variants known and proven to have and to deliver that known special technical effect. For this reason, the diverse mutations described in the appended claims do indeed relate only to a single invention and meet the requirements of unity.

In all instances, mentions of "increase" or "decrease" of stability, for example as reflected by increases or decreases in the Tm of particular polypeptides / scaffold proteins, are quoted relative to human Stefin A i.e. the properties of the wild-type human Stefin A polypeptide having the amino acid sequence as shown in SEQ ID No. 1. Similarly, all mutations are described with reference to the same wild-type human Stefin A sequence SEQ ID No. 1.

The following groups of mutations are especially advantageous for the following reasons.

- <u>Solvent Exposed</u>

  Mutations at these positions improve the network of charges on the protein's surface, helping to make it more stable. It can be easier to make charge changing amino acid substitutions on the surface of the Stefin A protein, which is advantageous. In addition, mutating residues on the protein surface can allow more freedom in the choice of substitutions. In addition, it is an advantage that the corresponding structural changes can be more predictable when altering residues on the protein surface compared to other locations.

One or more substitutions of solvent exposed residues may be selected from the group consisting of:

(E29K K30E E33K), N32G, N90T, K63R, T34V, T34K and T34R.

It is to be noted that throughout the text where a group of mutations is bracketed together (e.g. "(E29K K30E E33K)"), suitably those mutations are made together i.e. simultaneously as a group / as a single option from the list given.

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In particular, N32G offers improved stability of the helix.

In particular, K63R extends chain resulting in increased hydrophobic moiety, repositioning of charged atom.

- Partially Buried

Mutating these residues advantageously adds extra hydrophobic mass/area to the core of the protein. This increases the amount of hydrophobic interactions in the core of the protein. This has the advantage of improving the stability of the protein.

With regard to "Aliphatic chain extension" mutations, those advantageously add larger side chains, which again advantageously add extra hydrophobic mass to the core of the protein.

One or more substitutions of partially buried residues may be selected from the group consisting of:

Q42E, T51I, T51V, T51L and T51F.

Additional advantageous properties shared by particular subgroups of substitutions are set out below:

Aliphatic chain extension

T51I, T51L, T51F

Amphipathic to aliphatic

T51I, T51V, T51L

Amphipathic to aromatic

T51F

Amine to acid

Q42E

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With reference to the Q42E mutation, this advantageously keeps the chain length of the amino acid residue the same, and advantageously enables a change in chemistry to be isolated from the change to the overall size of the amino acid side chain.

- Hydrophobic Core (Tertiary Structure)

  These mutations increase burial of hydrophobic moieties, and therefore have the advantage of increasing stability in this manner.

One or more substitutions of hydrophobic core (tertiary structure) residues may be selected from the group consisting of:

T45V, T45I, A59V, A59I, A59L, M65V and M65I.

Additional advantageous properties shared by particular subgroups of substitutions are set out below:

Chain extension

T45I, A59V, A59I, A59L

Amphipathic to aliphatic

T45V, T45I

Beta-branching

M65V, M65I, A59V, A59I

With regard to "chain extension" mutations, these add additional hydrophobic contacts and therefore help to increase the stability further. This is especially true of longer and/or branched side chains in the amino acids which are introduced. Moreover, certain mutations remove polar groups from the amino acid residues and result in completely hydrophobic residue whereas the wild-type might have been amphipathic.

Without wishing to be bound by theory, it is believed that the gain in stability is due to the relative instability of exposed hydrophobic moieties in an unfolded state compared to the buried hydrophobic sidechains in the folded state. Therefore, provided a good packing occurs in the core of the folded state, the more hydrophobic parts the more stable the native state will be relative to the unfolded state - which is what we

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measure with Tm (a proxy for Gibbs free energy of folding (DG)). Thus the stability gain comes from maximising the amount of hydrophobics that are buried by packing with other hydrophobics efficiently.

With regard to both partially buried and hydrophobic core (tertiary structure) groups of mutations, these share the common property of advantage of tending to have more contacts within the protein structure. The effect of targeting these residues is that potentially larger effects on protein stability can be gained by a smaller number of substitutions compared to targeting other parts of the protein. Therefore, these groups of mutations together form a superset of substitutions all sharing the same single special technical feature.

- Secondary Structure

  It should be noted that deletion of amino acid 61 converts the 5 amino acid patch from residue 59 to 63 into a sequence consistent with a canonical β turn motif. This 4 amino acid motif created by the deletion of amino acid 61 presents a canonical β turn motif. This innovative approach taken by the inventors takes advantage of the existing residues in the wild-type protein to make considerable changes to the stability whilst enabling minimal mutation of the starting polypeptide. An advantage delivered by the group of mutations is the advantage of stabilising turns in the protein (and/or stabilising the helix in the case of N32G). In either case, targeting these residues delivers the advantage of promoting stable hydrogen bonding throughout the affected region of the protein, and thereby advantageously increasing stability.

One or more substitutions of secondary structure residues may be selected from the group consisting of:

N32G, ^{59}AN-GK, ^{59}IN-GK, ^{59}VN-GK, ^{59}LN-GK, ^{59}AG-NK, ^{59}VG-NK, A59V, A59I, A59L, T34K and T34R.

It is to be noted that throughout the text where a group of mutations is presented as a pair (e.g. "^{59}AN-GK"), suitably those mutations are made together i.e. simultaneously as a pair / as a single option from the list given.

Additional advantageous properties shared by particular subgroups of substitutions are set out below:

C-terminal helix cap

N32G, T34K, T34R

Turns

A59V, A59I, A59L, ^{59}AN-GK, ^{59}IN-GK, ^{59}VN-GK, ^{59}LN-GK, ^{59}AG-NK, ^{59}VG-NK

Type I' (type 1 prime)

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^{59}AN-GK, ^{59}IN-GK, ^{59}VN-GK, ^{59}LN-GK

Type II' (type 2 prime)

^{59}AG-NK, ^{59}VG-NK

Increased strand propensity

T45V, T45I, M65V, M65I

- Charge – Charge Interactions

These mutations deliver the technical advantage of improving charge interactions and thereby increasing stability.

One or more substitutions of charge-charge interaction residues may be selected from the group consisting of:

Q42E, (E29K K30E E33K), T34K and T34R.

Additional advantageous properties shared by particular subgroups of substitutions are set out below:

Surface

(E29K K30E E33K), T34K, T34R

Partially buried

Q42E

Acidic to basic and vice versa

(E29K K30E E33K)

Amine to acid

Q42E

Polar to basic

T34R, T34K

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In the particular case of Q42E, this improves the surface charge network. Without wishing to be bound by theory, Q42E adds charge and is believed to enables a salt bridge (most likely to a lysine residue) which is otherwise too far away in three-dimensional space of the protein to form such an interaction. A salt bridge is typically considered to be a charge-charge interaction between atoms less than or equal to 4 Å apart. Thus it is believed that this mutation promotes formation of a salt bridge from Q42E to a Lys.

- Location

It is advantageous that the overwhelming majority of substitutions taught herein are on the reverse face of the Stefin A based polypeptide/scaffold protein, i.e. the non-binding end of the protein. This is a surprise to the inventors. The expectation would be that the mutations would be scattered throughout the sequence of the polypeptide. However, the teachings presented are that by targeting the reverse face of the protein, significant gains in stability can be made whilst leaving the binding end of the protein un-mutated or only minimally mutated.

One or more substitutions of residues in particular locations may be selected from the group consisting of:

T51, A59, M65, N32, Q42, N90, K63, ^{59}AN-GK, ^{59}IN-GK, ^{59}VN-GK, ^{59}LN-GK, ^{59}AG-NK, ^{59}VG-NK and T34.

When it is desired to restrict the mutations to a particular location within the polypeptide, this may be advantageously accomplished by selecting mutation(s) from one of the individual groups set out below.

Obverse face (binding end)

T51.

Reverse face (non-binding end)

A59, M65, N32, Q42, N90, K63, ^{59}AN-GK, ^{59}IN-GK, ^{59}VN-GK, ^{59}LN-GK, ^{59}AG-NK, ^{59}VG-NK and T34.

In this regard, it should be noted that in the comprehensive analysis conducted by the inventors that every residue in the hydrophobic core of the protein was individually studied. It is surprising to the inventors that the most useful stability enhancing mutations are concentrated in the reverse face region of the protein.

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- Post Translational Modification

An additional advantage of certain of these mutations is to promote chemical stability of the molecule. For example, methionine residues can be oxidised, whereas valine/isoleucine residues cannot. Therefore, the mutations taught by the inventors have the additional advantage of avoiding oxidation at this point in the protein, which is undesirable, and so advantageously deliver a molecule which is resistant to changes which might otherwise be brought about by oxidation.

One or more substitutions of post-translational modification residues may be selected from the group consisting of:

N32G, M65V, M65I, T34V, T34K, and T34R.

When it is desired to eliminate a particular type of post-translational modification, this may be advantageously accomplished by selecting mutation(s) from one of the individual groups set out below:

Potential glycosylation

N32G, T34V, T34K, T34R

Potential oxidation

M65V, M65I

It should be further noted that the N32G mutant also removes the risk of deamidation. Degradation of N residues by deamidation can be a problem – this mutation advantageously removes such a problem. The same applies to Q42E, since Q residues are also prone to deamidation.

Potential deamidation

N32G, Q42E

- Different Between hSteA and SQT

It should be noted that N90T may be part of the turn at this region of the protein, and therefore delivers a further advantage of stabilising that turn.

Exemplary Scaffolds

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Exemplary Scaffolds for Research Applications:

named **3r**(esearch) scaffolds:

3r1 – hSteA Y35W N32G V48D M65I Q42E T51L (A59V ΔD61) (E29K K30E E33K)

3r2 – hSteA Y35W N32G V48D M65I Q42E T51L (A59V G60N ΔD61 N62G) (E29K K30E E33K)

Exemplary Scaffolds for Therapeutic Applications:

named **3t**(herapeutic) scaffolds:

3t1 – hSteA N32G V48D

3t2 – hSteA N32G V48D M65I

3t3 – hSteA N32G V48D M65I T51L

3t4 – hSteA N32G V48D M65I Q42E

3t5 – hSteA N32G V48D M65I Q42E T51L

Having regard to the standard/conventional nomenclature used herein, the sequences of the full scaffold proteins are disclosed with reference to SEQ ID NO: 1 and the stated mutations. However, for illustration purposes, the following are representative examples of preferred scaffold protein sequences:

3r1 with 2 heterologous peptide insertions. In this example n=9: SEQ ID NO: 18

MIPGGLSEAKPATPEIQEIVDKVKPQLEKETGKTWGKLEAVEYKTQVD(Xn)GLNYYIKVRVGNKYIHLKVFKS
L(Xn)EDLVLTGYQVDKNKDDELTGF

3r2 with 2 heterologous peptide insertions. In this example n=9: SEQ ID NO: 19

MIPGGLSEAKPATPEIQEIVDKVKPQLEKETGKTWGKLEAVEYKTQVD(Xn)GLNYYIKVRVNGKYIHLKVFKS
L(Xn)EDLVLTGYQVDKNKDDELTGF

3t1 with 2 heterologous peptide insertions. In this example n=9: SEQ ID NO: 20

MIPGGLSEAKPATPEIQEIVDKVKPQLEEKTGETYGKLEAVQYKTQVD(Xn)GTNYYIKVRAGDNKYMHLKVF
KSL(Xn)EDLVLTGYQVDKNKDDELTGF

3t2 with 2 heterologous peptide insertions. In this example n=9: SEQ ID NO: 21

MIPGGLSEAKPATPEIQEIVDKVKPQLEEKTGETYGKLEAVQYKTQVD(Xn)GTNYYIKVRAGDNKYIHLKVFK
SL(Xn)EDLVLTGYQVDKNKDDELTGF

3t3 with 2 heterologous peptide insertions. In this example n=9: SEQ ID NO: 22

MIPGGLSEAKPATPEIQEIVDKVKPQLEEKTGETYGKLEAVQYKTQVD(Xn)GLNYYIKVRAGDNKYIHLKVFK SL(Xn)EDLVLTGYQVDKNKDDELTGF

3t4 with 2 heterologous peptide insertions. In this example n=9: SEQ ID NO: 23

MIPGGLSEAKPATPEIQEIVDKVKPQLEEKTGETYGKLEAVEYKTQVD(Xn)GTNYYIKVRAGDNKYIHLKVFK SL(Xn)EDLVLTGYQVDKNKDDELTGF

3t5 with 2 heterologous peptide insertions. In this example n=9: SEQ ID NO: 24

MIPGGLSEAKPATPEIQEIVDKVKPQLEEKTGETYGKLEAVEYKTQVD(Xn)GLNYYIKVRAGDNKYIHLKVFKS L(Xn)EDLVLTGYQVDKNKDDELTGF

wherein X is any amino acid,

and wherein n is an integer number from 0 to 36.

Most suitably n is 9.

Cystatin A or Cystatin B

Human Stefin A belongs to family 1 of the cystatin superfamily. Cystatin As and Cystatin Bs are different. Cystatin Bs typically have a near neutral pI and have a cysteine residue near the C-terminus. It is believed that all cystatin Bs have this cysteine and all Cystatin Bs form disulfide bonded dimers, whereas Cystatin As have a more acidic pI and no Cystatin As have this C-Terminal cysteine.

Nucleic Acids, Promoters, Libraries, Host Cells

Manufacture/production of recombinant polypeptides and/or nucleic acids according to the present invention is well known to the person skilled in the art and requires only routine knowledge such as how to synthesise polypeptide or polynucleotide, and/or how to express a polynucleotide to produce a polypeptide in the laboratory or a scaled-up commercial bioreactor. Numerous companies around the world offer such routine production services and require only an indication of the sequence(s) to be produced.

Host cells, vectors for expression of polypeptide(s) according to the invention, promoters for use in such systems and the codon optimisation (if any) of the nucleic acid(s) encoding them are all well known to the person skilled in the art. Choice of particular vectors such as phage, phagemids, plasmids, or of promoters or host cells or other such 'tools' for production of the polypeptides or libraries described herein is a matter for the skilled person working the

invention. Similarly, PCR or cloning strategies, ligations, transformation/electroporation techniques are all routine and do not form part of the invention but are determined by the operator.

In case further guidance is needed, general molecular biological techniques are well known in the art, for example as in (2000 Current Protocols in Molecular Biology F.M. Ausubel et al, Eds. ISBN: 978-0-471-50338-5 published by John Wiley & Sons Ltd, Oldlands Way, Bognor Regis, West Sussex, PO22 9NQ, UK).

Exemplary cell strains:

TG1 (Lucigen, catalogue number 60502-2)

ER2738 (New England Biolabs, catalogue number E4104)

Exemplary phage strain:

M13KO7 (New England Biolabs, catalogue number N0315)

Exemplary phagemid vector:

pUC119 (Clontech, catalogue number 3319), which contains the lac promoter

Exemplary promoter:

lac promoter (see above)

The polypeptide / scaffold protein of the invention may further comprise a tag, such as for purification e.g. a 6his tag, MBP (maltose binding protein) tag, or any other suitable sequence to aid purification.

The polypeptide / scaffold protein of the invention may further comprise a linker, such as a glycine linker, for joining to another polypeptide.

The polypeptide / scaffold protein of the invention may further comprise a detection sequence, such as for detection by an antibody, e.g. a myc tag or flag tag or any other suitable sequence to facilitate detection.

The polypeptide / scaffold protein of the invention may be labelled, such as with a fluorescent label joined to the polypeptide / scaffold protein.

The polypeptide / scaffold protein of the invention may be joined to a carrier protein such as a transport protein to facilitate entry into cells.

The polypeptide / scaffold protein of the invention may be joined to a targeting protein such as an antibody or fragment thereof, or an aptamer, or Affimer® reagent, so as to direct the

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polypeptide / scaffold protein to a particular location such as to attach it to a target cell or any other entity to which the targeting protein is capable of binding.

The polypeptide / scaffold protein of the invention may be attached to a substrate or structure for example a bead, or nanosphere, or an electrode (for example as part of an array of electrodes), or a membrane (such as nitrocellulose membrane), or a reaction vessel such as an ELISA plate or microcentrifuge tube or any other such article.

Suitably the polypeptide / scaffold protein of the invention may be immobilised.

Joining of the polypeptide / scaffold protein to other moieties may be by any suitable means known in the art, for example by covalent joining, by disulfide bridging, by preparation as a single polypeptide (fusion protein), by conjugation to suitable amino acid residue(s) such as cysteine residue(s), joining to the N- or C- terminus of the polypeptide or any other suitable means known in the art.

Making Polypeptide(s)

Also disclosed is a method of making a polypeptide having an altered stability such as an increased thermal stability or a decreased thermal stability compared to SEQ ID NO: 1, said method comprising synthesising a polypeptide comprising one or more substitution(s) relative to SEQ ID NO: 1 selected from the group consisting of:

A12I,A12V,I16L,V20A,V20I,V20L,Q26E,E29M,

T31K,N32G,N32D,N32H,T34V,T34R,T34K,T34D,

T34P,L38A,L38V,L38F,A40I,A40V,Q42E,Q42D,

T45I,T45V,V48E,V48D,V48G,V48A,V48L,G50S,

T51F,T51V,T51L,T51I,T51A,A59L,A59I,A59V,

K63R,M65V,M65I,L67I,N90T,(V20I,L38A),

(V20L,L38A),(V20I,L38V),(V20L,L38V),

(E29K,K30E,E33K),(Y54D,T83D,Q86E),

(A59L,G60N,D61G,N62K),(A59V,D61N,N62K),

(G60N,D61G,N62K),(G60N,ΔD61,N62G),

ΔD61,(A59L,G60N,ΔD61,N62G),

(A59V,G60N,D61G,N62K),(A59I,G60N,D61G,N62K),

(A59I,G60N,ΔD61,N62G),(A59V,G60N,ΔD61,N62G),

(A59V,ΔD61),(G60P,ΔD61,N62P),

(G60P,D61P,N62K),(G60P,ΔD61,N62G),

(G60P,D61G,N62K),(D61N,N62K), and

(T83D,Q86E); more suitably the substitution(s) are selected from the group consisting of:

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A12I,A12V,I16L,V20I,V20L,Q26E,E29M,T31K
N32G,N32D,N32H,T34V,T34R,T34D,T34P,L38A
L38V,A40I,A40V,Q42D,T45I,T45V,V48E,V48G
V48A,T51F,T51V,T51L,T51A,A59L,A59I,M65V
L67I
(V20I,L38A),(V20L,L38A),(V20I,L38V),(V20L,L38V)
(E29K,K30E,E33K),(Y54D,T83D,Q86E)
(A59L,G60N,D61G,N62K),(A59V,D61N,N62K)
(G60N,D61G,N62K),(G60N,ΔD61,N62G)
ΔD61,(A59L,G60N,ΔD61,N62G)
(A59V,G60N,D61G,N62K),(A59I,G60N,D61G,N62K)
(A59I,G60N,ΔD61,N62G),(A59V,G60N,ΔD61,N62G)
(A59V,ΔD61),(G60P,ΔD61,N62P)
(G60P,D61P,N62K),(G60P,ΔD61,N62G)
(G60P,D61G,N62K),(D61N,N62K) and
(T83D,Q86E).

Also disclosed is a method of making a polypeptide having an altered stability such as an increased thermal stability compared to SEQ ID NO: 1, said method comprising synthesising a polypeptide comprising one or more substitution(s)relative to SEQ ID NO: 1 selected from the group consisting of:

E29M,N32G,T34V,T34R,T34K,Q42E,T45I,T45V
G50S,T51F,T51V,T51L,T51I,A59L,A59I,A59V
K63R,M65V,M65I,L67I,N90T
(E29K,K30E,E33K),(A59L,G60N,D61G,N62K)
(A59V,D61N,N62K),(G60N,D61G,N62K)
(G60N,ΔD61,N62G),ΔD61
(A59L,G60N,ΔD61,N62G),(A59V,G60N,D61G,N62K)
(A59I,G60N,D61G,N62K),(A59I,G60N,ΔD61,N62G)
(A59V,G60N,ΔD61,N62G), and (A59V,ΔD61); more suitably the substitution(s) are selected from the group consisting of:

E29M,N32G,T34V,T34R,T45I,T45V,T51F,T51V
T51L,A59L,A59I,M65V,L67I

(E29K,K30E,E33K),(A59L,G60N,D61G,N62K)
(A59V,D61N,N62K),(G60N,D61G,N62K)
(G60N,ΔD61,N62G),ΔD61
(A59L,G60N,ΔD61,N62G),(A59V,G60N,D61G,N62K)
(A59I,G60N,D61G,N62K),(A59I,G60N,ΔD61,N62G)
(A59V,G60N,ΔD61,N62G), and (A59V,ΔD61).

Also disclosed is a method of making a polypeptide having an altered stability such as a decreased thermal stability compared to SEQ ID NO: 1, said method comprising synthesising a polypeptide comprising one or more substitution(s) relative to SEQ ID NO: 1 selected from the group consisting of:

A12I,A12V,I16L,V20A,V20I,V20L,Q26E,T31K
N32D,N32H,T34D,T34P,L38A,L38V,L38F,A40I
A40V,Q42D,V48E,V48D,V48G,V48A,V48L,T51A
(V20I,L38A),(V20L,L38A),(V20I,L38V),(V20L,L38V)
(Y54D,T83D,Q86E),(G60P,ΔD61,N62P)
(G60P,D61P,N62K),(G60P,ΔD61,N62G)
(G60P,D61G,N62K),(D61N,N62K) and
(T83D,Q86E); more suitably the substitution(s) are selected from the group consisting of:
A12I,A12V,I16L,V20I,V20L,Q26E,T31K,N32D
N32H,T34D,T34P,L38A,L38V,A40I,A40V,Q42D
V48E,V48G,V48A,T51A
(V20I,L38A),(V20L,L38A),(V20I,L38V),(V20L,L38V)
(Y54D,T83D,Q86E),(G60P,ΔD61,N62P)
(G60P,D61P,N62K),(G60P,ΔD61,N62G)
(G60P,D61G,N62K),(D61N,N62K) and
(T83D,Q86E).

Suitably the step of synthesising comprises preparing a nucleic acid encoding said polypeptide and arranging for translation of said nucleic acid to produce the polypeptide. Suitably the nucleic acid may be comprised by a phage genome, such as one or more members of a phage display library.

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Additional mutations

There may be an additional advantage to making one or more further mutations selected from the group consisting of:

G4R,E18Q,P25S,N32Q,T34E,T34Q,G36E,M65F

M65L,E78A,(K91E,D92K),(K91P,D93G), NPDG

It should be noted that this group of mutations share the property of each contributing a small effect on the thermal stability. Care must be taken in measuring these if measured individually, as the experimental error in the measurements might give an indication of low effect, or even of no effect or a small reversal of effect (e.g. rather than showing as +0.5 °C the effect might show as zero or as -0.2 °C when the experimental error on individual measurements is within +/- 0.7 C of ^{0}C change). This is the case with all scientific measurements when experimental error approaches or exceeds the magnitude of the effect being measured.

Regarding the NPDG mutation beginning at amino acid 90 ('90NPDG'), this increases the statistical likelihood of forming a Type 1 turn at this point in the protein, and thereby increases stability via this mechanism.

- Small Effect - Increase In Thermal Stability

G4R +0.05 °C

E18Q +0.57 °C

P25S +0.01 °C

N32Q +0.37 °C

T34Q +0.29 °C

G36E +0.18 °C

M65F +0.19 °C

E78A +0.50 °C

(K91E, D92K) +0.56 °C

(K91P, D93G) +0.22 °C

90NPDG +0.7 °C

(N90 K91P D92 D93G) +0.22 °C

- Small Effect - Decrease In Thermal Stability

T34E -0.39 °C

M65L -0.23 °C

PK Effects

In certain embodiments, the polypeptide, such as an Affimer polypeptide, comprises a polypeptide portion, such as an Affimer polypeptide portion, that binds a target moiety, preferably a protein, more preferably a human protein, as a monomer with a dissociation constant (K_D) of about 1 μM or less, about 100 nM or less, about 40 nM or less, about 20 nM or less, about 10 nM or less, about 1 nM or less, or about 0.1 nM or less.

In certain embodiments, the polypeptide, such as an Affimer polypeptide, comprises a polypeptide portion, such as an Affimer polypeptide portion, that binds a target moiety, preferably a protein, more preferably a human protein, as a monomer with an off-rate constant (k_{off}), such as measured by Biacore, of about 10^{-3} s^{-1} (i.e., unit of 1/second) or slower; of about 10^{-4} s^{-1} or slower or even of about 10^{-5} s^{-1} or slower.

In certain embodiments, the polypeptide, such as an Affimer polypeptide, comprises a polypeptide portion, such as an Affimer polypeptide portion, that binds a target moiety, preferably a protein, more preferably a human protein, with an on-rate constant (k_{on}), such as measured by Biacore, of at least about 10^3 $M^{-1}s^{-1}$ or faster; at least about 10^4 $M^{-1}s^{-1}$ or faster; at least about 10^5 $M^{-1}s^{-1}$ or faster; or even at least about 10^6 $M^{-1}s^{-1}$ or faster.

In certain embodiments, the polypeptide, such as an Affimer polypeptide, comprises a polypeptide portion, such as an Affimer polypeptide portion, that binds a target moiety having a cognate binding partner, preferably a protein, more preferably a human protein, as a monomer with an IC50 in a competitive binding assay with cognate binding partner of 1 μM or less, about 100 nM or less, about 40 nM or less, about 20 nM or less, about 10 nM or less, about 1 nM or less, or about 0.1 nM or less.

Advantages

It is an advantage of the invention that a scaffold with excellent expression properties is provided.

It is an advantage of the invention that a scaffold with modest or reduced immunogenicity (low immunogenicity) is provided.

It is an advantage of the invention that the polypeptides / scaffold proteins described do not suffer from problems of aggregation/precipitation. In more detail, the scaffolds of the invention are observed not to aggregate/precipitate before they unfold, thus when the scaffold of the invention has an increased Tm relative to hSteA, that scaffold also advantageously possesses an increased resistance to aggregation/precipitation.

It is an advantage of the polypeptides / scaffold proteins described that they accept heterologous peptide insertions.

It is an advantage of the polypeptides /scaffold proteins of the invention that heterologous peptide insertions are properly displayed.

It is an advantage of the invention that the polypeptides /scaffold proteins described are not negatively affected regarding stability/protease resistance by the mutations which are introduced. In other words, the polypeptides /scaffold proteins of the invention advantageously retain their stability and/or protease resistance. This is another advantageous property of the particular mutations taught herein.

Further Embodiments

Also disclosed is use of a scaffold as described above in research applications such as screening for peptide(s) capable of binding a particular target, and/or screening for peptide(s) having particular activity/activities.

Also disclosed is use of a scaffold as described above in medical applications such as targeting compound(s) to particular cells or locations within the body, and/or use in inhibiting or promoting particular metabolic activities.

Also disclosed are compositions such as pharmaceutical compositions comprising polypeptide(s) / scaffold protein(s) as described above.

Also disclosed is a composition comprising one or more polypeptide(s) / scaffold protein(s) as described above together with a pharmaceutically acceptable carrier, diluent or excipient.

Also disclosed is use of a polypeptide / scaffold protein as described above as a diagnostic, a therapeutic, a biomarker, an agent to specifically detect a biomarker, a rational drug design template, a target or reagent for drug discovery, an antibody substitute, an aptamer, an Affimer® reagent, or a research tool.

Also disclosed is use of a polypeptide as described above as a scaffold protein.

In one embodiment described is a polypeptide, such as an Affimer polypeptide, comprising an amino acid sequence having at least 80% identity to amino acid residues 1 to 11, 13 to 15, 17 to 19, 21 to 25, 27 to 28, 35 to 37, 39, 41, 43 to 44, 46 to 47, 49 to 50, 52 to 53, 55 to 58, 63 to 64, 66, 68 to 82, 84 to 85, and 87 to 98 of SEQ ID NO: 3 (canine wild type SteA);

characterised in that said polypeptide comprises one or more mutations relative to SEQ ID NO: 3 selected from the group consisting of:

T51L, T51V, M65V, N32G, A59I, L38A, V20I, A40I, L38V, A12I, A12V, I16L, V20L, Q26E, E29M, T31K, N32D, N32H, T34V, T34R, T34D, T34P, A40V, Q42D, T45I, T45V, V48E, V48G, V48A, T51F, T51A, A59L, L67I, (V20I, L38A), (V20L, L38A), (V20I, L38V), (V20L, L38V), (E29K, K30E, E33K), (Y54D, T83D, Q86E), (A59L, G60N, D61G, N62K), (A59V, D61N, N62K), (G60N, D61G, N62K), (G60N, ΔD61, N62G), ΔD61, (A59L, G60N, ΔD61, N62G), (A59V, G60N, D61G, N62K), (A59I, G60N, D61G, N62K), (A59I, G60N, ΔD61, N62G), (A59V, G60N, ΔD61, N62G), (A59V, ΔD61), (G60P, ΔD61, N62P),

(G60P, D61P, N62K), (G60P, ΔD61, N62G), (G60P, D61G, N62K), (D61N, N62K) and (T83D, Q86E).

Suitably said one or more mutations relative to SEQ ID NO: 1 is or are selected from the group consisting of:

T51L, T51V, M65V, N32G, A59I, E29M, T34V, T34R, T45I, T45V, T51F, A59L, L67I, (E29K, K30E, E33K), (A59L, G60N, D61G, N62K), (A59V, D61N, N62K), (G60N, D61G, N62K), (G60N, ΔD61, N62G), ΔD61, (A59L, G60N, ΔD61, N62G), (A59V, G60N, D61G, N62K), (A59I, G60N, D61G, N62K), (A59I, G60N, ΔD61, N62G), (A59V, G60N, ΔD61, N62G), and (A59V, ΔD61);

preferably wherein said polypeptide has a Tm higher than the Tm of SEQ ID NO: 3; more preferably higher than the Tm of SEQ ID NO: 1. In another embodiment suitably said one or more mutations relative to SEQ ID NO: 1 is or are selected from the group consisting of:

L38A, V20I, A40I, L38V, A12I, A12V, I16L, V20L, Q26E, T31K, N32D, N32H, T34D, T34P, A40V, Q42D, V48E, V48G, V48A, T51A, (V20I, L38A), (V20L, L38A), (V20I, L38V), (V20L, L38V), (Y54D, T83D, Q86E), (G60P, ΔD61, N62P), (G60P, D61P, N62K), (G60P, ΔD61, N62G), (G60P, D61G, N62K), (D61N, N62K) and (T83D, Q86E);

preferably wherein said polypeptide has a Tm lower than the Tm of SEQ ID NO: 3, more preferably lower than the Tm of SEQ ID NO: 1.

In another embodiment described is a polypeptide, such as an Affimer polypeptide, comprising an amino acid sequence having at least 80% identity to amino acid residues 1 to 11, 13 to 15, 17 to 19, 21 to 25, 27 to 28, 35 to 37, 39, 41, 43 to 44, 46 to 47, 49 to 50, 52 to 53, 55 to 58, 63 to 64, 66, 68 to 82, 84 to 85, and 87 to 98 of SEQ ID NO: 3;

wherein said polypeptide comprises at least one heterologous peptide insertion;

characterised in that said polypeptide comprises one or more mutations relative to SEQ ID NO: 1 selected from the group consisting of:

M65I, T51I, T51L, T51V, M65V, A59V, N32G, A59I, L38A, V20A, V20I, A40I, L38V, G50S, L38F, A12I, A12V, I16L, V20L, Q26E, E29M, T31K, N32D, N32H, T34V, T34R, T34K, T34D, T34P, A40V, Q42E, Q42D, T45I, T45V, V48E, V48D, V48G, V48A, V48L, T51F, T51A, A59L, K63R, L67I, N90T, (V20I, L38A), (V20L, L38A), (V20I, L38V), (V20L, L38V), (E29K, K30E, E33K), (Y54D, T83D, Q86E), (A59L, G60N, D61G, N62K), (A59V, D61N, N62K), (G60N, D61G, N62K), (G60N, ΔD61, N62G), ΔD61, (A59L, G60N, ΔD61, N62G), (A59V, G60N, D61G, N62K), (A59I, G60N, D61G, N62K), (A59I, G60N, ΔD61, N62G), (A59V, G60N, ΔD61, N62G), (A59V, ΔD61), (G60P, ΔD61, N62P), (G60P, D61P, N62K), (G60P, ΔD61, N62G), (G60P, D61G, N62K), (D61N, N62K), and (T83D, Q86E);

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wherein said heterologous peptide insertion comprises a heterologous peptide inserted at one of the following positions relative to SEQ ID NO: 1:

d) 48-<heterologous peptide>-50,

e) 49-<heterologous peptide>-51,

f) 50-<heterologous peptide>-52,

q) 72-<heterologous peptide>-77,

r) 73-<heterologous peptide>-78; or

s) 74-<heterologous peptide>-79.

Brief Description of the Drawings

Figure 1 shows a bar chart.

Figure 2 shows a bar chart.

Figure 3 shows a bar chart.

Figures 4A and 4B show graphs.

Figure 5 shows graphs.

Figure 6 shows graphs.

Figure 7 shows graphs.

Figure 8 shows graphs.

Figure 9 shows graphs.

Figure 10 shows graphs.

Figure 11 shows a plot.

Figure 12 shows a table.

Figure 13 shows a table.

Figure 14 shows a table.

Figure 15 shows a plot.

Figure 16 shows a plot.

Figure 17 shows a table.

Figure 18 shows a graph.

Figure 19 shows a graph.

Description of Exemplary Embodiments

I. Definitions

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To facilitate an understanding of the present invention, a number of terms and phrases are defined below.

The term "Affimer" (or "Affimer Polypeptide") refers to small, highly stable proteins that are a recombinantly engineered variants of Stefin Polypeptides. Affimer proteins display peptide loops (typically two) and an N-terminal sequence that can all be randomised to bind to desired target proteins with high affinity and specificity, in a similar manner to monoclonal antibodies. Stabilisation of the peptide loop(s) by the Stefin protein scaffold constrains the possible conformations that the peptides can take, increasing the binding affinity and specificity compared to libraries of free peptides. These engineered non-antibody binding proteins are designed to mimic the molecular recognition characteristics of monoclonal antibodies in different applications. Variations to other parts of the Stefin polypeptide sequence can be carried out, with such variations improving the properties of these affinity reagents, such as increase stability, make them robust across a range of temperatures and pH and the like.

An "Encoded Affimer" refers to a nucleic acid construct which, when expressed by cells in a patient's body through a gene delivery process, produces an intended Affimer polypeptide in vivo.

An "Affimer-Linked Conjugate" refers to an Affimer polypeptide having one or more moieties conjugated thereto through a chemical conjugation other than through the formation of a continuous peptide bond through the C-terminus or N-terminus of the polypeptide portion of the Affimer polypeptide containing Affimer Polypeptide sequence. An Affimer-linked Conjugate may be an "Affimer-Drug Conjugate", which refers to an Affimer polypeptide including one or more pharmacologically active moieties conjugated thereto. An Affimer-linked Conjugate may also be an "Affimer-Tag Conjugate", which refers to an Affimer polypeptide including one or more detectable moieties (i.e., detectable labels) conjugated thereto.

The terms "polypeptide" and "peptide" and "protein" are used interchangeably herein and refer to polymers of amino acids of any length. The polymer may be linear or branched, it may comprise modified amino acids, and it may be interrupted by non-amino acids. The terms also encompass an amino acid polymer that has been modified naturally or by intervention; for example, disulfide bond formation, glycosylation, lipidation, acetylation, phosphorylation, or any other manipulation or modification, such as conjugation with a labelling component. Also included within the definition are, for example, polypeptides containing one or more analogues of an amino acid (including, for example, unnatural amino acids), as well as other modifications known in the art.

The terms "amino acid residue" and "amino acid" are used interchangeably and means, in the context of a polypeptide, an amino acid that is participating in one more peptide bonds of the polypeptide. In general, the abbreviations used herein for designating the amino acids are based on recommendations of the IUPAC-IUB Commission on Biochemical Nomenclature (see Biochemistry (1972) 11:1726-1732). For instance, Met, Ile, Leu, Ala and Gly represent "residues" of methionine, isoleucine, leucine, alanine and glycine, respectively. By the residue is meant a radical derived from the corresponding α–amino acid by eliminating the OH portion of the carboxyl group and the H portion of the α-amino group. The term "amino acid side

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chain" is that part of an amino acid exclusive of the --CH(NH2)COOH portion, as defined by K. D. Kopple, "Peptides and Amino Acids", W. A. Benjamin Inc., New York and Amsterdam, 1966, pages 2 and 33.

For the most part, the amino acids used in the application of this invention are those naturally occurring amino acids found in proteins, or the naturally occurring anabolic or catabolic products of such amino acids which contain amino and carboxyl groups. Particularly suitable amino acid side chains include side chains selected from those of the following amino acids: glycine, alanine, valine, cysteine, leucine, isoleucine, serine, threonine, methionine, glutamic acid, aspartic acid, glutamine, asparagine, lysine, arginine, proline, histidine, phenylalanine, tyrosine, and tryptophan, and those amino acids and amino acid analogues which have been identified as constituents of peptidoglycan bacterial cell walls.

Amino acid residues having “basic sidechains” include Arg, Lys and His. Amino acid residues having “acidic sidechains” include Glu and Asp. Amino acid residues having “neutral polar sidechains” include Ser, Thr, Asn, Gln, Cys and Tyr. Amino acid residues having “neutral non-polar sidechains” include Gly, Ala, Val, Ile, Leu, Met, Pro, Trp and Phe. Amino acid residues having “non-polar aliphatic sidechains” include Gly, Ala, Val, Ile and Leu. Amino acid residues having “hydrophobic sidechains” include Ala, Val, Ile, Leu, Met, Phe, Tyr and Trp. Amino acid residues having “small hydrophobic sidechains” include Ala and Val. Amino acid residues having “aromatic sidechains” include Tyr, Trp and Phe.

The term amino acid residue further includes analogues, derivatives and congeners of any specific amino acid referred to herein, as for instance, the subject polypeptides such as Affimers (particularly if generated by chemical synthesis) can include an amino acid analogue such as, for example, cyanoalanine, canavanine, djenkolic acid, norleucine, 3-phosphoserine, homoserine, dihydroxy-phenylalanine, 5-hydroxytryptophan, 1-methylhistidine, 3-methylhistidine, diaminiopimelic acid, ornithine, or diaminobutyric acid. Other naturally occurring amino acid metabolites or precursors having side chains which are suitable herein will be recognized by those skilled in the art and are included in the scope of the present invention.

The terms "identical" or percent "identity" in the context of two or more nucleic acids or polypeptides, refer to two or more sequences or subsequences that are the same or have a specified percentage of nucleotides or amino acid residues that are the same, when compared and aligned (introducing gaps, if necessary) for maximum correspondence, not considering any conservative amino acid substitutions as part of the sequence identity. The percent identity may be measured using sequence comparison software or algorithms or by visual inspection. Various algorithms and software that may be used to obtain alignments of amino acid or nucleotide sequences are well-known in the art. These include, but are not limited to, BLAST, ALIGN, Megalign, BestFit, GCG Wisconsin Package, and variants thereof. In some embodiments, two nucleic acids or polypeptides of the invention are substantially identical, meaning they have at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, and in some embodiments at least 95%, 96%, 97%, 98%, 99% nucleotide or amino acid residue

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identity, when compared and aligned for maximum correspondence, as measured using a sequence comparison algorithm or by visual inspection. In some embodiments, identity exists over a region of the amino acid sequences that is at least about 10 residues, at least about 20 residues, at least about 40-60 residues, at least about 60-80 residues in length or any integral value there between. In some embodiments, identity exists over a longer region than 60-80 residues, such as at least about 80-100 residues, and in some embodiments the sequences are substantially identical over the full length of the sequences being compared, such as the coding region of a target protein or an antibody. In some embodiments, identity exists over a region of the nucleotide sequences that is at least about 10 bases, at least about 20 bases, at least about 40-60 bases, at least about 60-80 bases in length or any integral value there between. In some embodiments, identity exists over a longer region than 60-80 bases, such as at least about 80-100 bases or more, and in some embodiments the sequences are substantially identical over the full length of the sequences being compared, such as a nucleotide sequence encoding a protein of interest.

A "conservative amino acid substitution" is one in which one amino acid residue is replaced with another amino acid residue having a similar side chain. Families of amino acid residues having similar side chains have been generally defined in the art, including basic side chains (e.g., lysine, arginine, histidine), acidic side chains (e.g., aspartic acid, glutamic acid), uncharged polar side chains (e.g., glycine, asparagine, glutamine, serine, threonine, tyrosine, cysteine), nonpolar side chains (e.g., alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan), beta-branched side chains (e.g., threonine, valine, isoleucine) and aromatic side chains (e.g., tyrosine, phenylalanine, tryptophan, histidine). For example, substitution of a phenylalanine for a tyrosine is a conservative substitution. Generally, conservative substitutions in the sequences of the polypeptides, soluble proteins, and/or antibodies of the invention do not abrogate the binding of the polypeptide, soluble protein, or antibody containing the amino acid sequence, to the target binding site. Methods of identifying amino acid conservative substitutions which do not eliminate binding are well-known in the art.

A polypeptide, soluble protein, antibody, polynucleotide, vector, cell, or composition which is "isolated" is a polypeptide, soluble protein, antibody, polynucleotide, vector, cell, or composition which is in a form not found in nature. Isolated polypeptides, soluble proteins, antibodies, polynucleotides, vectors, cells, or compositions include those which have been purified to a degree that they are no longer in a form in which they are found in nature. In some embodiments, a polypeptide, soluble protein, antibody, polynucleotide, vector, cell, or composition which is isolated is substantially pure.

The term "substantially pure" as used herein refers to material which is at least 50% pure (i.e., free from contaminants), at least 90% pure, at least 95% pure, at least 98% pure, or at least 99% pure.

The term "fusion protein" or "fusion polypeptide" as used herein refers to a hybrid protein expressed by a nucleic acid molecule comprising nucleotide sequences of at least two genes.

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The term "linker" or "linker region" as used herein refers to a linker inserted between a first polypeptide (e.g., copies of an Affimer) and a second polypeptide (e.g., another Affimer, an Fc domain, a ligand binding domain, etc). In some embodiments, the linker is a peptide linker. Linkers should not adversely affect the expression, secretion, or bioactivity of the polypeptides. Preferably, linkers are not antigenic and do not elicit an immune response.

The terms "polynucleotide" and "nucleic acid" and "nucleic acid molecule" are used interchangeably herein and refer to polymers of nucleotides of any length, and include DNA and RNA. The nucleotides can be deoxyribonucleotides, ribonucleotides, modified nucleotides or bases, and/or their analogues, or any substrate that can be incorporated into a polymer by DNA or RNA polymerase.

As used herein, the term "nucleic acid molecule encoding", "DNA sequence encoding," and "DNA encoding" refer to the order or sequence of nucleotides along a strand of deoxyribonucleic acid deoxyribonucleotides. The order of these deoxyribonucleotides determines the order of amino acids along the polypeptide (protein) chain. Thus, a nucleic acid sequence encoding the amino acid sequence.

When used in reference to nucleotide sequences, "sequence" as used herein, the term grammatical and other forms may comprise DNA or RNA, and may be single or double stranded. Nucleic acid sequences may be mutated. Nucleic acid sequence may have any length, for example 2 to 100,000 or more nucleotides (or any integral value above or between) a nucleic acid, for example a length of from about 100 to about 10,000, or from about 200 nucleotides to about 500 nucleotides.

The term "vector" as used herein means a construct, which is capable of delivering, and usually expressing, one or more gene(s) or sequence(s) of interest in a host cell. Examples of vectors include, but are not limited to, viral vectors, naked DNA or RNA expression vectors, plasmid, cosmid, or phage vectors, DNA or RNA expression vectors associated with cationic condensing agents, and DNA or RNA expression vectors encapsulated in liposomes.

As used herein, the term "transfection" refers to an exogenous nucleic acid into a eukaryotic cell. Transfection can be achieved by various means known in the art, including calcium phosphate -DNA co-precipitation, DEAE- dextran-mediated transfection, polybrene-mediated transfection, electroporation, microinjection, liposome fusion, lipofection, protoplast fusion, retroviral infection, and biolistics technology (biolistics).

The term "carrier" as used herein is an isolated nucleic acid comprising the isolated nucleic acid can be used to deliver a composition to the interior of the cell. It is known in the art a number of carriers including, but not limited to the linear polynucleotides, polynucleotides associated with ionic or amphiphilic compounds, plasmids, and viruses. Thus, the term "vector" includes an autonomously replicating plasmid or virus. The term should also be construed to include facilitate transfer of nucleic acid into cells of the non-plasmid and non-viral compounds, for example, polylysine compounds, liposomes, and the like. Examples of viral vectors include, but are not limited to adenoviral vectors, adeno-associated virus vectors, retroviral vectors and the like.

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As used herein, the term "expression vector" refers to a vector comprising a recombinant polynucleotide comprising expression control sequence and a nucleotide sequence to be expressed operably linked. The expression vector comprises sufficient cis-acting elements (cis-acting elements) used for expression; other elements for expression can be supplied by the host cell or in vitro expression system. Expression vectors include all those known in the art, such as cosmids, plasmids (e.g., naked or contained in liposomes) and viruses (e.g., lentivirus, retroviruses, adenoviruses and adeno-associated viruses).

As used herein, the term "operably linked" refers to functional linkage between the regulatory sequence and a heterologous nucleic acid sequence is connected to a connection results in the expression of the latter. For example, when the first nucleic acid sequence and a second nucleic acid sequence is a functional relationship between the first nucleic acid sequence and the second nucleic acid sequence is operably linked. For example, if the promoter affects the transcription or expression of the coding sequence, the promoter is operably linked to a coding sequence. Typically, DNA sequencing operably linked are contiguous, and to join two protein coding regions in the same reading frame as necessary.

As used herein, the term "promoter" is defined as a promoter DNA sequence recognized by the synthetic machinery required for the synthesis machinery of the cell specific transcription of a polynucleotide sequence or introduced.

The term "constitutive expression" as used herein refers to all expressed under physiological conditions.

The term "inducible expression" as used herein refers to expression under certain conditions, such as activation (or inactivation) of an intracellular signalling pathway or the contacting of the cells harbouring the expression construct with a small molecule that regulates the expression (or degree of expression) of a gene operably linked to an inducible promoter sensitive to the concentration of the small molecule.

The term "electroporation" refers to the use of a transmembrane electric field pulse to induce microscopic pathways (pores) in a bio-membrane; their presence allows biomolecules such as plasmids or other oligonucleotide to pass from one side of the cellular membrane to the other.

Suitably polypeptides of the invention comprise the amino acid sequences as described herein. Suitably polypeptides of the invention consist essentially of the amino acid sequences as described herein. Suitably polypeptides of the invention consist of the amino acid sequences as described herein.

II. Fusions Proteins - General

In some embodiments, the Affimer polypeptides may further comprise one or more additional polypeptide sequences at one or both ends of the Affimer sequence which modulate biological activity of the Affimer polypeptide. For example, the additions may modulate one or more properties or activities of modified Affimer such as affinity, e.g., for binding to and inhibiting a target molecule, modulate the circulating half-life, modulate the therapeutic half-life, modulate the stability of the Affimer polypeptide, modulate cleavage by proteases, modulate

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dose, modulate release or bio-availability, facilitate purification, decrease deamidation, improve shelf-life, or improve or alter a particular route of administration. Similarly, Affimer polypeptides may comprise protease cleavage sequences, reactive groups, antibody-binding domains (including but not limited to, FLAG or poly-His) or other affinity based sequences (including but not limited to, FLAG, poly-His, GST, etc.) or linked molecules (including but not limited to, biotin) that improve detection, purification or other traits of the polypeptide.

Accordingly, in certain aspects of the invention the Affimer polypeptide is a fusion protein having at least one Affimer polypeptide sequence and one or more heterologous polypeptide sequences ("fusion domain" herein). A fusion domain may be selected so as to confer a desired property, such as secretion from a cell or retention on the cell surface (i.e., for Encoded Affimers), to serve as substrate or other recognition sequences for post-translational modifications, to create multimeric structures aggregating through protein-protein interactions, to alter (often to extend) serum half-life, or to alter tissue localization or tissue exclusion and other ADME properties – merely as examples.

For example, some fusion domains are particularly useful for isolation and/or purification of the fusion proteins, such as by affinity chromatography. Well known examples of such fusion domains that facilitate expression or purification include, merely to illustrate, affinity tags such as polyhistidine (i.e., a His_6 tag), Strep II tag, streptavidin-binding peptide (SBP) tag, calmodulin-binding peptide (CBP), glutathione S-transferase (GST), maltose-binding protein (MBP), S-tag, HA tag, c-Myc tag, thioredoxin, protein A and protein G.

In order for the Affimer polypeptide to be secreted, it will generally contain a signal sequence that directs the transport of the protein to the lumen of the endoplasmic reticulum and ultimately to be secreted (or retained on the cell surface if a transmembrane domain or other cell surface retention signal). Signal sequences (also referred to as signal peptides or leader sequences) are located at the N-terminus of nascent polypeptides. They target the polypeptide to the endoplasmic reticulum and the proteins are sorted to their destinations, for example, to the inner space of an organelle, to an interior membrane, to the cell outer membrane, or to the cell exterior via secretion. Most signal sequences are cleaved from the protein by a signal peptidase after the proteins are transported to the endoplasmic reticulum. The cleavage of the signal sequence from the polypeptide usually occurs at a specific site in the amino acid sequence and is dependent upon amino acid residues within the signal sequence.

In some embodiments, the signal peptide is about 5 to about 40 amino acids in length (such as about 5 to about 7, about 7 to about 10, about 10 to about 15, about 15 to about 20, about 20 to about 25, or about 25 to about 30, about 30 to about 35, or about 35 to about 40 amino acids in length).

In some embodiments, the signal peptide is a native signal peptide from a human protein. In other embodiments, the signal peptide is a non-native signal peptide. For example, in some embodiments, the non-native signal peptide is a mutant native signal peptide from the corresponding native secreted human protein, and can include one or more (such as 2, 3, 4, 5, 6, 7, 8, 9, or 10 or more) substitutions insertions or deletions.

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In some embodiments, the signal peptide is a signal peptide or mutant thereof from an immunoglobulin (such as IgG heavy chain or IgG-kappa light chain), a cytokine (such as interleukin-2 (IL-2), or CD33), a serum albumin protein (e.g. HSA or albumin), a human azurocidin preprotein signal sequence, a luciferase, a trypsinogen (e.g. chymotrypsinogen or trypsinogen) or other signal peptide able to efficiently secrete a protein from a cell. Exemplary signal peptides include, but are not limited to:

Native Protein	Signal Sequence
HSA	MKWVTFISLLFLFSSAYS SEQ ID NO: 25
Ig kappa light chain	MDMRAPAGIFGFLLVLFPGYRS SEQ ID NO: 26
Human azurocidin preprotein	MTRLTVLALLAGLLASSRA SEQ ID NO: 27
IgG heavy chain	MELGLSWIFLLAILKGVQC SEQ ID NO: 28
IgG heavy chain	MELGLRWVFLVAILEGVQC SEQ ID NO: 29
IgG heavy chain	MKHLWFFLLLVAAPRWVLS SEQ ID NO: 30
IgG heavy chain	MDWTWRILFLVAAATGAHS SEQ ID NO: 31
IgG heavy chain	MDWTWRFLFVVAAATGVQS SEQ ID NO: 32
IgG heavy chain	MEFGLSWLFLVAILKGVQC SEQ ID NO: 33
IgG heavy chain	MEFGLSWVFLVALFRGVQC SEQ ID NO: 34
IgG heavy chain	MDLLHKNMKHLWFFLLLVAAPRWVLS SEQ ID NO: 35
IgG Kappa light	MDMRVPAQLLGLLLLWLSGARC SEQ ID NO: 36
IgG Kappa light	MKYLLPTAAAGLLLLAAQPAMA SEQ ID NO: 37
Gaussia luciferase	MGVKVLFALICIAVAEA SEQ ID NO: 38
Human albumin	MKWVTFISLLFLFSSAYS SEQ ID NO: 39
Human chymotrypsinogen	MAFLWLLSCWALLGTTFG SEQ ID NO: 40
Human interleukin-2	MQLLSCIALILALV SEQ ID NO: 41
Human trypsinogen-2	MNLLLILTFVAAAVA SEQ ID NO: 42
Human CD33	MPLLLLLPLLWAGALA SEQ ID NO: 43
Prolactin	MDSKGSSQKGSRLLLLLVVSNLLLCQGVVS SEQ ID NO: 44
Human tPA	MDAMKRGLCCVLLLCGAVFVSPS SEQ ID NO:45

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	Synthetic/Consensus	MLLLLLLLLLLALALA SEQ ID NO:46
	Synthetic/Consensus	MWWRLWWLLLLLLLLWPMVWA SEQ ID NO: 47

In some embodiments of a secreted Affimer polypeptide, the recombinant polypeptide comprises a signal peptide when expressed, and the signal peptide (or a portion thereof) is cleaved from the Affimer polypeptide upon secretion.

The subject fusion proteins may also include one or more linkers separating heterologous protein sequences or domains. As used herein, the term "linker" refers to a linker amino acid sequence inserted between a first polypeptide (e.g., an Affimer) and a second polypeptide (e.g., a second Affimer, an Fc region, a receptor trap, albumin, etc). Empirical linkers designed by researchers are generally classified into 3 categories according to their structures: flexible linkers, rigid linkers, and in vivo cleavable linkers. Besides the basic role in linking the functional domains together (as in flexible and rigid linkers) or releasing free functional domain in vivo (as in in vivo cleavable linkers), linkers may offer many other advantages for the production of fusion proteins, such as improving biological activity, increasing expression yield, and achieving desirable pharmacokinetic profiles. Linkers should not adversely affect the expression, secretion, or bioactivity of the fusion protein. Linkers should not be antigenic and should not elicit an immune response.

Suitable linkers are known to those of skill in the art and often include mixtures of glycine and serine residues and often include amino acids that are sterically unhindered. Other amino acids that can be incorporated into useful linkers include threonine and alanine residues. Linkers can range in length, for example from 1-50 amino acids in length, 1-22 amino acids in length, 1-10 amino acids in length, 1-5 amino acids in length, or 1-3 amino acids in length. In some embodiments, the linker may comprise a cleavage site. In some embodiments, the linker may comprise an enzyme cleavage site, so that the second polypeptide may be separated from the first polypeptide.

In certain preferred embodiments, the linker can be characterized as flexible. Flexible linkers are usually applied when the joined domains require a certain degree of movement or interaction. They are generally composed of small, non-polar (e.g. Gly) or polar (e.g. Ser or Thr) amino acids. See, for example, Argos P. (1990) “An investigation of oligopeptides linking domains in protein tertiary structures and possible candidates for general gene fusion” J Mol Biol. 211:943–958. The small size of these amino acids provides flexibility and allows for mobility of the connecting functional domains. The incorporation of Ser or Thr can maintain the stability of the linker in aqueous solutions by forming hydrogen bonds with the water molecules, and therefore reduces the unfavorable interaction between the linker and the protein moieties. The most commonly used flexible linkers have sequences consisting primarily of stretches of Gly and Ser residues (“GS” linker). An example of the most widely used flexible linker has the sequence of (Gly-Gly-Gly-Gly-Ser)n. By adjusting the copy number “n”, the length of this GS linker can be optimized to achieve appropriate separation of the functional domains, or to maintain necessary inter-domain interactions, and is preferably 1, 2, 3, 4, 5, 6, 7, 8, 9, or

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10. Besides the GS linkers, many other flexible linkers have been designed for recombinant fusion proteins. As These flexible linkers are also rich in small or polar amino acids such as Gly and Ser, but can contain additional amino acids such as Thr and Ala to maintain flexibility, as well as polar amino acids such as Lys and Glu to improve solubility.

In certain preferred embodiments, the linker can be characterized as rigid. While flexible linkers have the advantage to connect the functional domains passively and permitting certain degree of movements, the lack of rigidity of these linkers can be a limitation in certain fusion protein embodiments, such as in expression yield or biological activity. The ineffectiveness of flexible linkers in these instances was attributed to an inefficient separation of the protein domains or insufficient reduction of their interference with each other. Under these situations, rigid linkers have been successfully applied to keep a fixed distance between the domains and to maintain their independent functions.

Many natural linkers exhibited α-helical structures. The α-helical structure was rigid and stable, with intra-segment hydrogen bonds and a closely packed backbone. Therefore, the stiff α-helical linkers can act as rigid spacers between protein domains. George et al. (2002) "An analysis of protein domain linkers: their classification and role in protein folding" Protein Eng. 15(11):871-9. In general, rigid linkers exhibit relatively stiff structures by adopting α-helical structures or by containing multiple Pro residues. Under many circumstances, they separate the functional domains more efficiently than the flexible linkers. The length of the linkers can be easily adjusted by changing the copy number to achieve an optimal distance between domains. As a result, rigid linkers are chosen when the spatial separation of the domains is critical to preserve the stability or bioactivity of the fusion proteins. In this regard, alpha helix-forming linkers with the sequence of (EAAAK)n (where n is preferably 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10) have been applied to the construction of many recombinant fusion proteins. Another type of rigid linkers has a Pro-rich sequence, (XP)n, with X designating any amino acid, preferably Ala, Lys, or Glu and n is preferably 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10.

Merely to illustrate, exemplary linkers include:

Flexible	$(GGGGS)_n$ (i.e., n = 1-6)	SEQ ID NO: 48
Flexible	$(Gly)_8$	SEQ ID NO: 49
Flexible	$(Gly)_6$	SEQ ID NO: 50
Flexible	KESGSVSSEQLAQFRSLD	SEQ ID NO: 51
Flexible	EGKSSGSGSESKST	SEQ ID NO: 52
Flexible	GSAGSAAGSGEF	SEQ ID NO: 53
Rigid	$(EAAAK)_n$ (i.e., n = 1-6)	SEQ ID NO: 54
Rigid	$A(EAAAK)_4ALEA(EAAAK)_4A$	SEQ ID NO: 55
Rigid	PAPAP	SEQ ID NO: 56
Rigid	AEAAAKEAAAKA	SEQ ID NO: 57
Rigid	(Ala-Pro)n (10 to 34 aa)	SEQ ID NO: 58

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Other linkers that may be used in the subject fusion proteins include, but are not limited to, SerGly, GGSG (SEQ ID NO: 59), GSGS (SEQ ID NO: 60), GGGS(SEQ ID NO: 61), S(GGS)n (SEQ ID NO: 62) where n is 1-7, GRA, poly(Gly), poly(Ala), GGGSGGG (SEQ ID NO: 63), ESGGGGVT (SEQ ID NO: 64), LESGGGGVT (SEQ ID NO: 65), GRAQVT (SEQ ID NO: 66), WRAQVT (SEQ ID NO: 67), and ARGRAQVT (SEQ ID NO: 68). The hinge regions of the Fc fusions described below may also be considered linkers.

Various elements can be employed to anchor proteins on the plasma membrane of cells. For example, the transmembrane domains (TM) of type-I (oriented with the N-terminus outside the cell) and type-II (oriented with the N-terminus in the cytosol) integral membrane proteins can be used to target chimeric proteins to the plasma membrane. Proteins can also be attached to the cell surface by fusion of a GPI (glycophosphatidylinositol lipid) signal to the 3' end of genes. Cleavage of the short carboxy-terminal peptide allows attachment of a glycolipid to the newly exposed C-terminus through an amide linkage. See Udenfriend et al. (1995) "How Glycosylphoshpatidylinositol Anchored Membrane Proteins are Made" Annu Rev Biochem 64:563–591.

In certain embodiments, the fusion protein includes a transmembrane polypeptide sequence (a transmembrane domain). The distinguishing features of appropriate transmembrane polypeptides comprise the ability to be expressed at the surface of the cell on which the Affimer polypeptide is to be displayed. In certain embodiments, that may be an immune cell, in particular lymphocyte cells or Natural killer (NK) cells, and once there to interact with a tumor cell expressing a cell surface feature which the Affimer polypeptide on the immune cell surface binds so as to direct cellular response of the immune cell against a predefined target tumour cell. The transmembrane domain can be derived either from a natural or from a synthetic source. The transmembrane domain can be derived from any membrane-bound or transmembrane protein. As non-limiting examples, the transmembrane polypeptide can be a subunit of the T cell receptor such as α, β, γ or δ, polypeptide constituting CD3 complex, IL2 receptor p55 (a chain), p75 (β chain) or γ chain, subunit chain of Fc receptors, in particular Fey receptor III or CD proteins. Alternatively, the transmembrane domain can be synthetic and can comprise predominantly hydrophobic residues such as leucine and valine.

In certain other embodiments, the Affimer polypeptide is a fusion protein including, in addition to an Affimer polypeptide, a sequence that signals for the posttranslational addition of a glycosylphosphatidylinositol (GPI) anchor. GPI anchors are glycolipid structures that are added post-translationally to the C-terminus of many eukaryotic proteins. This modification to the Affimer polypeptide will cause it to be anchored (attached) on the extracellular surface of the cell membrane of the cell in which the Affimer polypeptide is re-expressed as a recombinant protein (i.e., an Encoded Affimer as described below). In these embodiments, the GPI anchor domain is C-terminal to the Affimer polypeptide sequence, and preferably occurs at the C-terminus of the fusion protein.

In one embodiment, the GPI anchor domain is a polypeptide that signals for the posttranslational addition of a GPI anchor when the fusion protein of which it is a part is

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expressed in a eukaryotic system. The GPI anchor signal sequence consists of a set of small amino acids at the site of anchor addition (the ω site) followed by a hydrophilic spacer and ending in a hydrophobic stretch (Low, (1989) FASEB J. 3:1600-1608). Cleavage of this signal sequence occurs in the ER before the addition of an anchor with conserved central components but with variable peripheral moieties (Homans et al., Nature, 333:269-272 (1988)). The C-terminus of a GPI-anchored protein is linked through a phosphoethanolamine bridge to the highly conserved core glycan, mannose(α1-2)mannose(α1-6)mannose(α1-4)glucosamine(α1-6)myo-inositol. A phospholipid tail attaches the GPI anchor to the cell membrane.

Exemplary GPI anchor domains that can be used in the subject Affimer-containing fusion proteins include:

SEQ ID NO: 69SGTTSGTTRLLSGHTCFTLTGLLGTLVTMGLLT

SEQ ID NO: 70SGTSPGLSAGATVGIMIGVLVGVALI

SEQ ID NO: 71SAPVLSAVATVGITIGVLARVALI

SEQ ID NO: 72SSPDLSAGTAVSIMIGVLAGMALI

SEQ ID NO: 73TLGGNSASYTFVSLLFSAVTLLLLC

SEQ ID NO: 74SGTSPGLSAGATVGIMIGVLVGVALI

GPI anchor attachment can be achieved by expression of the Affimer fusion protein containing the GPI anchor domain in a eukaryotic system capable of carrying out GPI posttranslational modifications. As with the transmembrane domain fusion proteins, human cells, including lymphocytes and other cells involved in initiating or promoting an antitumor are so capable and can be engineered to express and Encoded Affimer including a GPI anchor domain in order retain the expressed Affimer containing fusion on the surface of the engineered cell.

Still other modifications that can be made to the Affimer polypeptide sequence itself or to a flanking polypeptide moiety provided as part of a fusion protein is one or more sequences that are sites for post-translational modifications by enzymes. These can include, but are not limited to, glycosylation, acetylation, acylation, lipid-modification, palmitoylation, palmitate addition, phosphorylation, glycolipid-linkage modification, and the like.

a. Engineering PK and ADME Properties

In certain embodiment, the Affimer polypeptide may not have a half-life and/or PK profile that is optimal for the route of administration, such as parenteral therapeutic dosing. The term "half-life" refers to the amount of time it takes for a substance, such as an Affimer polypeptide of the present invention, to lose half of its pharmacologic or physiologic activity or concentration. Biological half-life can be affected by elimination, excretion, degradation (e.g., enzymatic) of the substance, or absorption and concentration in certain organs or tissues of the body. In some embodiments, biological half-life can be assessed by determining the time it takes for the blood plasma concentration of the substance to reach half its steady state level ("plasma half-life"). To address this shortcoming, there are a variety of general strategies for

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prolongation of half-life that have been used in the case of other protein therapeutics, including the incorporation of half-life extending moieties as part of the Affimer polypeptide.

The term "half-life extending moiety" refers to a pharmaceutically acceptable moiety, domain, or molecule covalently linked ("conjugated" or "fused") to the Affimer polypeptide to form the Affimer polypeptides described herein, optionally via a non-naturally encoded amino acid, directly or via a linker, that prevents or mitigates in vivo proteolytic degradation or other activity-diminishing modification of the Affimer polypeptide, increases half-life, and/or improves or alters other pharmacokinetic or biophysical properties including but not limited to increasing the rate of absorption, reducing toxicity, improving solubility, reducing protein aggregation, increasing biological activity and/or target selectivity of the modified Affimer polypeptide, increasing manufacturability, and/or reducing immunogenicity of the modified Affimer polypeptide, compared to a comparator such as an unconjugated form of the modified Affimer polypeptide. The term "half-life extending moiety" includes non-proteinaceous, half-life extending moieties, such as a water soluble polymer such as polyethylene glycol (PEG) or discrete PEG, hydroxyethyl starch (HES), a lipid, a branched or unbranched acyl group, a branched or unbranched C8-C30 acyl group, a branched or unbranched alkyl group, and a branched or unbranched C8-C30 alkyl group; and proteinaceous half-life extending moieties, such as serum albumin, transferrin, adnectins (e.g., albumin-binding or pharmacokinetics extending (PKE) adnectins), Fc domain, and unstructured polypeptide, such as XTEN and PAS polypeptide (e.g. conformationally disordered polypeptide sequences composed of the amino acids Pro, Ala, and/or Ser), and a fragment of any of the foregoing.

In certain embodiments, the half-life extending moiety extends the half-life of the resulting Affimer polypeptide circulating in mammalian blood serum compared to the half-life of the protein that is not so conjugated to the moiety (such as relative to the Affimer polypeptide alone). In some embodiments, half-life is extended by greater than about 1.2-fold, 1.5-fold, 2.0-fold, 3.0-fold, 4.0-fold., 5.0-fold, or 6.0-fold. In some embodiments, half-life is extended by more than 6 hours, more than 12 hours, more than 24 hours, more than 48 hours, more than 72 hours, more than 96 hours or more than 1 week after in vivo administration compared to the protein without the half-life extending moiety.

As means for further exemplification, half-life extending moieties that can be used in the generation of Affimer polypeptides of the invention include:

- Genetic fusion of the pharmacologically active Affimer sequence to a naturally long-half-life protein or protein domain (e.g., Fc fusion, transferrin [Tf] fusion, or albumin fusion. See, for example, Beck et al. (2011) "Therapeutic Fc-fusion proteins and peptides as successful alternatives to antibodies. MAbs. 3:1–2; Czajkowsky et al. (2012) "Fc-fusion proteins: new developments and future perspectives. EMBO Mol Med. 4:1015–28; Huang et al. (2009) "Receptor-Fc fusion therapeutics, traps, and Mimetibody technology" Curr Opin Biotechnol. 2009;20:692–9; Keefe et al. (2013) "Transferrin fusion protein therapies: acetylcholine receptor-transferrin fusion protein as a model. In: Schmidt S, editor. Fusion protein technologies for biopharmaceuticals: applications and challenges. Hoboken: Wiley; p. 345–56; Weimer et al. (2013) "Recombinant albumin fusion proteins. In: Schmidt S, editor. Fusion protein

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technologies for biopharmaceuticals: applications and challenges. Hoboken: Wiley; 2013. p. 297–323; Walker et al. (2013) "Albumin-binding fusion proteins in the development of novel long-acting therapeutics. In: Schmidt S, editor. Fusion protein technologies for biopharmaceuticals: applications and challenges. Hoboken: Wiley; 2013. p. 325–43.

- Genetic fusion of the pharmacologically active Affimer sequence to an inert polypeptide, e.g., XTEN (also known as recombinant PEG or ''rPEG''), a homoamino acid polymer (HAP; HAPylation), a proline-alanine-serine polymer (PAS; PASylation), or an elastin-like peptide (ELP; ELPylation). See, for example, Schellenberger et al. (2009) "A recombinant polypeptide extends the in vivo half-life of peptides and proteins in a tunable manner. Nat Biotechnol. 2009;27:1186–90; Schlapschy et al. Fusion of a recombinant antibody fragment with a homo-amino-acid polymer: effects on biophysical properties and prolonged plasma half-life. Protein Eng Des Sel. 2007;20:273–84; Schlapschy (2013) PASylation: a biological alternative to PEGylation for extending the plasma half-life of pharmaceutically active proteins. Protein Eng Des Sel. 26:489–501. Floss et al. (2012) "Elastin-like polypeptides revolutionize recombinant protein expression and their biomedical application. Trends Biotechnol. 28:37–45. Floss et al. "ELP-fusion technology for biopharmaceuticals. In: Schmidt S, editor. Fusion protein technologies for biopharmaceuticals: application and challenges. Hoboken: Wiley; 2013. p. 372–98.
- Increasing the hydrodynamic radius by chemical conjugation of the pharmacologically active peptide or protein to repeat chemical moieties, e.g., to PEG (PEGylation) or hyaluronic acid. See, for example, Caliceti et al. (2003) "Pharmacokinetic and biodistribution properties of poly(ethylene glycol)-protein conjugates" Adv Drug Delivery Rev. 55:1261–77; Jevsevar et al. (2010) PEGylation of therapeutic proteins. Biotechnol J 5:113–28; Kontermann (2009) "Strategies to extend plasma half-lives of recombinant antibodies" BioDrugs. 23:93–109; Kang et al. (2009) "Emerging PEGylated drugs" Expert Opin Emerg Drugs. 14:363–80; and Mero et al. (2013) "Conjugation of hyaluronan to proteins" Carb Polymers. 92:2163–70.
- Significantly increasing the negative charge of fusing the pharmacologically active peptide or protein by polysialylation; or, alternatively, (b) fusing a negatively charged, highly sialylated peptide (e.g., carboxy-terminal peptide [CTP; of chorionic gonadotropin (CG) b-chain]), known to extend the half-life of natural proteins such as human CG b-subunit, to the biological drug candidate. See, for example, Gregoriadis et al. (2005) "Improving the therapeutic efficacy of peptides and proteins: a role for polysialic acids" Int J Pharm. 2005; 300:125–30; Duijkers et al. "Single dose pharmacokinetics and effects on follicular growth and serum hormones of a long-acting recombinant FSH preparation (FSHCTP) in healthy pituitary-suppressed females" (2002) Hum Reprod. 17:1987–93; and Fares et al. "Design of a long-acting follitropin agonist by fusing the C-terminal sequence of the chorionic gonadotropin beta subunit to the follitropin beta subunit" (1992) Proc Natl Acad Sci USA. 89:4304–8. 35; and Fares "Half-life extension through O-glycosylation.

- Binding non-covalently, via attachment of a peptide or protein-binding domain to the bioactive protein, to normally long-half-life proteins such as HSA, human IgG, transferrin or fibronectin. See, for example, Andersen et al. (2011) "Extending half-life by indirect targeting of the neonatal Fc receptor (FcRn) using a minimal albumin binding domain" J Biol Chem. 286:5234–41; O'Connor-Semmes et al. (2014) "GSK2374697, a novel albumin-binding domain antibody (albudAb), extends systemic exposure of extendin-4: first study in humans—PK/PD and safety" Clin Pharmacol Ther. 2014;96:704–12. Sockolosky et al. (2014) "Fusion of a short peptide that binds immunoglobulin G to a recombinant protein substantially increases its plasma half-life in mice" PLoS One. 2014;9:e102566.

Classical genetic fusions to long-lived serum proteins offer an alternative method of half-life extension distinct from chemical conjugation to PEG or lipids. Two major proteins have traditionally been used as fusion partners: antibody Fc domains and human serum albumin (HSA). Fc fusions involve the fusion of peptides, proteins or receptor exodomains to the Fc portion of an antibody. Both Fc and albumin fusions achieve extended half-lives not only by increasing the size of the peptide drug, but both also take advantage of the body's natural recycling mechanism: the neonatal Fc receptor, FcRn. The pH-dependent binding of these proteins to FcRn prevents degradation of the fusion protein in the endosome. Fusions based on these proteins can have half-lives in the range of 3-16 days, much longer than typical PEGylated or lipidated peptides. Fusion to antibody Fc domains can improve the solubility and stability of the peptide or protein drug. An example of a peptide Fc fusion is dulaglutide, a GLP-1 receptor agonist currently in late-stage clinical trials. Human serum albumin, the same protein exploited by the fatty acylated peptides is the other popular fusion partner. Albiglutide is a GLP-1 receptor agonist based on this platform. A major difference between Fc and albumin is the dimeric nature of Fc versus the monomeric structure of HSA leading to presentation of a fused peptide as a dimer or a monomer depending on the choice of fusion partner. The dimeric nature of an Affimer-Fc fusion can produce an avidity effect if the Affimer target, such as cell surface protein on a target cell, is spaced closely enough together or are themselves dimers or higher order multimers. This may be desirable or not depending on the target.

(i) Fc Fusions

In some embodiments, the Affimer polypeptide may be part of a fusion protein with an immunoglobulin Fc domain ("Fc domain"), or a fragment or variant thereof, such as a functional Fc region. In this context, an Fc fusion ("Fc-fusion"), such as an Affimer polypeptide created as an Affimer-Fc fusion protein, is a polypeptide comprising one or more Affimer sequences covalently linked through a peptide backbone (directly or indirectly) to an Fc region of an immunoglobulin. An Fc-fusion may comprise, for example, the Fc region of an antibody (which facilitates effector functions and pharmacokinetics) and an Affimer sequence as part of the same polypeptide. An immunoglobulin Fc region may also be linked indirectly to one or more Affimers. Various linkers are known in the art and can optionally be used to link an Fc to

a polypeptide including an Affimer sequence to generate an Fc-fusion. In certain embodiments, Fc-fusions can be dimerized to form Fc-fusion homodimers, or using non-identical Fc domains, to form Fc-fusion heterodimers.

There are several reasons for choosing the Fc region of human antibodies for use in generating the subject Affimer polypeptides as Affimer fusion proteins. The principle rationale is to produce a stable protein, large enough to demonstrate a similar pharmacokinetic profile compared with those of antibodies, and to take advantage of the properties imparted by the Fc region; this includes the salvage neonatal FcRn receptor pathway involving FcRn-mediated recycling of the fusion protein to the cell surface post endocytosis, avoiding lysosomal degradation and resulting in release back into the bloodstream, thus contributing to an extended serum half-life. Another obvious advantage is the Fc domain's binding to Protein A, which can simplify downstream processing during production of the Affimer polypeptide and permit generation of highly pure preparation of the Affimer polypeptide.

In general, an Fc domain will include the constant region of an antibody excluding the first constant region immunoglobulin domain. Thus, Fc domain refers to the last two constant region immunoglobulin domains of IgA, IgD, and IgG, and the last three constant region immunoglobulin domains of IgE and IgM, and the flexible hinge N-terminal to these domains. For IgA and IgM Fc may include the J chain. For IgG, Fc comprises immunoglobulin domains Cγ2 and Cγ3 and the hinge between Cγ1 and Cγ2. Although the boundaries of the Fc domain may vary, the human IgG heavy chain Fc region is usually defined to comprise residues C226 or P230 to its carboxyl-terminus, wherein the numbering is according to the EU index as set forth in Kabat (Kabat et al., Sequences of Proteins of Immunological Interest, 5th Ed. Public Health Service, NIH, Bethesda, Md. (1991)). Fc may refer to this region in isolation, or this region in the context of a whole antibody, antibody fragment, or Fc fusion protein. Polymorphisms have been observed at a number of different Fc positions and are also included as Fc domains as used herein.

In certain embodiments, the Fc As used herein, a "functional Fc region" refers to an Fc domain or fragment thereof which retains the ability to bind FcRn. A functional Fc region binds to FcRn, but does not possess effector function. The ability of the Fc region or fragment thereof to bind to FcRn can be determined by standard binding assays known in the art. Exemplary "effector functions" include C1q binding; complement dependent cytotoxicity (CDC); Fc receptor binding; antibody-dependent cell-mediated cytotoxicity (ADCC); phagocytosis; down regulation of cell surface receptors (e.g., B cell receptor; BCR), etc. Such effector functions can be assessed using various assays known in the art for evaluating such antibody effector functions.

In an exemplary embodiment, the Fc domain is derived from an IgG1 subclass, however, other subclasses (e.g., IgG2, IgG3, and IgG4) may also be used. An exemplary sequence of a human IgG1 immunoglobulin Fc domain which can be used is:

DKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHE
DPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKE
YKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTC

LVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSR
WQQGNVFSCSVMHEALHNHYTQKSLSLSPGK (SEQ ID NO: 75)

In some embodiments, the Fc region used in the fusion protein may comprise the hinge region of an Fc molecule. An exemplary hinge region comprises the core hinge residues spanning positions 1-16 (i.e., DKTHTCPPCPAPELLG (SEQ ID NO: 76)) of the exemplary human IgG1 immunoglobulin Fc domain sequence provided above. In certain embodiments, the Affimer-containing fusion protein may adopt a multimeric structure (e.g., dimer) owing, in part, to the cysteine residues at positions 6 and 9 within the hinge region of the exemplary human IgG1 immunoglobulin Fc domain sequence provided above. In other embodiments, the hinge region as used herein, may further include residues derived from the CH1 and CH2 regions that flank the core hinge sequence of the exemplary human IgG1 immunoglobulin Fc domain sequence provided above. In yet other embodiments, the hinge sequence may comprise or consist of GSTHTCPPCPAPELLG (SEQ ID NO: 77) or EPKSCDKTHTCPPCPAPELLG (SEQ ID NO: 78).

In some embodiments, the hinge sequence may include one or more substitutions that confer desirable pharmacokinetic, biophysical, and/or biological properties. Some exemplary hinge sequences include:

SEQ ID NO: 79 EPKSCDKTHTCPPCPAPELLGGPS

SEQ ID NO: 80 EPKSSDKTHTCPPCPAPELLGGPS;

SEQ ID NO: 81 EPKSSDKTHTCPPCPAPELLGGSS;

SEQ ID NO: 82 EPKSSGSTHTCPPCPAPELLGGSS;

SEQ ID NO: 83 DKTHTCPPCPAPELLGGPS and

SEQ ID NO: 84 DKTHTCPPCPAPELLGGSS.

In one embodiment, the residue P at position 18 of the exemplary human IgG1 immunoglobulin Fc domain sequence provided above may be replaced with S to ablate Fc effector function; this replacement is exemplified in hinges having the sequences EPKSSDKTHTCPPCPAPELLGGSS (SEQ ID NO: 85), EPKSSGSTHTCPPCPAPELLGGSS (SEQ ID NO: 86), and DKTHTCPPCPAPELLGGSS (SEQ ID NO: 87). In another embodiment, the residues DK at positions 1-2 of the exemplary human IgG1 immunoglobulin Fc domain sequence provided above may be replaced with GS to remove a potential clip site; this replacement is exemplified in the sequence EPKSSGSTHTCPPCPAPELLGGSS (SEQ ID NO: 88). In another embodiment, the C at the position 103 of the heavy chain constant region of human IgG1 (i.e., domains CH1-CH3), may be replaced with S to prevent improper cysteine bond formation in the absence of a light chain; this replacement is exemplified by EPKSSDKTHTCPPCPAPELLGGPS (SEQ ID NO: 89), EPKSSDKTHTCPPCPAPELLGGSS (SEQ ID NO: 90), and EPKSSGSTHTCPPCPAPELLGGSS (SEQ ID NO: 91).

In some embodiments, the Fc is a mammalian Fc such as a human Fc, including Fc domains derived from IgG1, IgG2, IgG3 or IgG4. The Fc region may possess at least about 80%, 85%,

90%, 95%, 96%, 97%, 98%, or 99% sequence identity with a native Fc region and/or with an Fc region of a parent polypeptide. In some embodiments, the Fc region may have at least about 90% sequence identity with a native Fc region and/or with an Fc region of a parent polypeptide.

In some embodiments, the Fc domain comprises an amino acid sequence selected from the examples provided by SEQ ID Nos. 4-16. It should be understood that the C-terminal lysine of an Fc domain is an optional component of a fusion protein comprising an Fc domain. In some embodiments, the Fc domain comprises an amino acid sequence selected from SEQ ID NOs: 4 - 16, except that the C-terminal lysine thereof is omitted.

hIgG1a_191 [A subtype] **(SEQ ID NO: 4)**	DKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHE DPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKE YKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTC LVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSR WQQGNVFSCSVMHEALHNHYTQKSLSLSPGK
hIgG1a_189 [hIgG1a_191 sans "GK" on C term; A subtype] **(SEQ ID NO: 5)**	DKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHE DPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKE YKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTC LVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSR WQQGNVFSCSVMHEALHNHYTQKSLSLSP
hIgG1a_191b [A/F subtype] **(SEQ ID NO: 6)**	DKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHE DPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKE YKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDEMTKNQVSLTC LVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSR WQQGNVFSCSVMHEALHNHYTQKSLSLSPGK
hIgG1f_1.1_191 [Contains 5 point mutations to alter ADCC function, F subtype] **(SEQ ID NO: 7)**	DKTHTCPPCPAPEAEGAPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHE DPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKE YKCKVSNKALPSSIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTC LVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSR WQQGNVFSCSVMHEALHNHYTQKSLSLSPGK
hIgG1f_1.1_186 [Contains 5 point mutations to alter ADCC function and C225S (Edlemen numbering); F subtype] **(SEQ ID NO: 8)**	EPKSSDKTHTCPPCPAPEAEGAPSVFLFPPKPKDTLMISRTPEVTCVVV DVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDW LNGKEYKCKVSNKALPSSIEKTISKAKGQPREPQVYTLPPSREEMTKNQ VSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLT VDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK

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hIgG1a_(N297G)_191 [A subtype] **(SEQ ID NO: 9)**	DKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHE DPEVKFNWYVDGVEVHNAKTKPREEQYGSTYRVVSVLTVLHQDWLNGKE YKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTC LVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSR WQQGNVFSCSVMHEALHNHYTQKSLSLSPGK
hIgG1a_190 [hIgG1a_190 sans "K" on C term; A subtype] **(SEQ ID No. 10)**	DKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHE DPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKE YKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTC LVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSR WQQGNVFSCSVMHEALHNHYTQKSLSLSPG
hIgG1a_(N297Q)_191 [A subtype] **(SEQ ID NO: 11)**	DKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHE DPEVKFNWYVDGVEVHNAKTKPREEQYQSTYRVVSVLTVLHQDWLNGKE YKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTC LVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSR WQQGNVFSCSVMHEALHNHYTQKSLSLSPGK
hIgG1a_(N297S)_191 [A subtype] **(SEQ ID NO: 12)**	DKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHE DPEVKFNWYVDGVEVHNAKTKPREEQYSSTYRVVSVLTVLHQDWLNGKE YKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTC LVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSR WQQGNVFSCSVMHEALHNHYTQKSLSLSPGK
hIgG1a_(N297A)_191 [A subtype] **(SEQ ID NO: 13)**	DKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHE DPEVKFNWYVDGVEVHNAKTKPREEQYASTYRVVSVLTVLHQDWLNGKE YKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTC LVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSR WQQGNVFSCSVMHEALHNHYTQKSLSLSPGK
hIgG1a_(N297H)_191 [A subtype] **(SEQ ID NO: 14)**	DKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHE DPEVKFNWYVDGVEVHNAKTKPREEQYHSTYRVVSVLTVLHQDWLNGKE YKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTC LVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSR WQQGNVFSCSVMHEALHNHYTQKSLSLSPGK
hIgG4 **(SEQ ID No. 15)**	DKRVESKYGPPCPSCPAPEFLGGPSVFLFPPKPKDTLMISRTPEVTCVV VDVSQEDPEVQFNWYVDGVEVHNAKTKPREEQFNSTYRVVSVLTVLHQD WLNGKEYKCKVSNKGLPSSIEKTISKAKGQPREPQVYTLPPSQEEMTKN QVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSRL TVDKSRWQEGNVFSCSVMHEALHNHYTQKSLSLSLGK
hIgG4_(S241P) **(SEQ ID NO: 16)**	DKRVESKYGPPCPPCPAPEFLGGPSVFLFPPKPKDTLMISRTPEVTCVV VDVSQEDPEVQFNWYVDGVEVHNAKTKPREEQFNSTYRVVSVLTVLHQD WLNGKEYKCKVSNKGLPSSIEKTISKAKGQPREPQVYTLPPSQEEMTKN

QVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSRL
TVDKSRWQEGNVFSCSVMHEALHNHYTQKSLSLSLGK

The Affimer sequence can be placed at either the N-terminal or C-terminal end of the Fc domain, and may be attached directly or the fusion protein may have other polypeptide sequences intervening between the Fc domain and the Affimer polypeptide sequence.

"Antibody-dependent cell-mediated cytotoxicity" or "ADCC" refers to a form of cytotoxicity in which secreted Ig bound onto Fc receptors (FcRs) present on certain cytotoxic cells (e.g., Natural Killer (NK) cells, neutrophils, and macrophages) enables these cytotoxic effector cells to bind specifically to an antigen-bearing target cell and subsequently kill the target cell with cytotoxins.

In certain embodiments, the fusion protein includes an Fc domain sequence for which the resulting Affimer polypeptide has no (or reduced) ADCC and/or complement activation or effector functionality. For example, the Fc domain may comprise a naturally disabled constant region of IgG2 or IgG4 isotype or a mutated IgG1 constant region. Examples of suitable modifications are described in EP0307434. One example comprises the substitutions of alanine residues at positions 235 and 237 (EU index numbering).

In other embodiments, the fusion protein includes an Fc domain sequence for which the resulting Affimer polypeptide will retain some or all Fc functionality for example will be capable of one or both of ADCC and CDC activity, as for example if the fusion protein comprises the Fc domain from human IgG1 or IgG3. Levels of effector function can be varied according to known techniques, for example by mutations in the CH2 domain, for example wherein the IgG1 CH2 domain has one or more mutations at positions selected from 239 and 332 and 330, for example the mutations are selected from S239D and I332E and A330L such that the antibody has enhanced effector function, and/or for example altering the glycosylation profile of the antigen-binding protein of the invention such that there is a reduction in fucosylation of the Fc region.

(ii) Albumin fusion

In other embodiments, the Affimer polypeptide is a fusion protein comprising, in addition to at least one Affimer sequence, an albumin sequence or an albumin fragment. In other embodiments, the Affimer polypeptide is conjugated to the albumin sequence or an albumin fragment through chemical linkage other than incorporation into the polypeptide sequence including the Affimer. In some embodiments, the albumin, albumin variant, or albumin fragment is human serum albumin (HSA), a human serum albumin variant, or a human serum albumin fragment. Albumin serum proteins comparable to HSA are found in, for example, cynomolgus monkeys, cows, dogs, rabbits and rats. Of the non-human species, bovine serum albumin (BSA) is the most structurally similar to HSA. See, e.g., Kosa et al., (2007) J Pharm Sci. 96(11):3117-24. The present disclosure contemplates the use of albumin from non-human

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species, including, but not limited to, albumin sequence derived from cyno serum albumin or bovine serum albumin.

Mature HSA, a 585 amino acid polypeptide (approx. 67 kDa) having a serum half-life of about 20 days, is primarily responsible for the maintenance of colloidal osmotic blood pressure, blood pH, and transport and distribution of numerous endogenous and exogenous ligands. The protein has three structurally homologous domains (domains I, II and III), is almost entirely in the alpha-helical conformation, and is highly stabilized by 17 disulfide bridges. In certain preferred embodiments, the Affimer polypeptide can be an albumin fusion protein including one or more Affimer polypeptide sequences and the sequence for mature human serum albumin (SEQ ID No. 17) or a variant or fragment thereof which maintains the PK and/or biodistribution properties of mature albumin to the extent desired in the fusion protein.

DAHKSEVAHRFKDLGEENFKALVLIAFAQYLQQCPFEDHVKLVNEVTEF
AKTCVADESAENCDKSLHTLFGDKLCTVATLRETYGEMADCCAKQEPER
NECFLQHKDDNPNLPRLVRPEVDVMCTAFHDNEETFLKKYLYEIARRHP
YFYAPELLFFAKRYKAAFTECCQAADKAACLLPKLDELRDEGKASSAKQ
RLKCASLQKFGERAFKAWAVARLSQRFPKAEFAEVSKLVTDLTKVHTEC
CHGDLLECADDRADLAKYICENQDSISSKLKECCEKPLLEKSHCIAEVE
NDEMPADLPSLAADFVESKDVCKNYAEAKDVFLGMFLYEYARRHPDYSV
VLLLRLAKTYETTLEKCCAAADPHECYAKVFDEFKPLVEEPQNLIKQNC
ELFEQLGEYKFQNALLVRYTKKVPQVSTPTLVEVSRNLGKVGSKCCKHP
EAKRMPCAEDYLSVVLNQLCVLHEKTPVSDRVTKCCTESLVNRRPCFSA
LEVDETYVPKEFNAETFTFHADICTLSEKERQIKKQTALVELVKHKPKA
TKEQLKAVMDDFAAFVEKCCKADDKETCFAEEGKKLVAASQAALGL
(SEQ ID NO: 17)

The albumin sequence can be set off from the Affimer polypeptide sequence or other flanking sequences in the Affimer polypeptide by use of linker sequences as described above.

While unless otherwise indicated, reference herein to "albumin" or to "mature albumin" is meant to refer to HSA. However, it is noted that full-length HSA has a signal peptide of 18 amino acids (MKWVTFISLLFLFSSAYS) followed by a pro-domain of 6 amino acids (RGVFRR); this 24 amino acid residue peptide may be referred to as the pre-pro domain. The Affimer-HSA fusion proteins can be expressed and secreted using the HSA pre-pro-domain in the recombinant proteins coding sequence. Alternatively, the Affimer-HSA fusion can be expressed and secreted through inclusion of other secretion signal sequences, such as described above.

In alternative embodiments, rather than provided as part of a fusion protein with the Affimer polypeptide, the serum albumin polypeptide can be covalently coupled to the Affimer-containing polypeptide through a bond other than a backbone amide bond, such as cross-linked through chemical conjugation between amino acid sidechains on each of the albumin polypeptide and the Affimer-containing polypeptide.

(iii) Albumin binding domain

In certain embodiments, the Affimer polypeptide can include a serum-binding moiety – either as part of a fusion protein (if also a polypeptide) with the Affimer polypeptide sequence or chemically conjugated through a site other than being part of a contiguous polypeptide chain.

In certain embodiments, the serum-binding polypeptide is an albumin binding moiety. Albumin contains multiple hydrophobic binding pockets and naturally serves as a transporter of a variety of different ligands such as fatty acids and steroids as well as different drugs. Furthermore, the surface of albumin is negatively charged making it highly water-soluble.

The term "albumin binding moiety" as used herein refers to any chemical group capable of binding to albumin, i.e. has albumin binding affinity. Albumin binds to endogenous ligands such as fatty acids; however, it also interacts with exogenous ligands such as warfarin, penicillin and diazepam. As the binding of these drugs to albumin is reversible the albumin-drug complex serves as a drug reservoir that can enhance the drug biodistribution and bioavailability. Incorporation of components that mimic endogenous albumin-binding ligands, such as fatty acids, has been used to potentiate albumin association and increase drug efficacy.

In certain embodiments, a chemical modification method that can be applied in the generation of the subject Affimer polypeptides to increase protein half-life is lipidation, which involves the covalent binding of fatty acids to peptide side chains. Originally conceived of and developed as a method for extending the half-life of insulin, lipidation shares the same basic mechanism of half-life extension as PEGylation, namely increasing the hydrodynamic radius to reduce renal filtration. However, the lipid moiety is itself relatively small and the effect is mediated indirectly through the non-covalent binding of the lipid moiety to circulating albumin. One consequence of lipidation is that it reduces the water-solubility of the peptide but engineering of the linker between the peptide and the fatty acid can modulate this, for example by the use of glutamate or mini PEGs within the linker. Linker engineering and variation of the lipid moiety can affect self-aggregation which can contribute to increased half-life by slowing down biodistribution, independent of albumin. See, for example, Jonassen et al. (2012) Pharm Res. 29(8):2104-14.

Other examples of albumin binding moieties for use in the generation of certain Affimer polypeptides include albumin-binding (PKE2) adnectins (See WO2011140086 "Serum Albumin Binding Molecules", WO2015143199 "Serum albumin-binding Fibronectin Type III Domains" and WO2017053617 "Fast-off rate serum albumin binding fibronectin type iii domains"), the albumin binding domain 3 (ABD3) of protein G of Streptococcus strain G148, and the albumin binding domain antibody GSK2374697 ("AlbudAb") or albumin binding nanobody portion of ATN-103 (Ozoralizumab).

(iv) PEGylation, XTEN, PAS and Other Polymers

A wide variety of macromolecular polymers and other molecules can be linked to the Affimer containing polypeptides of the present disclosure to modulate biological properties of the resulting Affimer polypeptide, and/or provide new biological properties to the Affimer

polypeptide. These macromolecular polymers can be linked to the Affimer containing polypeptide via a naturally encoded amino acid, via a non-naturally encoded amino acid, or any functional substituent of a natural or non-natural amino acid, or any substituent or functional group added to a natural or non-natural amino acid. The molecular weight of the polymer may be of a wide range, including but not limited to, between about 100 Da and about 100,000 Da or more. The molecular weight of the polymer may be between about 100 Da and about 100,000 Da, including but not limited to, 100,000 Da, 95,000 Da, 90,000 Da, 85,000 Da, 80,000 Da, 75,000 Da, 70,000 Da, 65,000 Da, 60,000 Da, 55,000 Da, 50,000 Da, 45,000 Da, 40,000 Da, 35,000 Da, 30,000 Da, 25,000 Da, 20,000 Da, 15,000 Da, 10,000 Da, 9,000 Da, 8,000 Da, 7,000 Da, 6,000 Da, 5,000 Da, 4,000 Da, 3,000 Da, 2,000 Da, 1,000 Da, 900 Da, 800 Da, 700 Da, 600 Da, 500 Da, 400 Da, 300 Da, 200 Da, and 100 Da. In some embodiments, the molecular weight of the polymer is between about 100 Da and about 50,000 Da. In some embodiments, the molecular weight of the polymer is between about 100 Da and about 40,000 Da. In some embodiments, the molecular weight of the polymer is between about 1,000 Da and about 40,000 Da. In some embodiments, the molecular weight of the polymer is between about 5,000 Da and about 40,000 Da. In some embodiments, the molecular weight of the polymer is between about 10,000 Da and about 40,000 Da.

For this purpose, various methods including pegylation, polysialylation, HESylation, glycosylation, or recombinant PEG analogue fused to flexible and hydrophilic amino acid chain (500 to 600 amino acids) have been developed (See Chapman, (2002) Adv Drug Deliv Rev. 54. 531-545; Schlapschy et al., (2007) Prot Eng Des Sel. 20, 273-283; Contermann (2011) Curr Op Biotechnol. 22, 868-876; Jevsevar et al., (2012) Methods Mol Biol. 901, 233-246).

Examples of polymers include but are not limited to polyalkyl ethers and alkoxy-capped analogues thereof (e.g., polyoxyethylene glycol, polyoxyethylene/propylene glycol, and methoxy or ethoxy-capped analogues thereof, especially polyoxyethylene glycol, the latter is also known as polyethylene glycol or PEG); discrete PEG (dPEG); polyvinylpyrrolidones; polyvinylalkyl ethers; polyoxazolines, polyalkyl oxazolines and polyhydroxyalkyl oxazolines; polyacrylamides, polyalkyl acrylamides, and polyhydroxyalkyl acrylamides (e.g., polyhydroxypropylmethacrylamide and derivatives thereof); polyhydroxyalkyl acrylates; polysialic acids and analogues thereof; hydrophilic peptide sequences; polysaccharides and their derivatives, including dextran and dextran derivatives, e.g., carboxymethyldextran, dextran sulfates, aminodextran; cellulose and its derivatives, e.g., carboxymethyl cellulose, hydroxyalkyl celluloses; chitin and its derivatives, e.g., chitosan, succinyl chitosan, carboxymethylchitin, carboxymethylchitosan; hyaluronic acid and its derivatives; starches; alginates; chondroitin sulfate; albumin; pullulan and carboxymethyl pullulan; polyaminoacids and derivatives thereof, e.g., polyglutamic acids, polylysines, polyaspartic acids, polyaspartamides; maleic anhydride copolymers such as: styrene maleic anhydride copolymer, divinylethyl ether maleic anhydride copolymer; polyvinyl alcohols; copolymers thereof; terpolymers thereof; mixtures thereof; and derivatives of the foregoing.

The polymer selected may be water soluble so that the Affimer polypeptide to which it is attached does not precipitate in an aqueous environment, such as a physiological environment. The water-soluble polymer may be any structural form including but not limited

to linear, forked or branched. Typically, the water soluble polymer is a poly(alkylene glycol), such as poly(ethylene glycol) (PEG), but other water soluble polymers can also be employed. By way of example, PEG is used to describe certain embodiments of this disclosure. For therapeutic use of the Affimer polypeptide, the polymer may be pharmaceutically acceptable.

The term "PEG" is used broadly to encompass any polyethylene glycol molecule, without regard to size or to modification at an end of the PEG, and can be represented as linked to the Affimer containing polypeptide by the formula:

$$XO—(CH_2CH_2O)_n—CH_2CH_2—$$

or

$$XO—(CH_2CH_2O)_n—$$

where n is 2 to 10,000 and X is H or a terminal modification, including but not limited to, a C1-4 alkyl, a protecting group, or a terminal functional group. In some cases, a PEG used in the polypeptides of the disclosure terminates on one end with hydroxy or methoxy, i.e., X is H or CH3 ("methoxy PEG").

It is noted that the other end of the PEG, which is shown in the above formulas by a terminal "—", may attach to the Affimer containing polypeptide via a naturally-occurring or non-naturally encoded amino acid. For instance, the attachment may be through an amide, carbamate or urea linkage to an amine group (including but not limited to, the epsilon amine of lysine or the N-terminus) of the polypeptide. Alternatively, the polymer is linked by a maleimide linkage to a thiol group (including but not limited to, the thiol group of cysteine) – which in the case of attachment to the Affimer polypeptide sequence per se requires altering a residue in the Affimer sequence to a cysteine.

The number of water soluble polymers linked to the Affimer-containing polypeptide (i.e., the extent of PEGylation or glycosylation) can be adjusted to provide an altered (including but not limited to, increased or decreased) pharmacologic, pharmacokinetic or pharmacodynamic characteristic such as in vivo half-life in the resulting Affimer polypeptide. In some embodiments, the half-life of the resulting Affimer polypeptide is increased at least about 10, 20, 30, 40, 50, 60, 70, 80, 90 percent, 2-fold, 5-fold, 6-fold, 7-fold, 8-fold, 9-fold, 10-fold, 11-fold, 12-fold, 13-fold, 14-fold, 15-fold, 16-fold, 17-fold, 18-fold, 19-fold, 20-fold, 25-fold, 30-fold, 35-fold, 40-fold, 50-fold, or at least about 100-fold over an unmodified polypeptide.

Another variation of polymer system useful to modify the PK or other biological properties of the resulting Affimer polypeptide are the use of unstructured, hydrophilic amino acid polymers that are functional analogues of PEG, particularly as part of a fusion protein with the Affimer polypeptide sequence. The inherent biodegradability of the polypeptide platform makes it attractive as a potentially more benign alternative to PEG. Another advantage is the precise molecular structure of the recombinant molecule in contrast to the polydispersity of PEG. Unlike HSA and Fc peptide fusions, in which the three-dimensional folding of the fusion partner needs to be maintained, the recombinant fusions to unstructured partners can, in many cases, be subjected to higher temperatures or harsh conditions such as HPLC purification.

One of the more advanced of this class of polypeptides is termed XTEN (Amunix) and is 864 amino acids long and comprised of six amino acids (A, E, G, P, S and T). See Schellenberger et al. "A recombinant polypeptide extends the in vivo half-life of peptides and proteins in a tuneable manner" 2009 Nat Biotechnol. 27(12):1186-90. Enabled by the biodegradable nature of the polymer, this is much larger than the 40 kDa PEGs typically used and confers a concomitantly greater half-life extension. The fusion of XTEN to the Affimer containing polypeptide should result in half-life extension of the final Affimer polypeptide by 60- to 130-fold over the unmodified polypeptide.

A second polymer based on similar conceptual considerations is PAS (XL-Protein GmbH). Schlapschy et al. "PASYlation: a biological alternative to PEGylation for extending the plasma half-life of pharmaceutically active proteins" 2013 Protein Eng Des Sel. 26(8):489-501. A random coil polymer comprised of an even more restricted set of only three small uncharged amino acids, proline, alanine and serine. AS with Fc, HAS and XTEN, the PAS modification can be genetically encoded with the Affimer polypeptide sequence to produce an inline fusion protein when expressed.

b. Multi-specific Fusion Proteins

In certain embodiments, the Affimer polypeptide is a multi-specific and/or multivalent polypeptide including, for example, a first Affimer polypeptide that binds to a first target and at least one additional binding domain that binds to a second target that is difference from the first target – which may be a different molecule altogether (bispecific) or the same molecule type at the same site (multivalent) or the same molecule but at a different site (biparatopic or multiparatopic). The additional binding domain may be a polypeptide sequence selected from amongst, to illustrate, a second Affimer polypeptide sequence (which may be the same or different than the first Affimer polypeptide sequence), an antibody or fragment thereof or other antigen binding polypeptide, a ligand binding portion of a receptor (such as a receptor trap polypeptide), a receptor-binding ligand (such as a cytokine, growth factor or the like), engineered T-cell receptor, an enzyme or catalytic fragment thereof, or other polypeptide sequence that confers some

In certain embodiments, the Affimer polypeptide includes one or more antigen binding sites from an antibody. The resulting Affimer polypeptide can be a single chain including both the Affimer sequence and the sequence for the antibody antigen binding site (such as in the case of an scFV), or can be a multimeric protein complex such as in antibody assembled with heavy and/or light chains to which the sequence of the Affimer has also been fused.

In some embodiments with respect to a multi-specific Affimer polypeptide comprising a full-length immunoglobulin, the fusion of the Affimer polypeptide sequence to the antibody will preserve the Fc function of the Fc region of the immunoglobulin. For instance, in certain embodiments, the Affimer polypeptide will be capable of binding, via its Fc portion, to the Fc receptor of Fc receptor-positive cells. In some further embodiments, the Affimer polypeptide may activate the Fc receptor-positive cell by binding to the Fc receptor-positive cell, thereby initiating or increasing the expression of cytokines and/or co-stimulatory antigens.

Furthermore, the Affimer polypeptide may transfer at least a second activation signal required for physiological activation of the T cell to the T cell via the co-stimulatory antigens and/or cytokines.

In some embodiments, resulted from the binding of its Fc portion to other cells that express Fc receptors present on the surface of effector cells from the immune system, such as immune cells, hepatocytes, and endothelial cells, the Affimer polypeptide may possess antibody-dependent cellular cytotoxicity (ADCC) function, a mechanism of cell-mediated immune defence whereby an effector cell of the immune system actively lyses a target cell, whose membrane-surface antigen has been bound by an antibody, and therefore, trigger tumor cell death via ADCC. In some further embodiments, the Affimer polypeptide is capable of demonstrating ADCC function.

As described above, apart from the Fc-mediated cytotoxicity, the Fc portion may contribute to maintaining the serum levels of the Affimer polypeptide, critical for its stability and persistence in the body. For example, when the Fc portion binds to Fc receptors on endothelial cells and on phagocytes, the Affimer polypeptide may become internalized and recycled back to the blood stream, enhancing its half-life within the body.

Exemplary targets of the additional Affimer polypeptides include, but are not limited to, another immune checkpoint protein, and immune co-stimulatory receptor (particularly if the additional Affimer(s) can agonize the co-stimulatory receptor), a receptor, a cytokine, a growth factor, or a tumor-associated antigen, mere to illustrate.

c. Conjugates

The subject Affimer polypeptides may also include one or more Functional Moieties intended to impart detectability or additional pharmacologic activity to the Affimer polypeptide. Functional Moieties for detection are those which can be employed to detect association of the Affimer polypeptide with a cell or tissue (such as a tumour cell) in vivo. Functional Moieties with pharmacologic activity are those agents which are meant to be delivered to the tissue expressing the target of the Affimer polypeptide and in doing so have a pharmacologic consequence to the targeted tissues or cells.

The present disclosure provides Affimer polypeptides including conjugates of substances having a wide variety of functional groups, substituents or moieties, with those Functional Moieties including but not limited to a label; a dye; an immunoadhesion molecule; a radionuclide; a cytotoxic compound; a drug; an affinity label; a photoaffinity label; a reactive compound; a resin; a second protein or polypeptide or polypeptide analogue; an antibody or antibody fragment; a metal chelator; a cofactor; a fatty acid; a carbohydrate; a polynucleotide; a DNA; a RNA; an antisense polynucleotide; a saccharide; a water-soluble dendrimer; a cyclodextrin; an inhibitory ribonucleic acid; a biomaterial; a nanoparticle; a spin label; a fluorophore, a metal-containing moiety; a radioactive moiety; a novel functional group; a group that covalently or noncovalently interacts with other molecules; a photocaged moiety; an actinic radiation excitable moiety; a photoisomerizable moiety; biotin; a derivative of biotin; a biotin analogue; a moiety incorporating a heavy atom; a chemically cleavable group; a

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photocleavable group; an elongated side chain; a carbon-linked sugar; a redox-active agent; an amino thioacid; a toxic moiety; an isotopically labelled moiety; a biophysical probe; a phosphorescent group; a chemiluminescent group; an electron dense group; a magnetic group; an intercalating group; a chromophore; an energy transfer agent; a biologically active agent; a detectable label; a small molecule; a quantum dot; a nanotransmitter; a radionucleotide; a radiotransmitter; a neutron-capture agent; or any combination of the above, or any other desirable compound or substance.

(i) Labels and Detectable Moieties

Where the moiety is a detectable label, it can be a fluorescent label, radioactive label, enzymatic label or any other label known to the skilled person. In certain embodiments, the Functional Moiety is a detectable label that can be included as part of a conjugate to form certain Affimer polypeptides suitable for medical imaging. By "medical imaging" is meant any technique used to visualise an internal region of the human or animal body, for the purposes of diagnosis, research or therapeutic treatment. For instance, the Affimer polypeptide can be detected (and quantitated) by radio-scintigraphy, magnetic resonance imaging (MRI), computed tomography (CT scan), nuclear imaging, positron emission comprising a metal tomography (PET) contrast agent, optical imaging (such as fluorescence imaging including near-infrared fluorescence (NIRF) imaging), bioluminescence imaging, or combinations thereof. The Functional Moiety is optionally a contrast agent for X-ray imaging. Agents useful in enhancing such techniques are those materials that enable visualization of a particular locus, organ or disease site within the body, and/or that lead to some improvement in the quality of the images generated by the imaging techniques, providing improved or easier interpretation of those images. Such agents are referred to herein as contrast agents, the use of which facilitates the differentiation of different parts of the image, by increasing the "contrast" between those different regions of the image. The term "contrast agents" thus encompasses agents that are used to enhance the quality of an image that may nonetheless be generated in the absence of such an agent (as is the case, for instance, in MRI), as well as agents that are prerequisites for the generation of an image (as is the case, for instance, in nuclear imaging).

In certain preferred embodiments, the detectable label includes a chelate moiety for chelating a metal, e.g., a chelator for a radiometal or paramagnetic ion. In certain preferred embodiments, the detectable label is a chelator for a radionuclide useful for radiotherapy or imaging procedures. Radionuclides useful within the present invention include gamma-emitters, positron-emitters, Auger electron-emitters, X-ray emitters and fluorescence-emitters, with beta- or alpha-emitters preferred for therapeutic use. Examples of radionuclides useful as toxins in radiation therapy include: 43K, 47Sc, 51Cr, 57Co, 58Co, 59Fe, 64Cu, 67Ga, 67Cu, 68Ga, 71Ge, 75Br, 76Br, 77Br, 77As, 81Rb, 90Y, 97Ru, 99mTc, 100Pd, 101Rh, 103Pb, 105Rh, 109Pd, 111Ag, 111In, 113In, 119Sb 121Sn, 123I, 125I, 127Cs, 128Ba, 129Cs, 131I, 131Cs, 143Pr, 153Sm, 161Tb, 166Ho, 169Eu, 177Lu, 186Re, 188Re, 189Re, 191Os, 193Pt, 194Ir, 197Hg, 199Au, 203Pb, 211At, 212Pb, 212Bi and 213Bi. Conditions under which a chelator will coordinate a metal are described, for example, by Gansow et al., U.S. Pat. Nos. 4,831,175,

4,454,106 and 4,472,509. Examples of chelators includes, merely to illustrate, 1,4,7-triazacyclononane-N,N',N''-triacetic acid (NOTA) 1,4,7,10-tetraazacyclododecane-N,N',N'',N'''-tetraacetic acid (DOTA) 1 ,4,8,11-tetraazacyclotetradecane-N,N',N'',N'''-tetraacetic acid (TETA).

Other detectable isotopes that can be incorporated directly into the amino acid residues of the Affimer polypeptide or which otherwise do not require a chelator, include 3H, 14C, 32P, 35S and 36Cl.

Paramagnetic ions, useful for diagnostic procedures, may also be administered. Examples of paramagnetic ions include chromium (III), manganese (II), iron (III), iron (II), cobalt (II), nickel (II), copper (II), neodymium (III), samarium (III), ytterbium (III), gadolinium (III), vanadium (II), terbium (III), dysprosium (III), holmium (III), erbium (III), or combinations of these paramagnetic ions.

Examples of fluorescent labels include, but are not restricted to, organic dyes (e.g. cyanine, fluorescein, rhodamine, Alexa Fluors, Dylight fluors, ATTO Dyes, BODIPY Dyes, etc.), biological fluorophores (e.g. green fluorescent protein (GFP), R-Phycoerythrin, etc.), and quantum dots.

Non-limiting fluorescent compound that may be used in the present invention include, Cy5, Cy5.5 (also known as Cy5++), Cy2, fluorescein isothiocyanate (FITC), tetramethylrhodamine isothiocyanate (TRITC), phycoerythrin, Cy7, fluorescein (FAM), Cy3, Cy3.5 (also known as Cy3++), Texas Red, LightCycler-Red 640, LightCycler Red 705, tetramethylrhodamine (TMR), rhodamine, rhodamine derivative (ROX), hexachlorofluorescein (HEX), rhodamine 6G (R6G), the rhodamine derivative JA133, Alexa Fluorescent Dyes (such as Alexa Fluor 488, Alexa Fluor 546, Alexa Fluor 633, Alexa Fluor 555, and Alexa Fluor 647), 4',6-diamidino-2-phenylindole (DAPI), Propidium iodide, AMCA, Spectrum Green, Spectrum Orange, Spectrum Aqua, Lissamine, and fluorescent transition metal complexes, such as europium. Fluorescent compound that can be used also include fluorescent proteins, such as GFP (green fluorescent protein), enhanced GFP (EGFP), blue fluorescent protein and derivatives (BFP, EBFP, EBFP2, Azurite, mKalama1), cyan fluorescent protein and derivatives (CFP, ECFP, Cerulean, CyPet) and yellow fluorescent protein and derivatives (YFP, Citrine, Venus, YPet). WO2008142571, WO2009056282, WO9922026.

Examples of enzymatic labels include, but are not restricted to, horseradish peroxidase (HRP), alkaline phosphatase (AP), glucose oxidase and -galactosidase.

Another well-known label is biotin. Biotin labels are typically composed of the biotinyl group, a spacer arm and a reactive group that is responsible for attachment to target functional groups on proteins. Biotin can be useful for attaching the labelled protein to other moieties which comprise an avidin moiety.

(ii) Affimer-Drug Conjugates

In certain embodiments, the Affimer polypeptide includes one or more therapeutic agents, e.g., to form an Affimer-drug conjugate. As used herein, the term "therapeutic agent" refers to a substance that may be used in the cure, mitigation, treatment, or prevention of disease in a human or another animal. Such therapeutic agents include substances recognized in the

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official United States Pharmacopeia, official Homeopathic Pharmacopeia of the United States, official National Formulary, or any supplement thereof, and include but are not limited to small molecules, nucleotides, oligopeptides, polypeptides, etc. Therapeutic agents that may be attached to Affimer-containing polypeptides include, but are not limited to, cytotoxic agents, anti-metabolites, alkylating agents, antibiotics, growth factor, cytokines, anti-angiogenic agents, anti-mitotic agents, toxins, apoptotic agents or the like, such as DNA alkylating agents, topoisomerase inhibitors, endoplasmic reticulum stress inducing agents, platinum compounds, antimetabolites, vincalkaloids, taxanes, epothilones, enzyme inhibitors, receptor antagonists, therapeutic antibodies, tyrosine kinase inhibitors, radiosensitizers, and chemotherapeutic combination therapies, such as illustrations.

Non-limiting examples of DNA alkylating agents are nitrogen mustards, such as Mechlorethamine, Cyclophosphamide (Ifosfamide, Trofosfamide), Chlorambucil (Melphalan, Prednimustine), Bendamustine, Uramustine and Estramustine; nitrosoureas, such as Carmustine (BCNU), Lomustine (Semustine), Fotemustine, Nimustine, Ranimustine and Streptozocin; alkyl sulfonates, such as Busulfan (Mannosulfan, Treosulfan); Aziridines, such as Carboquone, ThioTEPA, Triaziquone, Triethylenemelamine; Hydrazines (Procarbazine); Triazenes such as Dacarbazine and Temozolomide; Altretamine and Mitobronitol.

Non-limiting examples of Topoisomerase I inhibitors include Campothecin derivatives including CPT-11 (irinotecan), SN-38, APC, NPC, campothecin, topotecan, exatecan mesylate, 9-nitrocamptothecin, 9-aminocamptothecin, lurtotecan, rubitecan, silatecan, gimatecan, diflomotecan, extatecan, BN-80927, DX-8951f, and MAG-CPT as described in Pommier Y. (2006) Nat. Rev. Cancer 6(10):789-802 and U.S. Patent Publication No. 200510250854; Protoberberine alkaloids and derivatives thereof including berberrubine and coralyne as described in Li et al. (2000) Biochemistry 39(24):7107-7116 and Gatto et al. (1996) Cancer Res. 15(12):2795-2800; Phenanthroline derivatives including Benzo[i]phenanthridine, Nitidine, and fagaronine as described in Makhey et al. (2003) Bioorg. Med. Chem. 11 (8): 1809-1820; Terbenzimidazole and derivatives thereof as described in Xu (1998) Biochemistry 37(10):3558-3566; and Anthracycline derivatives including Doxorubicin, Daunorubicin, and Mitoxantrone as described in Foglesong et al. (1992) Cancer Chemother. Pharmacol. 30(2):123-]25, Crow et al. (1994) J. Med. Chem. 37(19):31913194, and Crespi et al. (1986) Biochem. Biophys. Res. Commun. 136(2):521-8. Topoisomerase II inhibitors include, but are not limited to Etoposide and Teniposide. Dual topoisomerase I and II inhibitors include, but are not limited to, Saintopin and other Naphthecenediones, DACA and other Acridine-4-Carboxamindes, Intoplicine and other Benzopyridoindoles, TAS-103 and other 7H-indeno[2,1-c]Quinoline-7-ones, Pyrazoloacridine, XR 11576 and other Benzophenazines, XR 5944 and other Dimeric compounds, 7-oxo-7H-dibenz[f,ij]Isoquinolines and 7-oxo-7H-benzo[e]Perimidines, and Anthracenyl-amino Acid Conjugates as described in Denny and Baguley (2003) Curr. Top. Med. Chem. 3(3):339-353. Some agents inhibit Topoisomerase II and have DNA intercalation activity such as, but not limited to, Anthracyclines (Aclarubicin, Daunorubicin, Doxorubicin, Epirubicin, Idarubicin, Amrubicin, Pirarubicin, Valrubicin, Zorubicin) and Antracenediones (Mitoxantrone and Pixantrone).

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Examples of endoplasmic reticulum stress inducing agents include, but are not limited to, dimethyl-celecoxib (DMC), nelfinavir, celecoxib, and boron radiosensitizers (i.e. velcade (Bortezomib)).

Non-limiting examples of platinum-based compound include Carboplatin, Cisplatin, Nedaplatin, Oxaliplatin, Triplatin tetranitrate, Satraplatin, Aroplatin, Lobaplatin, and JM-216. (see McKeage et al. (1997) J. Clin. Oncol. 201:1232-1237 and in general, CHEMOTHERAPY FOR GYNECOLOGICAL NEOPLASM, CURRENT THERAPY AND NOVEL APPROACHES, in the Series Basic and Clinical Oncology, Angioli et al. Eds., 2004).

Non-limiting examples of antimetabolite agents include Folic acid based, i.e. dihydrofolate reductase inhibitors, such as Aminopterin, Methotrexate and Pemetrexed; thymidylate synthase inhibitors, such as Raltitrexed, Pemetrexed; Purine based, i.e. an adenosine deaminase inhibitor, such as Pentostatin, a thiopurine, such as Thioguanine and Mercaptopurine, a halogenated/ribonucleotide reductase inhibitor, such as Cladribine, Clofarabine, Fludarabine, or a guanine/guanosine: thiopurine, such as Thioguanine; or Pyrimidine based, i.e. cytosine/cytidine: hypomethylating agent, such as Azacitidine and Decitabine, a DNA polymerase inhibitor, such as Cytarabine, a ribonucleotide reductase inhibitor, such as Gemcitabine, or a thymine/thymidine: thymidylate synthase inhibitor, such as a Fluorouracil (5-FU). Equivalents to 5-FU include prodrugs, analogues and derivative thereof such as 5'-deoxy-5-fluorouridine (doxifluoroidine), 1-tetrahydrofuranyl-5-fluorouracil (ftorafur), Capecitabine (Xeloda), S-I (MBMS-247616, consisting of tegafur and two modulators, a 5-chloro-2,4-dihydroxypyridine and potassium oxonate), ralititrexed (tomudex), no latrexed (Thymitaq, AG337), LY231514 and ZD9331, as described for example in Papamicheal (1999) The Oncologist 4:478-487.

Examples of vincalkaloids, include, but are not limited to Vinblastine, Vincristine, Vinflunine, Vindesine and Vinorelbine.

Examples of taxanes include, but are not limited to docetaxel, Larotaxel, Ortataxel, Paclitaxel and Tesetaxel. An example of an epothilone is iabepilone.

Examples of enzyme inhibitors include, but are not limited to farnesyltransferase inhibitors (Tipifamib); CDK inhibitor (Alvocidib, Seliciclib); proteasome inhibitor (Bortezomib); phosphodiesterase inhibitor (Anagrelide; rolipram); IMP dehydrogenase inhibitor (Tiazofurine); and lipoxygenase inhibitor (Masoprocol). Examples of receptor antagonists include, but are not limited to ERA (Atrasentan); retinoid X receptor (Bexarotene); and a sex steroid (Testolactone).

Examples of therapeutic antibodies include, but are not limited to anti-HER1/EGFR (Cetuximab, Panitumumab); Anti-HER2/neu (erbB2) receptor (Trastuzumab); Anti-EpCAM (Catumaxomab, Edrecolomab) Anti-VEGF-A (Bevacizumab); Anti-CD20 (Rituximab, Tositumomab, Ibritumomab); Anti-CD52 (Alemtuzumab); and Anti-CD33 (Gemtuzumab). U.S. Pat. Nos. 5,776,427 and 7,601,355.

Examples of tyrosine kinase inhibitors include, but are not limited to inhibitors to ErbB: HER1/EGFR (Erlotinib, Gefitinib, Lapatinib, Vandetanib, Sunitinib, Neratinib); HER2/neu (Lapatinib, Neratinib); RTK class III: C-kit (Axitinib, Sunitinib, Sorafenib), FLT3 (Lestaurtinib), PDGFR (Axitinib, Sunitinib, Sorafenib); and VEGFR (Vandetanib, Semaxanib, Cediranib, Axitinib,

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Sorafenib); bcr-abl (Imatinib, Nilotinib, Dasatinib); Src (Bosutinib) and Janus kinase 2 (Lestaurtinib).

Chemotherapeutic agents that can be attached to the present Affimer-containing polypeptides may also include amsacrine, Trabectedin, retinoids (Alitretinoin, Tretinoin), Arsenic trioxide, asparagine depleter Asparaginase/Pegaspargase), Celecoxib, Demecolcine, Elesclomol, Elsamitrucin, Etoglucid, Lonidamine, Lucanthone, Mitoguazone, Mitotane, Oblimersen, Temsirolimus, and Vorinostat.

Examples of specific therapeutic agents that can be linked, ligated, or associated with the Affimer-containing polypeptides of the invention are flomoxef; fortimicin(s); gentamicin(s); glucosulfone solasulfone; gramicidin S; gramicidin(s); grepafloxacin; guamecycline; hetacillin; isepamicin; josamycin; kanamycin(s); flomoxef; fortimicin(s); gentamicin(s); glucosulfone solasulfone; gramicidin S; gramicidin(s); grepafloxacin; guamecycline; hetacillin; isepamicin; josamycin; kanamycin(s); bacitracin; bambermycin(s); biapenem; brodimoprim; butirosin; capreomycin; carbenicillin; carbomycin; carumonam; cefadroxil; cefamandole; cefatrizine; cefbuperazone; cefclidin; cefdinir; cefditoren; cefepime; cefetamet; cefixime; cefinenoxime; cefininox; cladribine; apalcillin; apicycline; apramycin; arbekacin; aspoxicillin; azidamfenicol; aztreonam; cefodizime; cefonicid; cefoperazone; ceforamide; cefotaxime; cefotetan; cefotiam; cefozopran; cefpimizole; cefpiramide; cefpirome; cefprozil; cefroxadine; cefteram; ceftibuten; cefuzonam; cephalexin; cephaloglycin; cephalosporin C; cephradine; chloramphenicol; chlortetracycline; clinafloxacin; clindamycin; clomocycline; colistin; cyclacillin; dapsone; demeclocycline; diathymosulfone; dibekacin; dihydrostreptomycin; 6-mercaptopurine; thioguanine; capecitabine; docetaxel; etoposide; gemcitabine; topotecan; vinorelbine; vincristine; vinblastine; teniposide; melphalan; methotrexate; 2-p-sulfanilyanilinoethanol; 4,4′-sulfinyldianiline; 4-sulfanilamidosalicylic acid; butorphanol; nalbuphine. streptozocin; doxorubicin; daunorubicin; plicamycin; idarubicin; mitomycin C; pentostatin; mitoxantrone; cytarabine; fludarabine phosphate; butorphanol; nalbuphine. streptozocin; doxorubicin; daunorubicin; plicamycin; idarubicin; mitomycin C; pentostatin; mitoxantrone; cytarabine; fludarabine phosphate; acediasulfone; acetosulfone; amikacin; amphotericin B; ampicillin; atorvastatin; enalapril; ranitidine; ciprofloxacin; pravastatin; clarithromycin; cyclosporin; famotidine; leuprolide; acyclovir; paclitaxel; azithromycin; lamivudine; budesonide; albuterol; indinavir; metformin; alendronate; nizatidine; zidovudine; carboplatin; metoprolol; amoxicillin; diclofenac; lisinopril; ceftriaxone; captopril; salmeterol; xinafoate; imipenem; cilastatin; benazepril; cefaclor; ceftazidime; morphine; dopamine; bialamicol; fluvastatin; phenamidine; podophyllinic acid 2-ethylhydrazine; acriflavine; chloroazodin; arsphenamine; amicarbilide; aminoquinuride; quinapril; oxymorphone; buprenorphine; floxuridine; dirithromycin; doxycycline; enoxacin; enviomycin; epicillin; erythromycin; leucomycin(s); lincomycin; lomefloxacin; lucensomycin; lymecycline; meclocycline; meropenem; methacycline; micronomicin; midecamycin(s); minocycline; moxalactam; mupirocin; nadifloxacin; natamycin; neomycin; netilmicin; norfloxacin; oleandomycin; oxytetracycline; p-sulfanilylbenzylamine; panipenem; paromomycin; pazufloxacin; penicillin N; pipacycline; pipemidic acid; polymyxin; primycin; quinacillin; ribostamycin; rifamide; rifampin; rifamycin SV; rifapentine; rifaximin; ristocetin; ritipenem; rokitamycin; rolitetracycline; rosaramycin; roxithromycin; salazosulfadimidine; sancycline; sisomicin; sparfloxacin; spectinomycin; spiramycin;

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streptomycin; succisulfone; sulfachrysoidine; sulfaloxic acid; sulfamidochrysoidine; sulfanilic acid; sulfoxone; teicoplanin; temafloxacin; temocillin; tetroxoprim; thiamphenicol; thiazolsulfone; thiostrepton; ticarcillin; tigemonam; tobramycin; tosufloxacin; trimethoprim; trospectomycin; trovafloxacin; tuberactinomycin; vancomycin; azaserine; candicidin(s); chlorphenesin; dermostatin(s); filipin; fungichromin; mepartricin; nystatin; oligomycin(s); perimycin A; tubercidin; 6-azauridine; 6-diazo-5-oxo-L-norleucine; aclacinomycin(s); ancitabine; anthramycin; azacitadine; azaserine; bleomycin(s); ethyl biscoumacetate; ethylidene dicoumarol; iloprost; lamifiban; taprostene; tioclomarol; tirofiban; amiprilose; bucillamine; gusperimus; gentisic acid; glucamethacin; glycol salicylate; meclofenamic acid; mefenamic acid; mesalamine; niflumic acid; olsalazine; oxaceprol; S-enosylmethionine; salicylic acid; salsalate; sulfasalazine; tolfenamic acid; carubicin; carzinophillin A; chlorozotocin; chromomycin(s); denopterin; doxifluridine; edatrexate; eflornithine; elliptinium; enocitabine; epirubicin; mannomustine; menogaril; mitobronitol; mitolactol; mopidamol; mycophenolic acid; nogalamycin; olivomycin(s); peplomycin; pirarubicin; piritrexim; prednimustine; procarbazine; pteropterin; puromycin; ranimustine; streptonigrin; thiamiprine; mycophenolic acid; procodazole; romurtide; sirolimus (rapamycin); tacrolimus; butethamine; fenalcomine; hydroxytetracaine; naepaine; orthocaine; piridocaine; salicyl alcohol; 3-amino-4-hydroxybutyric acid; aceclofenac; alminoprofen; amfenac; bromfenac; bromosaligenin; bumadizon; carprofen; diclofenac; diflunisal; ditazol; enfenamic acid; etodolac; etofenamate; fendosal; fepradinol; flufenamic acid; Tomudex (N-[[5-[[(1,4-Dihydro-2-methyl-4-oxo-6-quinazolinyl)methyl]methylamino]-2-thienyl]carbonyl]-L-glutamic acid), trimetrexate, tubercidin, ubenimex, vindesine, zorubicin; argatroban; coumetarol or dicoumarol.

In certain embodiments, the Affimer polypeptide includes a conjugated cytotoxic factor such as diptheria toxin, Pseudomonas aeruginosa exotoxin A chain, ricin A chain, abrin A chain, modeccin A chain, alpha-sarcin, Aleurites fordii proteins and compounds (e.g., fatty acids), dianthin proteins, Phytoiacca americana proteins PAPI, PAPII, and PAP-S, momordica charantia inhibitor, curcin, crotin, saponaria officinalis inhibitor, mitogellin, restrictocin, phenomycin, and enomycin.

Any method known in the art for conjugating to antibodies and other proteins may be employed in generating the conjugates of the present invention, including those methods described by Hunter, et al., (1962) Nature 144:945; David, et al., (1974) Biochemistry 13:1014; Pain, et al., (1981) J. Immunol. Meth. 40:219; and Nygren, J., (1982) Histochem. and Cytochem. 30:407. Methods for conjugating peptide, polypeptide and organic and inorganic moieties to antibodies and other proteins are conventional and very well known in the art and readily adapted for generating those versions of the subject Affimer polypeptides.

Where the conjugated moiety is a peptide or polypeptide, that moiety can be chemically cross-linked to the Affimer-containing polypeptide, or can be included as part of a fusion protein with the Affimer-containing polypeptide. And illustrative example would be a diphtheria toxin-Affimer fusion protein. In the case of non-peptide entities, the addition to the Affimer-containing polypeptide will generally be by way of chemical conjugation to the Affimer-containing polypeptide – such as through a functional group on an amino acid side chain or the carboxyl group at the C-terminal or amino group at the N-terminal end of the polypeptide. In

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certain embodiment, whether as a fusion protein or chemically cross-linked moiety, the conjugated moiety will include one or more sites that can be cleaved by an enzyme or are otherwise sensitive to an environmental condition (such as pH) that permits the conjugated moiety to be released from the Affimer-containing polypeptide, such as in the tumour or other diseased tissue (or tissue to be protected if the conjugated moiety functions to protect healthy tissue).

III. Expression Methods and Systems

Recombinant Affimer-containing proteins described herein can be produced by any suitable method known in the art. Such methods range from direct protein synthesis methods to constructing a DNA sequence encoding polypeptide sequences and expressing those sequences in a suitable host. For those recombinant Affimer polypeptides including further modifications, such as a chemical modifications or conjugation, the recombinant Affimer polypeptide can be further manipulated chemically or enzymatically after isolation form the host cell or chemical synthesis.

The present invention includes recombinant methods and nucleic acids for recombinantly expressing the recombinant Affimer polypeptides of the present invention comprising (i) introducing into a host cell a polynucleotide encoding the amino acid sequence of said Affimer polypeptide, for example, wherein the polynucleotide is in a vector and/or is operably linked to a promoter; (ii) culturing the host cell (e.g., eukaryotic or prokaryotic) under condition favorable to expression of the polynucleotide and, (iii) optionally, isolating the Affimer polypeptide from the host cell and/or medium in which the host cell is grown. See e.g., WO 04/041862, WO 2006/122786, WO 2008/020079, WO 2008/142164 or WO 2009/068627.

In some embodiments, a DNA sequence encoding a recombinant Affimer polypeptide of interest may be constructed by chemical synthesis using an oligonucleotide synthesizer. Oligonucleotides can be designed based on the amino acid sequence of the desired polypeptide and selecting those codons that are favored in the host cell in which the recombinant polypeptide of interest will be produced. Standard methods can be applied to synthesize a polynucleotide sequence encoding an isolated polypeptide of interest. For example, a complete amino acid sequence can be used to construct a back-translated gene. Further, a DNA oligomer containing a nucleotide sequence coding for the particular isolated polypeptide can be synthesized. For example, several small oligonucleotides coding for portions of the desired polypeptide can be synthesized and then ligated. The individual oligonucleotides typically contain 5' or 3' overhangs for complementary assembly.

Once a nucleic acid sequence encoding a recombinant Affimer polypeptide of the invention has been obtained, the vector for the production of the recombinant Affimer polypeptide may be produced by recombinant DNA technology using techniques well known in the art. Methods which are well known to those skilled in the art can be used to construct expression vectors containing the recombinant Affimer polypeptide coding sequences and appropriate transcriptional and translational control signals. These methods include, for example, in vitro recombinant DNA techniques, synthetic techniques, and in vivo genetic recombination. (See,

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for example, the techniques described in Sambrook et al, 1990, MOLECULAR CLONING, A LABORATORY MANUAL, 2d Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. and Ausubel et al. eds., 1998, CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, John Wiley & Sons, NY).

An expression vector comprising the nucleotide sequence of a recombinant Affimer polypeptide can be transferred to a host cell by conventional techniques (e.g., electroporation, liposomal transfection, and calcium phosphate precipitation) and the transfected cells are then cultured by conventional techniques to produce the recombinant Affimer polypeptide of the invention. In specific embodiments, the expression of the recombinant Affimer polypeptide is regulated by a constitutive, an inducible or a tissue, specific promoter.

The expression vector may include an origin of replication, such as may be selected based upon the type of host cell being used for expression. By way of example, the origin of replication from the plasmid pBR322 (Product No. 303-3s, New England Biolabs, Beverly, Mass.) is useful for most Gram- negative bacteria while various origins from SV40, polyoma, adenovirus, vesicular stomatitus virus (VSV) or papillomaviruses (such as HPV or BPV) are useful for cloning vectors in mammalian cells. Generally, the origin of replication component is not needed for mammalian expression vectors (for example, the SV40 origin is often used because it contains the early promoter).

The vector may include one or more selectable marker genes, e.g., genetic elements that encode a protein necessary for the survival and growth of a host cell grown in a selective culture medium. Typical selection marker genes encode proteins that (a) confer resistance to antibiotics or other toxins, e.g., ampicillin, tetracycline, or kanamycin for prokaryotic host cells, (b) complement auxotrophic deficiencies of the cell; or (c) supply critical nutrients not available from complex media. Preferred selectable markers are the kanamycin resistance gene, the ampicillin resistance gene, and the tetracycline resistance gene. A neomycin resistance gene may also be used for selection in prokaryotic and eukaryotic host cells. Other selection genes may be used to amplify the gene which will be expressed. Amplification is a process where genes which are in greater demand for the production of a protein critical for growth are reiterated in tandem within the chromosomes of successive generations of recombinant cells. Examples of selectable markers for mammalian cells include dihydrofolate reductase (DHFR) and thymidine kinase. The mammalian cell transformants are placed under selection pressure which only the transformants are uniquely adapted to survive by virtue of the marker present in the vector. Selection pressure is imposed by culturing the transformed cells under conditions in which the concentration of selection agent in the medium is successively changed, thereby leading to amplification of both the selection gene and the DNA that encodes the recombinant Affimer polypeptide. As a result, increased quantities of the recombinant Affimer polypeptide are synthesized from the amplified DNA.

The vector may also include one or more ribosome binding site, which will be transcribed into the mRNA including the coding sequence for the recombinant Affimer polypeptide. For example, such a site is characterized by a Shine-Dalgarno sequence (prokaryotes) or a Kozak sequence (eukaryotes). The element is typically located 3' to the promoter and 5' to the coding sequence of the polypeptide to be expressed. The Shine-Dalgarno sequence is varied but is

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typically a polypurine (having a high A-G content). Many Shine-Dalgarno sequences have been identified, each of which can be readily synthesized using methods set forth above and used in a prokaryotic vector.

The expression vectors will typically contain a promoter that is recognized by the host organism and operably linked to a nucleic acid molecule encoding the recombinant Affimer polypeptide. Either a native or heterologous promoter may be used depending the host cell used for expression and the yield desired.

Promoters for use with prokaryotic hosts include the beta-lactamase and lactose promoter systems; alkaline phosphatase, a tryptophan (trp) promoter system; and hybrid promoters such as the tac promoter. Other known bacterial promoters are also suitable. Their sequences have been published, and they can be ligated to a desired nucleic acid sequence(s), using linkers or adapters as desired to supply restriction sites.

Promoters for use with yeast hosts are also known in the art. Yeast enhancers are advantageously used with yeast promoters. Suitable promoters for use with mammalian host cells are well known and include those obtained from the genomes of viruses such as polyoma virus, fowlpox virus, adenovirus (such as Adenovirus 2), bovine papilloma virus, avian sarcoma virus, cytomegalovirus, a retrovirus, hepatitis-B virus and most preferably Simian Virus 40 (SV40). Other suitable mammalian promoters include heterologous mammalian promoters, e.g., heat-shock promoters and the actin promoter.

Additional promoters which may be used for expressing the selective binding agents of the invention include, but are not limited to: the SV40 early promoter region (Bernoist and Chambon, Nature, 290:304-310, 1981); the CMV promoter; the promoter contained in the 3' long terminal repeat of Rous sarcoma virus (Yamamoto et al. (1980), Cell 22: 787-97); the herpes thymidine kinase promoter (Wagner et al. (1981), Proc. Natl. Acad. Sci. U.S.A. 78: 1444-5); the regulatory sequences of the metallothionine gene (Brinster et al, Nature, 296; 39-42, 1982); prokaryotic expression vectors such as the beta- lactamase promoter (Villa-Kamaroff, et al., Proc. Natl. Acad. Sci. U.S.A., 75; 3727-3731, 1978); or the tac promoter (DeBoer, et al. (1983), Proc. Natl. Acad. Sci. U.S.A., 80: 21-5). Also of interest are the following animal transcriptional control regions, which exhibit tissue specificity and have been utilized in transgenic animals: the elastase I gene control region which is active in pancreatic acinar cells (Swift et al. (1984), Cell 38: 639-46; Ornitz et al. (1986), Cold Spring Harbor Symp. Quant. Biol. 50: 399-409; MacDonald (1987), Hepatology 7: 425-515); the insulin gene control region which is active in pancreatic beta cells (Hanahan (1985), Nature 315: 115-22); the immunoglobulin gene control region which is active in lymphoid cells (Grosschedl et al. (1984), Cell 38; 647-58; Adames et al. (1985), Nature 318; 533-8; Alexander et al. (1987), Mol. Cell. Biol. 7: 1436-44); the mouse mammary tumor virus control region which is active in testicular, breast, lymphoid and mast cells (Leder et al. (1986), Cell 45: 485-95), albumin gene control region which is active in liver (Pinkert et al. (1987), Genes and Devel. 1: 268-76); the alphafetoprotein gene control region which is active in liver (Krumlauf et al. (1985), Mol. Cell. Biol. 5: 1639-48; Hammer et al. (1987), Science, 235: 53-8); the alpha 1- antitrypsin gene control region which is active in the liver (Kelsey et al. (1987), Genes and Devel. 1: 161-71); the beta-globin gene control region which is active in myeloid cells (Mogram et al., Nature, 315 338-340, 1985; Kollias et al. (1986),

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Cell 46: 89-94); the myelin basic protein gene control region which is active in oligodendrocyte cells in the brain (Readhead et al. (1987), Cell, 48: 703-12); the myosin light chain-2 gene control region which is active in skeletal muscle (Sani (1985), Nature, 314: 283-6); and the gonadotropic releasing hormone gene control region which is active in the hypothalamus (Mason et al. (1986), Science 234: 1372-8).

An enhancer sequence may be inserted into the vector to increase transcription in eukaryotic host cells. Several enhancer sequences available from mammalian genes are known (e.g., globin, elastase, albumin, alpha-feto-protein and insulin). Typically, however, an enhancer from a virus will be used. The SV40 enhancer, the cytomegalovirus early promoter enhancer, the polyoma enhancer, and adenovirus enhancers are exemplary enhancing elements for the activation of eukaryotic promoters.

While an enhancer may be spliced into the vector at a position 5' or 3' to the polypeptide coding region, it is typically located at a site 5' from the promoter.

Vectors for expressing nucleic acids include those which are compatible with bacterial, insect, and mammalian host cells. Such vectors include, inter alia, pCRII, pCR3, and pcDNA3.1 (Invitrogen Company, San Diego, Calif.), pBSII (Stratagene Company, La Jolla, Calif.), pET15 (Novagen, Madison, Wis.), pGEX (Pharmacia Biotech, Piscataway, N.J.), pEGFP-N2 (Clontech, Palo Alto, Calif.), pETL (BlueBacII; Invitrogen), pDSR- alpha (PCT Publication No. WO90/14363) and pFastBacDual (Gibco/BRL, Grand Island, N.Y.).

Additional possible vectors include, but are not limited to, cosmids, plasmids or modified viruses, but the vector system must be compatible with the selected host cell. Such vectors include, but are not limited to plasmids such as Bluescript® plasmid derivatives (a high copy number ColEl-based phagemid, Stratagene Cloning Systems Inc., La Jolla Calif.), PCR cloning plasmids designed for cloning Taq-amplified PCR products (e.g., TOPO™. TA Cloning® Kit, PCR2.1 plasmid derivatives, Invitrogen, Carlsbad, Calif.), and mammalian, yeast or virus vectors such as a baculovirus expression system (pBacPAK plasmid derivatives, Clontech, Palo Alto, Calif.). The recombinant molecules can be introduced into host cells via transformation, transfection, infection, electroporation, or other known techniques

Eukaryotic and prokaryotic host cells, including mammalian cells as hosts for expression of the recombinant Affimer polypeptide disclosed herein are well known in the art and include many immortalized cell lines available from the American Type Culture Collection (ATCC). These include, inter alia, Chinese hamster ovary (CHO) cells, NSO, SP2 cells, HeLa cells, baby hamster kidney (BHK) cells, monkey kidney cells (COS), human hepatocellular carcinoma cells (e.g., Hep G2), A549 cells, 3T3 cells, HEK-293 cells and a number of other cell lines. Mammalian host cells include human, mouse, rat, dog, monkey, pig, goat, bovine, horse and hamster cells. Cell lines of particular preference are selected through determining which cell lines have high expression levels. Other cell lines that may be used are insect cell lines, such as Sf9 cells, amphibian cells, bacterial cells, plant cells and fungal cells. Fungal cells include yeast and filamentous fungus cells including, for example, Pichia pastoris, Pichia finlandica, Pichia trehalophila, Pichia koclamae, Pichia membranaefaciens, Pichia minuta (Ogataea minuta, Pichia lindneri), Pichia opuntiae, Pichia thermotolerans, Pichia salictaria, Pichia guercuum, Pichia pijperi, Pichia stiptis,

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Pichia methanolica, Pichia sp., Saccharomyces cerevisiae, Saccharomyces sp., Hansenula polymorpha, Kluyveromyces sp., Kluyveromyces lactis, Candida albicans, Aspergillus nidulans, Aspergillus niger, Aspergillus oryzae, Trichoderma reesei, Chrysosporium lucknowense, Fusarium sp., Fusarium gramineum, Fusarium venenatum, Physcomitrella patens and Neurospora crassa. Pichia sp., any Saccharomyces sp., Hansenula polymorpha, any Kluyveromyces sp., Candida albicans, any Aspergillus sp., Trichoderma reesei, Chrysosporium lucknowense, any Fusarium sp., Yarrowia lipolytica, and Neurospora crassa.

A variety of host-expression vector systems may be utilized to express the recombinant Affimer polypeptide of the invention. Such host-expression systems represent vehicles by which the coding sequences of the recombinant Affimer polypeptide may be produced and subsequently purified, but also represent cells which may, when transformed or transfected with the appropriate nucleotide coding sequences, express the recombinant Affimer polypeptide of the invention in situ. These include, but are not limited to, microorganisms such as bacteria (e.g., E. coli and B. subtilis) transformed with recombinant bacteriophage DNA, plasmid DNA or cosmid DNA expression vectors containing Affimer polypeptide coding sequences; yeast (e.g., Saccharomyces pichia) transformed with recombinant yeast expression vectors containing Affimer polypeptide coding sequences; insect cell systems infected with recombinant virus expression vectors (e.g., baculovirus) containing the Affimer polypeptide coding sequences; plant cell systems infected with recombinant virus expression vectors (e.g., cauliflower mosaic virus (CμMV) and tobacco mosaic virus (TMV)) or transformed with recombinant plasmid expression vectors (e.g., Ti plasmid) containing Affimer polypeptide coding sequences; or mammalian cell systems (e.g., COS, CHO, BHK, 293, 293T, 3T3 cells, lymphotic cells (see U.S. Pat. No. 5,807,715), Per C.6 cells (rat retinal cells developed by Crucell)) harboring recombinant expression constructs containing promoters derived from the genome of mammalian cells (e.g., metallothionein promoter) or from mammalian viruses (e.g., the adenovirus late promoter; the vaccinia virus 7.5K promoter).

In bacterial systems, a number of expression vectors may be advantageously selected depending upon the use intended for the recombinant Affimer polypeptide being expressed. For example, when a large quantity of such a protein is to be produced, for the generation of pharmaceutical compositions of the recombinant Affimer polypeptide, vectors which direct the expression of high levels of fusion protein products that are readily purified may be desirable. Such vectors include, but are not limited, to the E. coli expression vector pUR278 (Ruther et al. (1983) "Easy Identification Of cDNA Clones," EMBO J. 2:1791-1794), in which the Affimer polypeptide coding sequence may be ligated individually into the vector in frame with the lac Z coding region so that a fusion protein is produced; pIN vectors (Inouye et al. (1985) "Up-Promoter Mutations In The Lpp Gene Of Escherichia coli," Nucleic Acids Res. 13:3101-3110; Van Heeke et al. (1989) "Expression Of Human Asparagine Synthetase In Escherichia coli," J. Biol. Chem. 24:5503-5509); and the like. pGEX vectors may also be used to express foreign polypeptides as fusion proteins with glutathione S-transferase (GST). In general, such fusion proteins are soluble and can easily be purified from lysed cells by adsorption and binding to a matrix glutathione-agarose beads followed by elution in the presence of free glutathione. The pGEX vectors are designed to include thrombin or factor Xa protease cleavage sites so that the cloned target gene product can be released from the GST moiety.

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In an insect system, Autographa californica nuclear polyhedrosis virus (AcNPV) is used as a vector to express foreign genes. The virus grows in Spodoptera frugiperda cells. The Affimer polypeptide coding sequence may be cloned individually into non-essential regions (e.g., the polyhedrin gene) of the virus and placed under control of an AcNPV promoter (e.g., the polyhedrin promoter).

In mammalian host cells, a number of viral-based expression systems may be utilized. In cases where an adenovirus is used as an expression vector, the Affimer polypeptide coding sequence of interest may be ligated to an adenovirus transcription/translation control complex, e.g., the late promoter and tripartite leader sequence. This chimeric gene may then be inserted in the adenovirus genome by in vitro or in vivo recombination. Insertion in a non-essential region of the viral genome (e.g., region E1 or E3) will result in a recombinant virus that is viable and capable of expressing the immunoglobulin molecule in infected hosts. (see e.g., see Logan et al. (1984) "Adenovirus Tripartite Leader Sequence Enhances Translation Of mRNAs Late After Infection," Proc. Natl. Acad. Sci. (U.S.A.) 81:3655-3659). Specific initiation signals may also be required for efficient translation of inserted Affimer polypeptide coding sequences. These signals include the ATG initiation codon and adjacent sequences. Furthermore, the initiation codon must be in phase with the reading frame of the desired coding sequence to ensure translation of the entire insert. These exogenous translational control signals and initiation codons can be of a variety of origins, both natural and synthetic. The efficiency of expression may be enhanced by the inclusion of appropriate transcription enhancer elements, transcription terminators, etc. (see Bitter et al. (1987) "Expression And Secretion Vectors For Yeast," Methods in Enzymol. 153:516-544).

In addition, a host cell strain may be chosen which modulates the expression of the inserted sequences, or modifies and processes the gene product in the specific fashion desired. Such modifications (e.g., glycosylation) and processing (e.g., cleavage) of protein products may be important for the function of the protein. Different host cells have characteristic and specific mechanisms for the post-translational processing and modification of proteins and gene products. Appropriate cell lines or host systems can be chosen to ensure the correct modification and processing of the foreign protein expressed. To this end, eukaryotic host cells which possess the cellular machinery for proper processing of the primary transcript, glycosylation, and phosphorylation of the gene product may be used. Such mammalian host cells include but are not limited to CHO, VERY, BHK, Hela, COS, MDCK, 293, 293T, 3T3, WI38, BT483, Hs578T, HTB2, BT20 and T47D, CRL7030 and Hs578Bst.

For long-term, high-yield production of recombinant proteins, stable expression is preferred. For example, cell lines which stably express an antibody of the invention may be engineered. Rather than using expression vectors which contain viral origins of replication, host cells can be transformed with DNA controlled by appropriate expression control elements (e.g., promoter, enhancer, sequences, transcription terminators, polyadenylation sites, etc.), and a selectable marker. Following the introduction of the foreign DNA, engineered cells may be allowed to grow for 1-2 days in an enriched media, and then are switched to a selective media. The selectable marker in the recombinant plasmid confers resistance to the selection and allows cells to stably integrate the plasmid into their chromosomes and grow to form foci which in

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turn can be cloned and expanded into cell lines. This method may advantageously be used to engineer cell lines which express the recombinant Affimer polypeptides of the invention. Such engineered cell lines may be particularly useful in screening and evaluation of compounds that interact directly or indirectly with the recombinant Affimer polypeptides.

A number of selection systems may be used, including but not limited to the herpes simplex virus thymidine kinase (Wigler et al. (1977) "Transfer Of Purified Herpes Virus Thymidine Kinase Gene To Cultured Mouse Cells," Cell 11:223-232), hypoxanthine-guanine phosphoribosyltransferase (Szybalska et al. (1962) "Genetics Of Human Cess Line. IV. DNA-Mediated Heritable Transformation Of A Biochemical Trait," Proc. Natl. Acad. Sci. (U.S.A.) 48:2026-2034), and adenine phosphoribosyltransferase (Lowy et al. (1980) "Isolation Of Transforming DNA: Cloning The Hamster Aprt Gene," Cell 22:817-823) genes can be employed in tk-, hgprt- or aprt- cells, respectively. Also, antimetabolite resistance can be used as the basis of selection for the following genes: dhfr, which confers resistance to methotrexate (Wigler et al. (1980) "Transformation Of Mammalian Cells With An Amplfiable Dominant-Acting Gene," Proc. Natl. Acad. Sci. (U.S.A.) 77:3567-3570; O'Hare et al. (1981) "Transformation Of Mouse Fibroblasts To Methotrexate Resistance By A Recombinant Plasmid Expressing A Prokaryotic Dihydrofolate Reductase," Proc. Natl. Acad. Sci. (U.S.A.) 78:1527-1531); gpt, which confers resistance to mycophenolic acid (Mulligan et al. (1981) "Selection For Animal Cells That Express The Escherichia coli Gene Coding For Xanthine-Guanine Phosphoribosyltransferase," Proc. Natl. Acad. Sci. (U.S.A.) 78:2072-2076); neo, which confers resistance to the aminoglycoside G-418 (Tachibana et al. (1991) "Altered Reactivity Of Immunoglobutin Produced By Human-Human Hybridoma Cells Transfected By pSV2-Neo Gene," Cytotechnology 6(3):219-226; Tolstoshev (1993) "Gene Therapy, Concepts, Current Trials And Future Directions," Ann. Rev. Pharmacol. Toxicol. 32:573-596; Mulligan (1993) "The Basic Science Of Gene Therapy," Science 260:926-932; and Morgan et al. (1993) "Human gene therapy," Ann. Rev. Biochem. 62:191-217). Methods commonly known in the art of recombinant DNA technology which can be used are described in Ausubel et al. (eds.), 1993, CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, John Wiley & Sons, NY; Kriegler, 1990, GENE TRANSFER AND EXPRESSION, A LABORATORY MANUAL, Stockton Press, NY; and in Chapters 12 and 13, Dracopoli et al. (eds), 1994, CURRENT PROTOCOLS IN HUMAN GENETICS, John Wiley & Sons, NY.; Colbere-Garapin et al. (1981) "A New Dominant Hybrid Selective Marker For Higher Eukaryotic Cells," J. Mol. Biol. 150:1-14; and hygro, which confers resistance to hygromycin (Santerre et al. (1984) "Expression Of Prokaryotic Genes For Hygromycin B And G418 Resistance As Dominant-Selection Markers In Mouse L Cells," Gene 30:147-156).

The expression levels of a recombinant Affimer polypeptide can be increased by vector amplification (for a review, see Bebbington and Hentschel, "The Use Of Vectors Based On Gene Amplification For The Expression Of Cloned Genes In Mammaian Cells," in DNA CLONING, Vol. 3. (Academic Press, New York, 1987)). When a marker in the vector system expressing a recombinant Affimer polypeptide is amplifiable, increase in the level of inhibitor present in culture of host cell will increase the number of copies of the marker gene. Since the amplified region is associated with the nucleotide sequence of the recombinant Affimer polypeptide, production of the recombinant Affimer polypeptide will also increase (Crouse et al. (1983)

"Expression And Amplification Of Engineered Mouse Dihydrofolate Reductase Minigenes," Mol. Cell. Biol. 3:257-266).

Where the Affimer polypeptide is an Affimer antibody fusion or other multiprotein complex, the host cell may be co-transfected with two expression vectors, for instance the first vector encoding a heavy chain and the second vector encoding a light chain derived polypeptide, one or both of which includes an Affimer polypeptide coding sequence. The two vectors may contain identical selectable markers which enable equal expression of heavy and light chain polypeptides. Alternatively, a single vector may be used which encodes both heavy and light chain polypeptides. In such situations, the light chain should be placed before the heavy chain to avoid an excess of toxic free heavy chain (Proudfoot (1986) "Expression And Amplification Of Engineered Mouse Dihydrofolate Reductase Minigenes," Nature 322:562-565; Kohler (1980) "Immunoglobulin Chain Loss In Hybridoma Lines," Proc. Natl. Acad. Sci. (U.S.A.) 77:2197-2199). The coding sequences for the heavy and light chains may comprise cDNA or genomic DNA.

In general, glycoproteins produced in a particular cell line or transgenic animal will have a glycosylation pattern that is characteristic for glycoproteins produced in the cell line or transgenic animal. Therefore, the particular glycosylation pattern of the recombinant Affimer polypeptide will depend on the particular cell line or transgenic animal used to produce the protein. In certain embodiments of Affimer/antibody fusions, a glycosylation pattern comprising only non-fucosylated N-glycans may be advantageous, because in the case of antibodies this has been shown to typically exhibit more potent efficacy than fucosylated counterparts both in vitro and in vivo (See for example, Shinkawa et al., J. Biol. Chem. 278: 3466-3473 (2003); U.S. Pat. Nos. 6,946,292 and 7,214,775).

Further, expression of an Affimer polypeptide from production cell lines can be enhanced using a number of known techniques. For example, the glutamine synthetase gene expression system (the GS system) is a common approach for enhancing expression under certain conditions. The GS system is discussed in whole or part in connection with European Patent Nos. 0216846, 0256055, and 0323997 and European Patent Application No. 89303964.4. Thus, in an embodiment of the invention, the mammalian host cells (e.g., CHO) lack a glutamine synthetase gene and are grown in the absence of glutamine in the medium wherein, however, the polynucleotide encoding the immunoglobulin chain comprises a glutamine synthetase gene which complements the lack of the gene in the host cell. Such host cells containing the binder or polynucleotide or vector as discussed herein as well as expression methods, as discussed herein, for making the binder using such a host cell are part of the present invention.

Expression of recombinant proteins in insect cell culture systems (e.g., baculovirus) also offers a robust method for producing correctly folded and biologically functional proteins. Baculovirus systems for production of heterologous proteins in insect cells are well-known to those of skill in the art.

The recombinant Affimer polypeptides produced by a transformed host can be purified according to any suitable method. Standard methods include chromatography (e.g., ion exchange, affinity, and sizing column chromatography), centrifugation, differential solubility, or by any other standard technique for protein purification. Affinity tags such as hexa-histidine,

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maltose binding domain, influenza coat sequence, and glutathione-S-transferase can be attached to the protein to allow easy purification by passage over an appropriate affinity column. Isolated proteins can also be physically characterized using such techniques as proteolysis, mass spectrometry (MS), nuclear magnetic resonance (NMR), high performance liquid chromatography (HPLC), and x-ray crystallography.

In some embodiments, recombinant Affimer polypeptides produced in bacterial culture can be isolated, for example, by initial extraction from cell pellets, followed by one or more concentration, salting-out, aqueous ion exchange, or size exclusion chromatography steps. HPLC can be employed for final purification steps. Microbial cells employed in expression of a recombinant protein can be disrupted by any convenient method, including freeze-thaw cycling, sonication, mechanical disruption, or use of cell lysing agents.

IV. Encoded Affimers for In Vivo Delivery

An alternative approach to the delivery of therapeutic Affimer polypeptides would be to leave the production of the therapeutic polypeptide to the body itself. A multitude of clinical studies have illustrated the utility of in vivo gene transfer into cells using a variety of different delivery systems. In vivo gene transfer seeks to administer to patients the "Encoded Affimer" nucleotide sequence, rather than the Affimer polypeptide. This allows the patient's body to produce the therapeutic Affimer polypeptide of interest for a prolonged period of time, and secrete it either systemically or locally, depending on the production site. Gene-based Encoded Affimers can present a labor- and cost-effective alternative to the conventional production, purification and administration of the polypeptide version of the Affimer polypeptide. A number of antibody expression platforms have been pursued in vivo to which delivery of Encoded Affimers can be adapted: these include viral vectors, naked DNA and RNA. Encoded Affimer gene transfer can not only enable cost-savings by reducing the cost of goods and of production, but may also be able to reduce the frequency of drug administration. Overall, a prolonged in vivo production of the therapeutic Affimer polypeptide by expression of the Encoded Affimer can contribute to (i) a broader therapeutic or prophylactic application of Affimer polypeptides in price-sensitive conditions, (ii) an improved accessibility to therapy in both developed and developing countries, and (iii) more effective and affordable treatment modalities. In addition to in vivo gene transfer, cells can be harvested from the host (or a donor), engineered with Encoded Affimer sequences to produce Affimer polypeptides and re-administered to patients.

Intramuscular antibody gene administration has been most widely evaluated (reviewed in Deal et al. (2015) "Engineering humoral immunity as prophylaxis or therapy" Curr Opin Immunol. 35:113–22.), and also carries the highest clinical translatability and application when applied to Encoded Affimers. Indeed, the inherent anatomical, cellular and physiological properties of skeletal muscle make it a stable environment for long-term Encoded Affimer expression and systemic circulation. Skeletal muscle is easily accessible, allowing multiple or repeated administrations. The abundant blood vascular supply provides an efficient transport system for secreted therapeutic Affimer polypeptides into the circulation. The syncytial nature of muscle

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fibers allows dispersal of nucleotides from a limited site of penetration to a large number of neighboring nuclei within the fiber. Skeletal muscle fibers are also terminally differentiated cells, and nuclei within the fibers are post-mitotic. Consequently, integration in the host genome is not a prerequisite to attain prolonged mAb expression. The liver is another site often used for pre-clinical antibody gene transfer, and is typically transfected via i.v. injection, and can also be a site of gene transfer for Encoded Affimers either for local delivery of Affimer polypeptides (such as in the treatment of liver cancer and/or metaplasias) or for the generation of Affimer polypeptides that are secreted into the vascular for systemic circulation. This organ has various physiological functions, including the synthesis of plasma proteins. This organ can be particularly well suited for in vivo Encoded Affimer expression.

The tumor presents another site for Encoded Affimer transfer, targeted either via i.v. or direct injection/electroporation. Indeed, intratumoral Encoded Affimer expression can allow for a local production of the therapeutic Affimer polypeptides, waiving the need for high systemic Affimer polypeptide levels that might otherwise be required to penetrate and impact solid tumors. A similar rationale applies for the brain, which is frequently targeted in the context of antibody gene transfer to avoid the difficulties with blood–brain barrier trafficking and would likewise be a target for delivery of Encoded Affimers. See, for example, Beckman et al. (2015) "Antibody constructs in cancer therapy: protein engineering strategies to improve exposure in solid tumors" Cancer 109(2):170–9; Dronca et al. (2015) "Immunomodulatory antibody therapy of cancer: the closer, the better" Clin Cancer Res. 21(5):944–6; and Neves et al. (2016) "Antibody approaches to treat brain diseases" Trends Biotechnol. 34(1):36–48.

The success of gene therapy has largely been driven by improvements in nonviral and viral gene transfer vectors. An array of physical and chemical nonviral methods have been used to transfer DNA and mRNA to mammalian cells and a substantial number of these have been developed as clinical stage technologies for gene therapy, both ex vivo and in vivo, and are readily adapted for delivery of the Encoded Affimers of the present invention. To illustrate, cationic liposome technology can be employed, which is based on the ability of amphipathic lipids, possessing a positively charged head group and a hydrophobic lipid tail, to bind to negatively charged DNA or RNA and form particles that generally enter cells by endocytosis. Some cationic liposomes also contain a neutral co-lipid, thought to enhance liposome uptake by mammalian cells. See, for example, Felgner et al. (1987) Lipofection: a highly efficient, lipid-mediated DNA-transfection procedure. MNAS 84:7413–7417; San et al. (1983) "Safety and short term toxicity of a novel cationic lipid formulation for human gene therapy" Hum. Gene Ther. 4:781–788; Xu et al. (1996) "Mechanism of DNA release from cationic liposome/DNA complexes used in cell transfection" Biochemistry 35,:5616–5623; and Legendre et al. (1992) "Delivery of plasmid DNA into mammalian cell lines using pH-sensitive liposomes: comparison with cationic liposomes" Pharm. Res. 9, 1235-1242.

Similarly, other polycations, such as poly-l-lysine and polyethylene-imine, can be used to deliver Encoded Affimers. These polycations complex with nucleic acids via charge interaction and aid in the condensation of DNA or RNA into nanoparticles, which are then substrates for endosome-mediated uptake. Several of these cationic nucleic acid complex technologies have been developed as potential clinical products, including complexes with plasmid DNA,

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oligodeoxynucleotides, and various forms of synthetic RNA. Modified (and unmodified or "naked") DNA and RNA have also been shown to mediate successful gene transfer in a number of circumstances and can also be used as systems for delivery of Encoded Affimers. These include the use of plasmid DNA by direct intramuscular injection, the use of intratumoral injection of plasmid DNA. See, for example, Rodrigo et al. (2012) "De novo automated design of small RNA circuits for engineering synthetic riboregulation in living cells" PNAS 109:15271–15276; Oishi et al. (2005) "Smart polyion complex micelles for targeted intracellular delivery of PEGylated antisense oligonucleotides containing acid-labile linkages" Chembiochem. 6:718–725; Bhatt et al. (2015) "Microbeads mediated oral plasmid DNA delivery using polymethacrylate vectors: an effectual groundwork for colorectal cancer" Drug Deliv. 22:849–861; Ulmer et al. (1994) Protective immunity by intramuscular injection of low doses of influenza virus DNA vaccines" Vaccine 12: 1541–1544; and Heinzerling et al. (2005) "Intratumoral injection of DNA encoding human interleukin 12 into patients with metastatic melanoma: clinical efficacy" Hum. Gene Ther. 16:35–48.

Viral vectors are currently used as a delivery vehicle in the vast majority of pre-clinical and clinical gene therapy trials and in the first to be approved directed gene therapy. See Gene Therapy Clinical Trials Worldwide 2017 (http://www.abedia.com/wiley/). The main driver thereto is their exceptional gene delivery efficiency, which reflects a natural evolutionary development; viral vector systems are attractive for gene delivery, because viruses have evolved the ability to cross through cellular membranes by infection, thereby delivering nucleic acids such as Encoded Affimers to target cells. Pioneered by adenoviral systems, the field of viral vector-mediated antibody gene transfer made significant strides in the past decades. The myriad of successfully evaluated administration routes, pre-clinical models and disease indications puts the capabilities of antibody gene transfer at full display through which the skilled artisan would readily be able to identify and adapt antibody gene transfer systems and techniques for in vivo delivery of Encoded Affimer constructs. Muscle has emerged as the administration site of choice for prolonged mAb expression and would similarly be a suitable target tissue for prolonged Affimer polypeptide expression. In the context of vectored intratumoral Encoded Affimer gene transfer, oncolytic viruses have a distinct advantage, as they can specifically target tumor cells, boost Affimer polypeptide expression, and amplify therapeutic responses – such as to a checkpoint inhibitory or a costimulatory agonist Affimer polypeptide.

In vivo gene transfer of Encoded Affimers can also be accomplished by use of nonviral vectors, such as expression plasmids. Nonviral vectors are easily produced and do not seem to induce specific immune responses. Muscle tissue is most often used as target tissue for transfection, because muscle tissue is well vascularized and easily accessible, and myocytes are long-lived cells. Intramuscular injection of naked plasmid DNA results in transfection of a certain percentage of myocytes. Using this approach, plasmid DNA encoding cytokines and cytokine/IgG1 chimeric proteins has been introduced in vivo and has positively influenced (autoimmune) disease outcome.

In some instances, in order to increase transfection efficiency via so-called intravascular delivery in which increased gene delivery and expression levels are achieved by inducing a

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short-lived transient high pressure in the veins. Special blood-pressure cuffs that may facilitate localized uptake by temporarily increasing vascular pressure and can be adapted for use in human patients for this type of gene delivery. See, for example, Zhang et al. (2001) "Efficient expression of naked DNA delivered intraarterially to limb muscles of nonhuman primates" Hum. Gene Ther., 12:427-438

Increased efficiency can also be gained through other techniques, such as in which delivery of the nucleic acid is improved by use of chemical carriers—cationic polymers or lipids—or via a physical approach—gene gun delivery or electroporation. See Tranchant et al. (2004) "Physicochemical optimisation of plasmid delivery by cationic lipids" J. Gene Med., 6 (Suppl. 1):S24-S35; and Niidome et al. (2002) "Gene therapy progress and prospects: nonviral vectors" Gene Ther., 9:1647-1652. Electroporation is especially regarded as an interesting technique for nonviral gene delivery. Somiari, et al. (2000) "Theory and in vivo application of electroporative gene delivery" Mol. Ther. 2:178-187; and Jaroszeski et al. (1999) "In vivo gene delivery by electroporation" Adv. Drug Delivery Rev., 35:131-137. With electroporation, pulsed electrical currents are applied to a local tissue area to enhance cell permeability, resulting in gene transfer across the membrane. Research has shown that in vivo gene delivery can be at least 10–100 times more efficient with electroporation than without. See, for example, Aihara et al. (1998) "Gene transfer into muscle by electroporation in vivo" Nat. Biotechnol. 16:867-870; Mir, et al. (1999) "High-efficiency gene transfer into skeletal muscle mediated by electric pulses" PNAS 96:4262-4267; Rizzuto, et al. (1999) "Efficient and regulated erythropoietin production by naked DNA injection and muscle electroporation" PNAS 96: 6417-6422; and Mathiesen (1999) "Electropermeabilization of skeletal muscle enhances gene transfer in vivo" Gene Ther., 6:508-514.

Encoded Affimers can be delivered by a wide range of gene delivery system commonly used for gene therapy including viral, non-viral, or physical. See, for example, Rosenberg et al., Science, 242:1575-1578, 1988, and Wolff et al., Proc. Natl. Acad. Sci. USA 86:9011-9014 (1989). Discussion of methods and compositions for use in gene therapy include Eck et al., in Goodman & Gilman's The Pharmacological Basis of Therapeutics, Ninth Edition, Hardman et al., eds., McGraw-Hill, New York, (1996), Chapter 5, pp. 77-101; Wilson, Clin. Exp. Immunol. 107 (Suppl. 1):31-32, 1997; Wivel et al., Hematology/Oncology Clinics of North America, Gene Therapy, S. L. Eck, ed., 12(3):483-501, 1998; Romano et al., Stem Cells, 18:19-39, 2000, and the references cited therein. U.S. Pat. No. 6,080,728 also provides a discussion of a wide variety of gene delivery methods and compositions. The routes of delivery include, for example, systemic administration and administration in situ.

An effective Encoded Affimer gene transfer approach must be directed to the specific tissues/cells where it is needed, and the resulting transgene expression should be at a level that is appropriate to the specific application. Promoters are a major cis-acting element within the vector genome design that can dictate the overall strength of expression as well as cell-specificity,

Table 1. Exemplary Ubiquitous and Cell-specific Promoters.

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Promoter	Specificity	Relative Strength	Size (bps)	Reference(s)
CMV	Ubiquitous	+++	750–800	Xu et al. Gene Ther. 2001 8:1323–1332; Gray et al., Hum Gene Ther. 2011 22:1143–1153
CBA (including derivatives: CAG, CBh, etc.)	Ubiquitous	+++	248–1,600	Klein et al. Exp Neurol. 2002 176(1):66–74; Ohlfest et al. Blood. 2005 105:2691–2698; and Gray et al. Hum Gene Ther. 2011 22:1143–1153.
EF-1α	Ubiquitous	++	2,500	Gill et al. Gene Ther. 2001 8(20):1539–1546; Xu et al. Gene Ther. 2001 8:1323–1332; and Gilham et al. J Gene Med. 2010 12(2):129–136.
PGK	Ubiquitous	++	426	Gilham et al. J Gene Med. 2010 12(2):129–136.
UBC	Ubiquitous	+	403	Gill et al. Gene Ther. 2001 8(20):1539–1546; Qin et al. PLoS One. 2010 5(5):e10611.
GUSB (hGBp)	Ubiquitous	+	378	Husain et al. Gene Ther. 2009 16:927–932.
UCOE (Promoter of HNRPA2B1-CBX3)	Ubiquitous	++	600–2,500	Antoniou et al. Hum Gene Ther. 2013 24(4):363–374.
hAAT	Liver	++	347–1,500	Van Linthout et al. Hum Gene Ther. 2002 13(7):829–840; Cunningham et al. Mol Ther. 2008 16(6):1081–1088
TBG	Liver	++	400	Yan et al. Gene. 2012 506(2):289–294.
Desmin	Skeletal muscle	+++	1,700	Talbot et al. Mol Ther. 2010 18:601–608.
MCK	Skeletal muscle	++	595–1,089	Talbot et al. Mol Ther. 2010 18:601–608; Wang et al. Gene Ther. 2008 15:1489–1499; Katwal et al. Gene Ther. 2013 20(9):930–938.

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Promoter	Specificity	Relative Strength	Size (bps)	Reference(s)
C5-12	Skeletal, cardiac, and diaphragm	++	312	Wang et al. Gene Ther. 2008 15:1489–1499
NSE	Neuron	+++	300–2,200	Xu et al. Gene Ther. 2001 8:1323–1332
Synapsin	Neuron	+	470	Kügler et al. Virology. 2003 311:89–95; Hioki et al. Gene Ther. 2007 14:872–882; Kuroda et al. J Gene Med. 2008 10:1163–1175.
PDGF	Neuron	+++	1,400	Patterna et al. Gene Ther. 2000 7(15):1304–1311; Hioki et al. Gene Ther. 2007 14:872–882
MecP2	Neuron	+	229	Rastegar et al. LoS One. 2009 4:e6810; Gray et al., Hum Gene Ther. 2011 22:1143–1153
CaMKII	Neuron	++	364–2,300	Hioki et al. Gene Ther. 2007 14:872–882; Kuroda et al. J Gene Med. 2008 10:1163–1175
mGluR2	Neuron	+	1,400	Brené et al. Eur J Neurosci. 2000 12:1525–1533; Kuroda et al. J Gene Med. 2008 10:1163–1175
NFL	Neuron	+	650	Xu et al. Gene Ther. 2001 8:1323–1332
NFH	Neuron	+	920	Xu et al. Gene Ther. 2001 8:1323–1332
nβ2	Neuron	+	650	Xu et al. Gene Ther. 2001 8:1323–1332
PPE	Neuron	+	2700	Xu et al. Gene Ther. 2001 8:1323–1332
Enk	Neuron	+	412	Xu et al. Gene Ther. 2001 8:1323–1332
EAAT2	Neuron and astrocyte	++	966	Su et al. Proc Natl Acad Sci U S A. 2003 100:1955–1960; Kuroda et al. J Gene Med. 2008 10:1163–1175
GFAP	Astrocyte	++	681–2,200	Brenner et al. J Neurosci. 1994 14:1030–1037; Xu et al. Gene Ther. 2001 8:1323–1332; Lee et al. Glia. 2008 56:481–

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Promoter	Specificity	Relative Strength	Size (bps)	Reference(s)
				493; Dirren et al. Hum Gene Ther. 2014 25:109–120
MBP	Oligodendrocytes	++	1,900	Chen et al. Gene Ther. 1998 5(1):50–58

In some cases, ubiquitous expression of the Encoded Affimer construct in all cell types is desired. Constitutive promoters such as the human elongation factor 1α-subunit (EF1α), immediate-early cytomegalovirus (CMV), chicken β-actin (CBA) and its derivative CAG, the β glucuronidase (GUSB), or ubiquitin C (UBC) can be used to promote expression of the Encoded Affimer Construct in most tissues. Generally, CBA and CAG promote the larger expression among the constitutive promoters; however, their size of ~1.7 kbs in comparison to CMV (~0.8 kbs) or EF1α (~1.2 kbs) may limit use in vectors with packaging constraints such as AAV, particularly where Affimer polypeptide produced by expression of the Encoded Affimer construct is large. The GUSB or UBC promoters can provide ubiquitous gene expression with a smaller size of 378 bps and 403 bps, respectively, but they are considerably weaker than the CMV or CBA promoter. Thus, modifications to constitutive promoters in order to reduce the size without affecting its expression have been pursued and examples such as the CBh (~800 bps) and the miniCBA (~800 bps) can promote expression comparable and even higher in selected tissues (Gray et al., Hum Gene Ther. 2011 22:1143–1153).

When expression of the Encoded Affimer construct should be restricted to certain cell types within an organ, promoters can be used to mediate this specificity. For example, within the nervous system promoters have been used to restrict expression to neurons, astrocytes, or oligodendrocytes. In neurons, the neuron-specific enolase (NSE) promoter drives stronger expression than ubiquitous promoters. Additionally, the platelet-derived growth factor B-chain (PDGF-β), the synapsin (Syn), and the methyl-CpG binding protein 2 (MeCP2) promoters can drive neuron-specific expression at lower levels than NSE. In astrocytes, the 680 bps-long shortened version [gfaABC(1)D] of the glial fibrillary acidic protein (GFAP, 2.2 kbs) promoter can confer higher levels of expression with the same astrocyte-specificity as the GFAP promoter. Targeting oligodendrocytes can also be accomplished by the selection of the myelin basic protein (MBP) promoter, whose expression is restricted to this glial cell; however, its size of 1.9 kbs and low expression levels limit its use.

In the case of expressing the Encoded Affimer constructs in skeletal muscle cells, exemplary promoters based on muscle creatine kinase (MCK) and desmin (1.7 kbs) have showed a high rate of specificity (with minimal expression in the liver if desired). The promoter of the α-myosin heavy chain (α-MHC; 1.2 kbs) has shown significant cardiac specificity in comparison with other muscle promoters (Lee et al., 2011 J Cardiol. 57(1):115-22). In hematopoietic stem cells the synthetic MND promoter (Li et al., 2010 J Neurosci Methods. 189(1):56-64) and the promoter contained in the 2AUCOE (ubiquitous chromatin opening element) have shown to drive a higher transgene expression in all cell lineages when compared to the EF1α and CMV

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promoters, respectively (Zhang et al., 2007 Blood. 110(5):1448-57; Koldej 2013 Hum Gene Ther Clin Dev. 24(2):77-85; Dighe et al., 2014 PLoS One. 9(8):e104805.). Conversely, using promoters to restrict expression to only liver hepatocytes after vector-mediated gene transfer has been shown to reduce transgene-specific immune responses in systems where that is a risk, and to even induce immune tolerance to the expressed protein (Zhang et al., 2012 Hum Gene Ther. 23(5):460-72), which for certain Affimer polypeptides may be beneficial. The α1-antitrypsin (hAAT; 347 bps) and the thyroxine binding globulin (TBG; ~400 bps) promoters drive gene expression restricted to the liver with minimal invasion to other tissues (Yan et al., 2012 Gene. 506(2):289-94; Cunningham et al., 2008 Mol Ther. 16(6):1081-8).

In certain embodiments, a mechanism to control the duration and amount of in vivo Encoded Affimer expression will typically be desired. There are a variety of inducible promoters which can be adapted for use with viral vectored- and plasmid DNA-based Encoded Affimer gene transfer. See Fang et al. (2007) "An antibody delivery system for regulated expression of therapeutic levels of monoclonal antibodies in vivo" Mol Ther. 5(6):1153–9; and Perez et al. (2004) "Regulatable systemic production of monoclonal antibodies by in vivo muscle electroporation" Genet Vaccines Ther. 2(1):2. An exemplary a regulatable mechanism currently under clinical evaluation is an ecdysone-based gene switch activated by a small molecule ligand. Cai et al. (2016) "Plasma pharmacokinetics of veledimex, a small-molecule activator ligand for a proprietary gene therapy promoter system, in healthy subjects" Clin Pharmacol Drug Dev. 2016.

In certain embodiments of the Encoded Affimer constructs, viral post-transcriptional regulatory elements (PREs) may be used; these cis-acting elements are required for nuclear export of intronless viral RNA (Huang and Yen, 1994 J Virol. 68(5):3193-9; and 1995 Mol Cell Biol. 15(7):3864-9). Examples include HPRE (Hepatitis B Virus PRE, 533 bps) and WPRE (Woodchuck Hepatitis Virus PRE, 600 bps), which can increase the level of transgene expression by almost 10-fold in certain instances (Donello et al., 1998 J Virol. 72(6):5085-92). To further illustrate, using lentiviral and AAV vectors, WPRE was found to increase CMV promoter driven transgene expression, as well as increase PPE, PDGF and NSE promoter-driven transgene expression. Another effect of the WPRE can be to protect Encoded Affimer constructs transgenes from silencing (Paterna et al., 2000 Gene Ther. 7(15):1304-11; Xia et al., 2007 Stem Cells Dev. 2007 Feb; 16(1):167-76).

The polyadenylation of a transcribed Encoded Affimer transcript can also be important for nuclear export, translation, and mRNA stability. Therefore, in certain embodiments, the Encoded Affimer construct will include a polyadenylation signal sequence. A variety of studies are available that have determined the effects of different polyA signals on gene expression and mRNA stability. Exemplary polyadenylation signal sequences include SV40 late or bovine growth hormone polyA (bGHpA) signal sequences, as well as minimal synthetic polyA (SPA) signal (Levitt et al., 1989 Genes Dev. 3(7):1019-25; Yew et al., 1997 Hum Gene Ther. 1997 8(5):575-84). The efficiency of polyadenylation is increased by the SV40 late polyA signal upstream enhancer (USE) placed upstream of other polyA signals (Schek et al., 1992 Mol Cell Biol. 12(12):5386-93). In certain embodiments, merely to illustrate, the Encoded Affimer construct will include an SV40 late + 2xUSE polyA signal.

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Table 2: Exemplary Polyadenylation Signals

PolyA Signal and USE	Relative Strength	Size (bps)	Source	Reference(s)
hGH	+	624	Human growth hormone	Ostedgaard et al. Proc Natl Acad Sci U S A. 2005 102(8):2952–2957
SV40 late	+++	135	Simian virus 40	Choi et al. Mol Brain. 2014 7:17
SPA (synthetic polyA)	+	49	Rabbit β-globin	Levitt et al. Genes Dev. 3(7):1019–1025; Yew et al. Hum Gene Ther. 1997 8(5):575–584; Ostedgaard et al. Proc Natl Acad Sci U S A. 2005 102(8):2952–2957; Choi et al. Mol Brain. 2014 7:17
bGH	++	250	Bovine growth hormone	Yew et al. Hum Gene Ther. 1997 8(5):575–584; Xu et al. Gene Ther. 2001 8:1323–1332; Wu et al. Mol Ther. 2008 16(2):280–289; Gray et al., Hum Gene Ther. 2011 22:1143–1153; Choi et al. Mol Brain. 2014 7:17
SV40 late 2xUSE	++	100	Simian virus 40	Schambach et al. Mol Ther. 2007 15(6):1167–1173; Choi et al. Mol Brain. 2014 7:17
HIV-1 USE	+	35	Human immunodeficiency virus 1	Schambach et al. Mol Ther. 2007 15(6):1167–1173
GHV USE	+	39	Ground squirrel hepatitis virus	Schambach et al. Mol Ther. 2007 15(6):1167–1173
Adenovirus (L3) USE	+	21	Adenovirus	Schambach et al. Mol Ther. 2007 15(6):1167–1173
hTHGB USE	+	21	Human prothrombin	Schambach et al. Mol Ther. 2007 15(6):1167–1173
hC2 USE	+	53	Human C2 complement gene	Schambach et al. Mol Ther. 2007 15(6):1167–1173

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In certain embodiments, it may be desirable for the Encoded Affimer construct to include one or more regulatory enhancers, i.e., in addition to any promoter sequences. The CMV enhancer is upstream of the CMV promoter at −598 to −68 (Boshart et al., 1985 Cell. 41(2):521-30) (~600 bps) and contains transcription binding sites. In certain embodiments, a CMV enhancer can be included in the construct to increase tissue-specific promoter-driven transgene expression, such as using the ANF (atrial natriuretic factor) promoter, the CC10 (club cell 10) promoter, SP-C (surfactant protein C) promoter, or the PDGF-β (platelet-derived growth factor-β) promoter (merely as examples). Altogether, the CMV enhancer increases transgene expression under different cell-specific promoters and different cell types making it a broadly applicable tool to increase transgene expression levels. In muscle, for example, in AAV expression systems transgene expression using the CMV enhancer with a muscle-specific promoter can increase expression levels of the protein encoded by the transgene, so would be particularly useful in the current invention for expressing Affimer polypeptides from Encoded Affimer constructs introduced into muscle cells of a patient.

The subject Encoded Affimer constructs may also include one or more intronic sequences. The presence of an intron or intervening sequence in mRNA was first described, in vitro, to be important for mRNA processing and increased transgene expression (Huang and Gorman, 1990 Mol Cell Biol. 10(4):1805-10; Niwa et al., 1990 Genes Dev. 4(9):1552-9). The intron(s) can be placed within the coding sequence for the Affimer polypeptide and/or can be placed between the promoter and transgene. A variety of introns (Table 3) placed between the promoter and transgene were compared, in mice using AAV2, for liver transgene expression (Wu et al., 2008). The MVM (minute virus of mice) intron increased transgene expression more than any other intron tested and more than 80-fold over no intron (Wu et al., 2008). However, in cultured neurons using AAV expression cassettes, transgene expression was less under a CaMPKII promoter with a chimeric intron (human β-globin donor and immunoglobulin heavy chain acceptor) between the transgene and polyA signal compared to a WPRE (Choi et al., 2014). Together, an intron can be a valuable element to include in an expression cassette to increase transgene expression.

Table 3: Exemplary Introns

Itron	Relative Strength	Size (bps)	Source	Reference(s)
MVM	+++	67–97	Minute virus of mice	Wu et al. Mol Ther. 2008 16(2):280–289
F.IX truncated intron 1	+	300	Human factor IX	Wu et al. Mol Ther. 2008 16(2):280–289; Kurachi et al. J Biol Chem. 1995 270(10):5276–5281

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Itron	Relative Strength	Size (bps)	Source	Reference(s)
β-globin SD / immunoglobin heavy chain SA	+	250	Human, pZac2.1	Wu et al. Mol Ther. 2008 16(2):280–289; Choi et al. Mol Brain. 2014;7:17
Adenovirus SD# / immunoglobulin SA*	++	500	pAdβ	Wong et al. Chromosoma. 1985 92(2):124–135; Yew et al. Hum Gene Ther. 1997 8(5):575–584
SV40 late SD# / SA* (19S/16S)	+	180	pCMVβ	Yew et al. Hum Gene Ther. 1997 8(5):575–584
Hybrid adenovirus SD# / IgG SA*	+++	230	Adenovirus	Choi et al. Mol Brain. 2014;7:17; Huang et al. Mol Cell Biol. 1990 10(4):1805–1810

In the case of episomal vectors, the subject Encoded Affimer constructs may also include one or more origins of replication, minichromosome maintenance elements (MME) and/or nuclear localization elements. Episomal vectors of the invention comprise a portion of a virus genomic DNA that encodes an origin of replication (ori), which is required for such vectors to be self-replicating and, thus, to persist in a host cell over several generations. In addition, an episomal vector of the invention also may contain one or more genes encoding viral proteins that are required for replication, i.e., replicator protein (s). Optionally, the replicator protein(s) which help initiate replication may be expressed in trans on another DNA molecule, such as on another vector or on the host genomic DNA, in the host cell containing a self-replicating episomal expression vector of this invention. Preferred self-replicating episomal LCR-containing expression vectors of the invention do not contain viral sequences that are not required for long-term stable maintenance in a eukaryotic host cell such as regions of a viral genome DNA encoding core or capsid proteins that would produce infectious viral particles or viral oncogenic sequences which may be present in the full-length viral genomic DNA molecule. The term "stable maintenance" herein, refers to the ability of a self-replicating episomal expression vector of this invention to persist or be maintained in non-dividing cells or in progeny cells of dividing cells in the absence of continuous selection without a significant loss (e.g., >50%) in copy number of the vector for two and preferably five or more generations. The most preferred vectors will be maintained over 10-15 or more cell generations. In contrast, "transient" or "short-term" persistence of a plasmid in a host cell refers to the inability of a vector to replicate and segregate in a host cell in a stable manner; that is, the vector will be lost after one or two generations, or will undergo a loss of >51% of its copy number between successive generations.

Several representative self-replicating, LCR-containing, episomal vectors useful in the context of the present invention are described further below. The self-replicating function may

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alternatively be provided by one or more mammalian sequences such as described by Wohlgeuth et al., 1996, Gene Therapy 3:503; Vos et al., 1995, Jour. Cell. Biol., Supp. 21A, 433; and Sun et al., 1994, Nature Genetics 8:33, optionally in combination with one or more sequence which may be required for nuclear retention. The advantage of using mammalian, especially human sequences for providing the self- replicating function is that no extraneous activation factors are required which could have toxic or oncogenic properties. It will be understood by one of skill in the art that the invention is not limited to any one origin of replication or any one episomal vector, but encompasses the combination of the tissue-restricted control of an LCR in an episomal vector. See also WO1998007876 "Self-replicating episomal expression vectors conferring tissue-specific gene expression" and US Patent 7790446 "Vectors, cell lines and their use in obtaining extended episomal maintenance replication of hybrid plasmids and expression of gene products"

Epstein-Barr Virus-Based Self-Replicating Episomal Expression Vectors. The latent origin oriP from Epstein-Barr Virus (EBV) is described in Yates et. al., Proc . Natl . Acad . Sci . USA 81:3806-3810 (1984); Yates et al., Nature 313:812-815 (1985); Krysan et al., Mol . Cell . Biol . 9:1026-1033 (1989); James et al.. Gene 86: 233-239 (1990), Peterson and Legerski, Gene 107:279-284 (1991); and Pan et al., Som . Cell Molec. Genet . 18:163-177 (1992)). An EBV-based episomal vector useful according to the invention can contain the oriP region of EBV which is carried on a 2.61 kb fragment of EBV and the EBNA-1 gene which is carried on a 2.18 kb fragment of EBV. The EBNA-1 protein, which is the only viral gene product required to support in trans episomal replication of vectors containing oriP, may be provided on the same episomal expression vector containing oriP. It is also understood, that as with any protein such as EBNA-1 known to be required to support replication of viral plasmid in trans, the gene also may be expressed on another DNA molecule, such as a different DNA vector.

Papilloma Virus-Based, Self-Replicating, Episomal Expression Vectors. The episomal expression vectors of the invention also may be based on replication functions of the papilloma family of virus, including but not limited to Bovine Papilloma Virus (BPV) and Human Papilloma Viruses (HPVs). BPV and HPVs persist as stably maintained plasmids in mammalian cells. -S trans-acting factors encoded by BPV and HPVs, namely El and E2, have also been identified which are necessary and sufficient for mediate replication in many cell types via minimal origin of replication (Ustav et al., EMBO J. 10: 449-457 (1991); Ustavet al., EMBO J . 10:4231-4329, (1991); Ustav et al., Proc . Natl . Acad . Sci . USA 90: 898-902 (1993)).

An episomal vector useful according to the invention is the BPV-I vector system described in Piirsoo et al., EMBO J. , 15:1 (1996) and in WO 94/12629. The BPV-1 vector system described in Piirsoo et al. comprises a plasmid harboring the BPV-1 origin of replication (minimal origin plus extrachro osomal maintenance element) and optionally the El and E2 genes. The BPV-I El and E2 genes are required for stable maintenance of a BPV episomal vector. These factors ensure that the plasmid is replicated to a stable copy number of up to thirty copies per cell independent of cell cycle status. The gene construct therefore persists stably in both dividing and non-dividing cells. This allows the maintenance of the gene construct in cells such as hemopoietic stem cells and more committed precursor cells.

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The BPV origin of replication has been located at the 3' end of the upstream regulatory region within a 60 base pair (bp) DNA fragment (nucleotides (nt) 7914 - 7927) which includes binding sites for the El and E2 replication factors. The minimal origin of replication of HPV has also been characterized and located in the URR fragment (nt 7022- 7927) of HPV (see, for example, Chiang et al., Proc . Natl . Acad. Sci . USA 89:5799-5803 (1992)). As used herein, "El" refers to the protein encoded by nucleotides (nt) 849-2663 of BPV subtype 1 or by nt 832- 2779 of HPV of subtype 11, to equivalent El proteins of other papilloma viruses, or to functional fragments or mutants of a papilloma virus El protein, i.e., fragments or mutants of El which possess the replicating properties of El.

As used herein, "E2H refers to the protein encoded by nt 2594-3837 of BPV subtype 1 or by nt 2723-3823 of HPV subtype 11, to equivalent E2 proteins of other papilloma viruses, or to functional fragments or mutants of a papilloma virus E2 protein, i.e., fragments or mutants of E2 which possess the replicating properties of E2. "Minichromosomal maintenance element" (MME) refers to the extrachromosomal maintenance element of the papilloma viral genome to which viral or human proteins essential for papilloma viral replication bind, which region is essential for stable episomal maintenance of the papilloma viral MO in a host cell, as described in Piirsoo et al. (supra). Preferably, the MME is a sequence containing multiple binding sites for the transcriptional activator E2. The MME in BPV is herein defined as the region of BPV located within the upstream regulatory region which includes a minimum of about six sequential E2 binding sites, and which gives optimum stable maintenance with about ten sequential E2 binding sites. E2 binding site 9 is a preferred sequence for this site, as described hereinbelow, wherein the sequential sites are separated by a spacer of about 4-10 nucleotides, and optimally 6 nucleotides. El and E2 can be provided to the plasmid either in cis or in trans, also as described in WO 94/12629 and in Piirsoo et al. (supra).

"E2 binding site" refers to the minimum sequence of papillomavirus double-stranded DNA to which the E2 protein binds. An E2 binding site may include the sequence 5* ACCGTTGCCGGT 3', which is high affinity E2 binding site 9 of the BPV-1 URR; alternatively, an E2 binding site may include permutations of binding site 9, which permutations are found within the URR, and fall within the generic E2 binding sequence 5' ACCN6GGT 3'. One or more transcriptional activator E2 binding sites are, in most papillomaviruses, located in the upstream regulatory region, as in BPV and HPV. A vector which also is useful according to the invention may include a region of BPV between 6959 - 7945/1 - 470 on the BPV genetic map (as described in WO 94/12629), which region includes an origin of replication, a first promoter operatively associated with a gene of interest, the BPV El gene operatively associated with a second promoter to drive transcription of the El gene; and the BPV E2 gene operatively associated with a third promoter to drive transcription of the E2 gene.

El and E2 from BPV will replicate vectors containing the BPV origin or the origin of many HPV subtypes (Chiang et al., supra). El and E2 from HPV will replicate vectors via the BPV origin and via the origin of many HPV subtypes (Chiang et al., supra). As with all vectors of the invention, the BPV-based episomal expression vectors of the invention must persist through 2-5 or more divisions of the host cell.

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See also US Patent 7790446 and Abroi et al. (2004) "Analysis of chromatin attachment and partitioning functions of bovine papillomavirus type 1 E2 protein. Journal of Virology 78:2100-13 which have shown that the BPV1 E2 protein dependent MME and EBV EBNA1 dependent FR segregation/partitioning activities function independently from replication of the plasmids. The stable-maintenance function of EBNA1/FR and E2/MME can be used to ensure long-time episomal maintenance for cellular replication origins.

Papovavirus-Based, Self-Replicating, Episomal Expression Vectors. The vectors of the invention also may be derived from a human papovavirus BK genomic DNA molecule. For example, the BK viral genome can be digested with restriction enzymes EcoRI and BamHI to produce a 5 kilobase (kb) fragment that contains the BK viral origin of replication sequences that can confer stable maintenance on vectors (see, for example, De Benedetti and Rhoads, Nucleic Acids Res . 19:1925 (1991), as can a 3.2 kb fragment of the BK virus (Cooper and Miron, Human Gene Therapy 4:557 (1993)).

The Encoded Affimer constructs of the present invention can be provided as circular or linear nucleic acids. The circular and linear nucleic acids are capable of directing expression of the Affimer polypeptide coding sequence in an appropriate subject cell. The one or more nucleic acid systems for expressing an Affimer polypeptide may be chimeric, meaning that at least one of its components is heterologous with respect to at least one of its other components.

Viral Vectors

Exemplary viral gene therapy system that are readily adapted for use in the present invention include plasmid, adenovirus, adeno-associated virus (AAV), retrovirus, lentivirus, herpes simplex virus, vaccinia virus, poxvirus, reovirus, measles virus, Semliki Forest virus, and the like. Preferred viral vectors are based on non-cytopathic eukaryotic viruses in which non-essential genes have been replaced with the nucleic acid construct carrying the nucleic acid sequences encoding the epitopes and targeting sequences of interest.

To further illustrate, encoded affimers can be delivered in vivo using adenoviruses and adeno-associated (AAV) viruses, which are double-stranded DNA viruses that have already been approved for human use in gene therapy.

Adenovirus Vectors

One illustrative method for in vivo delivery of one or more nucleic acid sequences involves the use of an adenovirus ("AdV") expression vector. AdVs are non-enveloped, double-stranded DNA viruses that neither integrate in the host genome nor replicate during cell division. AdV-mediated antibody gene transfer has shown therapeutic efficacy in a variety of different disease models advancing towards the clinic. Systemic mAb expression has mostly been pursued, via s.c. and especially i.v. and intramuscular AdV injection. See Wold et al. (2013) "Adenovirus vectors for gene therapy, vaccination and cancer gene therapy" Curr Gene Ther. 13(6):421–33; and Deal et al. "Engineering humoral immunity as prophylaxis or therapy" 2015 Curr Opin Immunol. 35:113–22. Other routes of delivery have focused on more local mAb

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production, such as via intranasal, intratracheal or intrapleural administration of the encoding AdV. The use of AdVs as oncolytic vectors is a popular approach particularly for generation of encoded antibodies at the site of tumors. Foreign genes delivered by current adenoviral gene delivery system are episomal, and therefore, have low genotoxicity to host cells. Therefore, gene therapy using adenoviral gene delivery systems may be considerably safe. The present invention specifically contemplates the delivery of Affimer polypeptides by expression of Encoded Affimer constructs delivered in the form of an adenoviral vector and delivery system.

Adenovirus has been usually employed as a gene delivery vector because of its mid-sized genome, ease of manipulation, high titer, wide target-cell range and high infectivity. Both ends of the viral genome contains 100-200 bp ITRs (inverted terminal repeats), which are cis elements necessary for viral DNA replication and packaging. The E1 region (E1A and E1B) of genome encodes proteins responsible for the regulation of transcription of the viral genome and a few cellular genes. The E2 region (E2A and E2B) encodes proteins responsible for viral DNA replication. Of adenoviral vectors developed so far, the replication incompetent adenovirus having the deleted E1 region is usually used and represent one exemplary choice of AdV for generating the Encoded Affimer constructs of the present invention. The deleted E3 region in adenoviral vectors may provide an insertion site for transgenes (Thimmappaya, B. et al., Cell, 31:543-551(1982); and Riordan, J. R. et al., Science, 245:1066-1073(1989)).

An "adenovirus expression vector" is meant to include those constructs containing adenovirus sequences sufficient to (a) support packaging of the construct and (b) to express a polynucleotide that encodes a polypeptide including an Affimer polypeptide (the Encoded Affimer sequence). In certain embodiments, the sequence for an Encoded Affimer may be inserted into the DA promoter region. According to an exemplary embodiment, the recombinant adenovirus comprises deleted E1B and E3 region and the nucleotide sequence for an encoded Affimer is inserted into the deleted E1B and E3 region.

Adeno-Associated Virus Vectors (AAV)

AAVs (or "rAAV" for recombinant AAV) are non-enveloped small, single-stranded DNA viruses capable of infecting both dividing and non-dividing cells. Similar to AdV, AAV-based vectors remain in an episomal state in the nucleus and display a limited risk of integration. In contrast to the generally limited durability of AdV-mediated gene transfer, transgene expression can persist for years following intramuscular recombinant AAV (rAAV) vector delivery.

Alipogene tiparvovec (Glybera™), an rAAV encoding the human lipoprotein lipase gene, was approved in 2012 as the first gene therapy product in Europe. Since then, various rAAV-based gene therapy products are currently under clinical evaluation. In the context of antibody gene transfer, a variety of reports have demonstrated in vivo production of an anti-human immune deficiency virus (HIV) mAb in mice following intramuscular injection of the mAb-encoding rAAV. The rAAV vector's potential for combination therapy has also been demonstrated, i.e. by expressing two mAbs. Similar to AdV, intramuscular and i.v. rAAV administration have been most often pursued. Reviewed in Deal et al. "Engineering humoral immunity as prophylaxis or therapy" 2015 Curr Opin Immunol. 35:113–22. A variety of additional delivery sites have also

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been demonstrated to achieve more local therapeutic effects, including intracranial, intranasal, intravitreal, intrathecal, intrapleural, and intraperitoneal routes. With the utility of rAAV demonstrated for antibody gene transfer, the present invention also specifically contemplates the use of rAAV systems for the delivery of Encoded Affimer sequences in vivo and the production of Affimer polypeptides in the body of a patient as a consequence to expression of the rAAV construct.

One important feature to AAV is that these gene transfer viruses are capable of infecting non-dividing cells and various types of cells, making them useful in constructing the Encoded Affimer delivery system of this invention. The detailed descriptions for use and preparation of exemplary AAV vectors are found in, for example, U.S. Pat. Nos. 5,139,941 and 4,797,368, as well as LaFace et al, Viology, 162:483486 (1988), Zhou et al., Exp. Hematol. (NY), 21:928-933 (1993), Walsh et al, J. Clin. Invest., 94:1440-1448(1994) and Flotte et al., Gene Therapy, 2:29-37(1995). AAV is a good choice of delivery vehicles due to its safety, i.e., genetically engineered (recombinant) does not integrate into the host genome. Likewise, AAV is not pathogenic and not associated with any disease. The removal of viral coding sequences minimizes immune reactions to viral gene expression, and therefore, recombinant AAV does not evoke an inflammatory response.

Typically, a recombinant AAV virus is made by co-transfecting a plasmid containing the gene of interest (i.e., the coding sequence for an Affimer polypeptide) flanked by the two AAV terminal repeats (McLaughlin et al., J. Virol., 62:1963-1973(1988); Samulski et al., J. Virol., 63:3822-3828(1989)) and an expression plasmid containing the wild type AAV coding sequences without the terminal repeats (McCarty et al., J. Virol., 65:2936-2945(1991)). Typically, viral vectors containing an Encoded Affimer construct are assembled from polynucleotides encoding the Affimer containing polypeptide, suitable regulatory elements and elements necessary for expression of the encoded Affimer which mediate cell transduction. In one embodiment, adeno-associated viral (AAV) vectors are employed. In a more specific embodiment, the AAV vector is an AAV1, AAV6, or AAV8.

The AAV expression vector which harbors the Encoded Affimer sequence bounded by AAV ITRs, can be constructed by directly inserting the selected sequence(s) into an AAV genome which has had the major AAV open reading frames ("ORFs") excised therefrom.

For eukaryotic cells, expression control sequences typically include a promoter, an enhancer, such as one derived from an immunoglobulin gene, SV40, cytomegalovirus, etc. (see above), and a polyadenylation sequence which may include splice donor and acceptor sites. The polyadenylation sequence generally is inserted following the transgene sequences and before the 3′ ITR sequence.

Selection of these and other common vector and regulatory elements are conventional, and many such sequences are available. See, e.g., Sambrook et al., and references cited therein at, for example, pages 3.18-3.26 and 16.17-16.27 and Ausubel et al., Current Protocols in Molecular Biology, John Wiley & Sons, New York, 1989). Of course, not all vectors and expression control sequences will function equally well to express all of the transgenes of this invention. However, one of skill in the art may make a selection among these expression

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control sequences without departing from the scope of this invention. Suitable promoter/enhancer sequences may be selected by one of skill in the art using the guidance provided by this application. Such selection is a routine matter and is not a limitation of the molecule or construct.

Retrovirus Vectors

Non-cytopathic viruses useful in the context of delivery of Encoded Affimer constructs include retroviruses, the life cycle of which involves reverse transcription of genomic viral RNA into DNA with subsequent proviral integration into host cellular DNA. Retroviruses have been approved for human gene therapy trials. Most useful are those retroviruses that are replication-deficient (i.e., capable of directing synthesis of the desired proteins, but incapable of manufacturing an infectious particle). Such genetically altered retroviral expression vectors have general utility for the high-efficiency transduction of genes in vivo. Standard protocols for producing replication-deficient retroviruses (including the steps of incorporation of exogenous genetic material into a plasmid, transfection of a packaging cell lined with plasmid, production of recombinant retroviruses by the packaging cell line, collection of viral particles from tissue culture media, and infection of the target cells with viral particles) are known to those of skill in the art.

In order to construct a retroviral vector, the Affimer polypeptide coding sequence is inserted into the viral genome in the place of certain viral sequences to produce a replication-defective virus. To produce virions, a packaging cell line containing the gag, pol and env genes but without the LTR (long terminal repeat) and psi ( ) components is constructed (Mann et al., Cell, 33:153-159(1983)). When a recombinant plasmid containing the cytokine gene, LTR and psi is introduced into this cell line, the psi sequence allows the RNA transcript of the recombinant plasmid to be packaged into viral particles, which are then secreted into the culture media (Nicolas and Rubinstein "Retroviral vectors," In: Vectors: A survey of molecular cloning vectors and their uses, Rodriguez and Denhardt (eds.), Stoneham: Butterworth, 494-513(1988)). The media containing the recombinant retroviruses is then collected, optionally concentrated and used for gene delivery system.

Successful gene transfer using such second-generation retroviral vectors has been reported. Kasahara et al. (Science, 266:1373-1376(1994)) prepared variants of Moloney murine leukemia virus in which the EPO (erythropoietin) sequence is inserted in the place of the envelope region, consequently, producing chimeric proteins having novel binding properties. Likely, the present gene delivery system can be constructed in accordance with the construction strategies for the second-generation retroviral vector.

In certain embodiments, the retrovirus is a "gammaretroviruses", which refers to a genus of the retroviridae family. Exemplary gammaretroviruses include mouse stem cell virus, murine leukemia virus, feline leukemia virus, feline sarcoma virus, and avian reticuloendotheliosis viruses.

In certain preferred embodiments, the retroviral vector for use in the present invention is a lentiviral vector, which refers to a genus of retroviruses that are capable of infecting dividing

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and non-dividing cells and typically produce high viral titers. Several examples of lentiviruses include HIV (human immunodeficiency virus: including HIV type 1, and HIV type 2); equine infectious anemia virus; feline immunodeficiency virus (FIV); bovine immune deficiency virus (BIV); and simian immunodeficiency virus (SIV).

Another class of widely used retroviral vectors that can be used for the delivery and expression of an Encoded Affimer include those based upon murine leukemia virus (MuLV), gibbon ape leukemia virus (GaLV) and combinations thereof (see, e.g., Buchscher et al., J. Virol. 66:2731-2739, 1992; Johann et al., J. Virol. 66: 1635-1640, 1992; Sommerfelt et al., Virol. 176:58-59, 1990; Wilson et al., J. Virol. 63:2374-2378, 1989; Miller et al., J. Virol. 65:2220-2224, 1991 ; and PCT/US94/05700).

Still other retroviral vectors that can be also be used in the present invention include, e.g., vectors based on human foamy virus (HFV) or other viruses in the Spumavirus genera. Foamy viruses (FVes) are the largest retroviruses known today and are widespread among different mammals, including all non-human primate species, however are absent in humans. This complete apathogenicity qualifies FV vectors as ideal gene transfer vehicles for genetic therapies in humans and clearly distinguishes FV vectors as gene delivery system from HIV-derived and also gammaretrovirus-derived vectors.

Suitable retroviral vectors for use herein are described, for example, in U.S. Pat. Nos. 5,399,346 and 5,252,479; and in WIPO publications WO 92/07573, WO 90/06997, WO 89/05345, WO 92/05266 and WO 92/14829, which provide a description of methods for efficiently introducing nucleic acids into human cells using such retroviral vectors. Other retroviral vectors include, for example, mouse mammary tumor virus vectors (e.g., Shackleford et al., Proc. Natl. Acad. Sci. U.S.A. 85:9655-9659, 1998), lentiviruses, and the like.

Additional retroviral viral delivery systems that can be readily adapted for delivery of a transgene encoding an Affimer polypeptide include, merely to illustrate Published PCT Applications WO/2010/045002, WO/2010/148203, WO/2011/126864, WO/2012/058673, WO/2014/066700, WO/2015/021077, WO/2015/148683, WO/2017/040815 - the specifications and figures of each of which are incorporated by reference herein.

In certain embodiments, a retroviral vector contains all of the cis-acting sequences necessary for the packaging and integration of the viral genome, i.e., (a) a long terminal repeat (LTR), or portions thereof, at each end of the vector; (b) primer binding sites for negative and positive strand DNA synthesis; and (c) a packaging signal, necessary for the incorporation of genomic RNA into virions. More detail regarding retroviral vectors can be found in Boesen, et al., 1994, Biotherapy 6:291-302; Clowes, et ai, 1994, J. Clin. Invest. 93:644-651 ; Kiem, et al., 1994, Blood 83: 1467-1473; Salmons and Gunzberg, 1993, Human Gene Therapy 4: 129-141 ; Miller, et al., 1993, Meth. Enzymol. 217:581- 599; and Grossman and Wilson, 1993, Curr. Opin. in Genetics and Devel. 3: 110-1 14.

In certain embodiments, the retrovirus is a recombinant replication competent retrovirus comprising: a nucleic acid sequence encoding a retroviral GAG protein; a nucleic acid sequence encoding a retroviral POL protein; a nucleic acid sequence encoding a retroviral envelope; an oncoretroviral polynucleotide sequence comprising Long-Terminal Repeat (LTR) sequences at

the 5' and 3' end of the oncoretroviral polynucleotide sequence; a cassette comprising an internal ribosome entry site (IRES) operably linked to a coding sequence for an Affimer polypeptide wherein the cassette is positioned 5' to the U3 region of the 3' LTR and 3' to the sequence encoding the retroviral envelope; and cis-acting sequences for reverse transcription, packaging and integration in a target cell.

In certain embodiments, the retrovirus is a recombinant replication competent retrovirus comprising: a retroviral GAG protein; a retroviral POL protein; a retroviral envelope; a retroviral polynucleotide comprising Long-Terminal Repeat (LTR) sequences at the 3' end of the retroviral polynucleotide sequence, a promoter sequence at the 5' end of the retroviral polynucleotide, the promoter being suitable for expression in a mammalian cell, a gag nucleic acid domain, a pol nucleic acid domain and an env nucleic acid domain; a cassette comprising an Encoded Affimer sequence, wherein the cassette is positioned 5' to the 3' LTR and is operably linked and 3' to the env nucleic acid domain encoding the retroviral envelope; and cis-acting sequences necessary for reverse transcription, packaging and integration in a target cell.

In certain preferred embodiments of the recombinant replication competent retrovirus, the envelope is chosen from one of amphotropic, polytropic, xenotropic, 10A1, GALV, Baboon endogenous virus, RD114, rhabdovirus, alphavirus, measles or influenza virus envelopes.

In certain preferred embodiments of the recombinant replication competent retrovirus, the retroviral polynucleotide sequence is engineered from a virus selected from the group consisting of murine leukemia virus (MLV) , Moloney murine leukemia virus (MoMLV) , Feline leukemia virus (FeLV) , Baboon endogenous retrovirus (BEV) , porcine endogenous virus (PERV) , the cat derived retrovirus RD114, squirrel monkey retrovirus, Xenotropic murine leukemia virus-related virus (XMRV) , avian reticuloendotheliosis virus (REV) , or Gibbon ape leukemia virus (GALV) .

In certain preferred embodiments of the recombinant replication competent retrovirus, retrovirus is a gammaretrovirus.

In certain preferred embodiments of the recombinant replication competent retrovirus, there is a second cassette comprising a coding sequence for a second therapeutic protein, such as another checkpoint inhibitor polypeptide, a co-stimulatory polypeptide and/or a immunostimulatory cytokine (merely as examples), e.g., downstream of the cassette. In certain instances, the second cassette can include an internal ribosome entry site (IRES) or a minipromoter or a polIII promoter operably linked to the coding sequence for the second therapeutic protein.

In certain preferred embodiments of the recombinant replication competent retrovirus, it is a nonlytic, amphotropic retroviral replicating vector which, preferably, selectively infects and replicates in the cells of the tumor microenvironment.

Other Viral Vectors as Expression Constructs

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In the context of vectored intratumoral Encoded Affimer gene transfer, oncolytic viruses have a distinct advantage, as they can specifically target tumor cells, boost therapeutic Affimer polypeptide expression, and amplify antitumor therapeutic responses. Oncolytic viruses, which overlap with certain viral systems described above, promote anti-tumor responses through selective tumor cell killing and induction of systemic anti-tumor immunity. The mechanisms of action are not fully elucidated but are likely to depend on viral replication within transformed cells, induction of primary cell death, interaction with tumor cell anti-viral elements and initiation of innate and adaptive anti-tumor immunity. Reviewed in Kaufman et al. 2015 "Oncolytic viruses: a new class of immunotherapy drugs" Nat Rev Drug Discov. 14(9):642–62. Many of the oncolytic viruses that are currently in the clinic have a natural tropism for cell surface proteins that are aberrantly expressed by cancer cells. To date, AdV, poxviruses, coxsackieviruses, poliovirus, measles virus, Newcastle disease virus, reovirus, and others have entered into early-phase clinical trials. In 2015, the FDA and EMA approved talimogene laherparepvec (T-VEC, Imlygic™), an oncolytic herpes virus armed with the gene for granulocyte–macrophage colony-stimulating factor (GM-CSF). The self-perpetuating nature of oncolytic viruses makes them an appealing platform for Encoded Affimer gene transfer of the present invention, as transgene products can be amplified along with viral replication, thereby maximizing therapeutic effect. Liu et al. 2008 "Oncolytic adenoviruses for cancer gene therapy" Methods Mol Biol. 433:243–58.

In the case of Affimer polypeptides that are large fusion proteins, i.e., which comprise other protein domains beyond a single Affimer domain, local intratumoral expression can present an appealing strategy to overcome poor penetration in solid tumors if and where that might be an issue. Beckman et al. (2007) "Antibody constructs in cancer therapy: protein engineering strategies to improve exposure in solid tumors" Cancer 109(2):170–9; and Dronca et al. 2015 "Immunomodulatory antibody therapy of cancer: the closer, the better" Clin Cancer Res. 21(5):944–6. Likewise, intratumoral delivery of the Encoded Affimer construct and concomitant local expression of the Affimer polypeptide can create a better therapeutic index where dose-limiting toxicities might otherwise prevent reaching the effective intratumoral concentration for efficacy when the Affimer polypeptide is delivered (or expressed) systemically.

In the case of the immuno-oncology active Affimer polypeptides of the present invention, such as checkpoint inhibitors or costimulatory agonists, the immunomodulatory nature of these Affimers are very relevant to the use of oncolytic viruses. Indeed, for oncolytic virus therapy, it is desirable to override immune checkpoint inhibitor networks and thereby create a pro-inflammatory environment within the cancer. Numerous clinical trials are currently underway to evaluate the combination of oncolytic viruses and conventional immunomodulatory mAb administration. Kaufman et al. 2015 "Oncolytic viruses: a new class of immunotherapy drugs" Nat Rev Drug Discov. 14(9):642–62; and Lichty et al. 2014 "Going viral with cancer immunotherapy" Nat Rev Cancer. 14(8):559–67. However, systemic treatment with checkpoint-blocking mAbs can lead to severe immune-related adverse effects, highlighting the opportunity for local therapies, e.g. via Encoded Affimer-armed oncolytic viruses. Different studies have pursued this approach and can be readily adapted for use with the subject Encoded Affimers. Dias et al. armed a replication-deficient and -competent oncolytic AdV with

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an anti-human CTLA-4 mAb. Dias et al. 2012 "Targeted cancer immunotherapy with oncolytic adenovirus coding for a fully human monoclonal antibody specific for CTLA-4" Gene Ther. 19(10):988–98. Another system recently described (and that can be adapted for use with the Encoded Affimers of the present invention) involved armed oncolytic vaccinia viruses with anti-murine programmed cell death protein 1 (PD-1) Fab, scFv or full-length mAb. Reflecting virus replication, mAb levels in the tumor peaked 3–5 days after intratumoral injection at 9 or 30 μg/ml, depending on the tumor model. Serum mAb levels followed the same trend, albeit threefold or more lower, although mAb detection was lost after 5 days. Intratumorally expressed mAbs lasted longer compared to intratumoral injection of anti-PD-1 mAb protein, with follow-up limited to 11 days after injection. Fab and scFv expression were not reported. Anti-tumor responses of the virus armed with either the anti-PD-1 scFv or mAb were superior to the unarmed virus and as effective as the combination of the unarmed virus and systemic anti-PD-1 mAb protein injections. Kleinpeter et al. 2016 "Vectorization in an oncolytic vaccinia virus of an antibody, a Fab and a scFv against programmed cell death-1 (PD-1) allows their intratumoral delivery and an improved tumor-growth inhibition" Oncoimmunology. 5(10):e1220467 (online). Also recently, intratumoral administration of a combination of an oncolytic AdV and a helper-dependent AdV, armed with an anti-PD-L1 mini-antibody (a scFv CH2-CH3 fusion protein), improved the anti-tumor effect of chimeric antigen receptor (CAR) T cell therapy in mice. The benefits of locally produced anti-PD-L1 mini-antibody could not be achieved by anti-PD-L1 IgG infusion plus CAR T-cells and co-administration of an unarmed AdV. Tanoue et al 2017 "Armed oncolytic adenovirus expressing PD-L1 mini-body enhances anti-tumor effects of chimeric antigen receptor T-cells in solid tumors" Cancer Res. 77(8):2040–51. The use of that system, particularly in combination with CAR-T cell therapy, is also contemplated for use in delivering an Encoded Affimer to a target tumor.

Other viral vectors may be employed as a gene delivery system in the present invention. Vectors derived from viruses such as vaccinia virus (Puhlmann M. et al., Human Gene Therapy, 10:649-657(1999); Ridgeway, "Mammalian expression vectors," In: Vectors: A survey of molecular cloning vectors and their uses. Rodriguez and Denhardt, eds. Stoneham: Butterworth, 467-492(1988); Baichwal and Sugden, "Vectors for gene transfer derived from animal DNA viruses: Transient and stable expression of transferred genes," In: Kucherlapati R, ed. Gene transfer. New York: Plenum Press, 117-148(1986) and Coupar et al., Gene, 68:1-10(1988)), lentivirus (Wang G. et al., J. Clin. Invest., 104(11):R55-62(1999)), herpes simplex virus (Chamber R., et al., Proc. Natl. Acad. Sci USA, 92:1411-1415(1995)), poxvirus (GCE, NJL, Krupa M, Esteban M., The poxvirus vectors MVA and NYVAC as gene delivery systems for vaccination against infectious diseases and cancer Curr Gene Ther 8(2):97-120(2008)), reovirus, measles virus, Semliki Forest virus, and polioviruses may be used in the present delivery systems for transferring the gene of interest into cells. They offer several attractive features for various mammalian cells. Also included are hepatitis B viruses.

b. Non-Viral Vectors

In 1990, Wolff et al. showed how injection of naked plasmid DNA (pDNA) into the skeletal muscle of mice led to the local expression of the encoded protein, kick-starting the field of

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DNA-based therapeutics. See Wolff et al. 1990 "Direct gene transfer into mouse muscle in vivo" Science. 247(4949 Pt 1):1465–8. The use of "pDNA" for delivering Encoded Affimers of the present invention waives the need for a virus as biological vector, and presents an appealing platform for Encoded Affimer gene transfer. Compared to viral vectors, pDNA is considered low-immunogenic (allowing e.g. repeated dosing), is cheaper to produce, ship, and store, and has a much longer shelf-life. After entry in the nucleus, pDNA remains in a non-replicating non-integrating episomal state, and is lost during the breakdown of the nuclear envelope at mitosis. pDNA has no defined restrictions regarding the size of the transgene compared to viral vectors, and its modular nature allows for straightforward molecular cloning, making them easy to manipulate and design for therapeutic use. Hardee et al. 2017 "Advances in non-viral DNA vectors for gene therapy" Genes. 8(2):65. Plasmids are used in about 17% of the ongoing or completed gene therapy clinical trials, and showed to be well-tolerated and safe.

The method of DNA administration can greatly impact transgene expression. In vivo DNA-mediated Encoded Affimer gene transfer can utilize such physical methods of transfection used for antibody gene transfer, such as electroporation or hydrodynamic injection. Electroporation presents the propagation of electrical fields within tissues, which induces a transient increase in cell membrane permeability. Electrotransfer of DNA is a multistep process, involving (i) electrophoretic migration of DNA towards the plasma membrane, (ii) DNA accumulation and interaction with the plasma membrane, and (iii) intracellular trafficking of the DNA to the nucleus, after which gene expression can commence. Heller LC. 2015 "Gene electrotransfer clinical trials" Adv Genet. 89:235–62. Intramuscular, intratumoral and intradermal administration have been evaluated in clinical trials and are also suitable target tissues for electroporation of Encoded Affimer constructs.

Hydrodynamic-based transfection utilizes the i.v. injection of high volumes of pDNA, driving DNA molecules out of the blood circulation and into tissue. Other potentially less invasive physical delivery methods include sonoporation and magnetofection. DNA uptake can also be improved by complexing the molecules with chemical delivery vehicles (e.g. cationic lipids or polymers and lipid nanoparticles). Such techniques can also be applied to in vivo DNA-mediated Encoded Affimer gene transfer.

In addition to the choice of delivery method, Encoded Affimer transgene expression can be improved by modifying the make-up of pDNA constructs. See, for example, Hardee et al. 2017 "Advances in non-viral DNA vectors for gene therapy" Genes 8(2):65; and Simcikova et al. 2015 "Towards effective non-viral gene delivery vector" Biotechnol Genet Eng Rev. 31(1–2):82–107. Conventional pDNA consists of a transcription unit and bacterial backbone. The transcription unit carries the Encoded Affimer sequence along with regulatory elements. The bacterial backbone includes elements like an antibiotic resistance gene, an origin of replication, unmethylated CpG motifs, and potentially cryptic expression signals. Some of these sequences are required for the production of plasmid DNA. However, in general, for therapeutic Encoded Affimer gene therapy the presence of a bacterial backbone will likely be counterproductive. However, there are a variety of different types of available minimal vectors that can be selected, including minicircle DNA (mcDNA) which already been used for antibody gene

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transfer and can be readily adapted for Encoded Affimer gene transfer. Minicircles are plasmid molecules devoid of bacterial sequences, generated via a process of recombination, restriction and/or purification. Simcikova et al. 2015 supra. Elimination of the bacterial backbone has shown higher transfection efficiency and prolonged transgene expression in a variety of tissues.

Also provided herein is a linear nucleic acid, or linear expression cassette ("LEC"), that is capable of being efficiently delivered to a subject via electroporation and expressing the Encoded Affimer sequence included therein. The LEC may be any linear DNA devoid of any phosphate backbone. The LEC may contain a promoter, an intron, a stop codon, and/or a polyadenylation signal. The expression of the Encoded Affimer coding sequence may be controlled by the promoter.

Plasmid Vectors

In certain embodiments, the subject Encoded Affimers constructs are delivered as plasmid vectors. Plasmid vectors have been extensively described in the art and are well known to those of skill in the art. See e.g. Sambrook et al., 1989, cited above. In the last few years, plasmid vectors have been used as DNA vaccines for delivering antigen-encoding genes to cells in vivo. They are particularly advantageous for this because they reduced safety concerns relative to other vectors. These plasmids, however, having a promoter compatible with the host cell, can express a peptide epitope encoded by nucleic acid within the plasmid. Other plasmids are well known to those of ordinary skill in the art. Additionally, plasmids may be custom designed using restriction enzymes and ligation reactions to remove and add specific fragments of DNA. Plasmids may be delivered by a variety of parenteral, mucosal and topical routes. For example, the DNA plasmid can be injected by intramuscular, intradermal, subcutaneous, or other routes. It may also be administered by intranasal sprays or drops, rectal suppository and orally. It may also be administered into the epidermis or a mucosal surface using a gene-gun. The plasmids may be given in an aqueous solution, dried onto gold particles or in association with another DNA delivery system including but not limited to liposomes, dendrimers, cochleate and microencapsulation.

To expand the application and efficiency of using plasmid DNA to deliver an Encoded Affimer construct to tissue in vivo, different approaches can be pursued based on principles producing higher mAb expression or overall efficacy in prior art reports. A first strategy simply relies on giving multiple or repeated pDNA doses. Kitaguchi et al. 2005 “Immune deficiency enhances expression of recombinant human antibody in mice after nonviral in vivo gene transfer” Int J Mol Med 16(4):683–8; and Yamazaki et al. 2011 “Passive immune-prophylaxis against influenza virus infection by the expression of neutralizing anti-hemagglutinin monoclonal antibodies from plasmids” Jpn J Infect Dis. 64(1):40–9. Another approach relates to the use of a delivery adjuvant. pDNA electrotransfer can be enhanced by pre-treating the muscle with hyaluronidase, an enzyme that transiently breaks down hyaluronic acid, decreasing the viscosity of the extracellular matrix and facilitating DNA diffusion. Yamazaki et al. 2011, supra; and McMahon et al. 2001 “Optimisation of electrotransfer of plasmid into skeletal muscle by

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pretreatment with hyaluronidase: increased expression with reduced muscle damage" Gene Ther. 8(16):1264–70. For antibody gene transfer, this led to an increase in mAb expression by approximately 3.5-fold, achieving plasma peak titers of 3.5 µg/ml with 30 µg pDNA, and would can be adapted by one skilled in the art for Encoded Affimer gene transfer. Still another strategy focuses on antibody or cassette engineering. Following codon-, RNA- and leader sequence-optimization, peak serum mAb or Fab titers have been attained with intramuscular electrotransfer of 'optimized' pDNA. See, for example, Flingai et al. 2015 "Protection against dengue disease by synthetic nucleic acid antibody prophylaxis/immunotherapy" Sci Rep. 5:12616.

The purpose of the plasmid is the efficient delivery of nucleic acid sequences to and expression of therapeutic Affimer polypeptides in a cell or tissue. In particular, the purpose of the plasmid may be to achieve high copy number, avoid potential causes of plasmid instability and provide a means for plasmid selection. As for expression, the nucleic acid cassette contains the necessary elements for expression of the Encoded Affimer within the cassette. Expression includes the efficient transcription of an inserted gene, nucleic acid sequence, or nucleic acid cassette with the plasmid. Thus, in one aspect, a plasmid is provided for expression of Encoded Affimer construct which includes an expression cassette comprising the coding sequence for the Affimer polypeptide; also referred to as a transcription unit. When a plasmid is placed in an environment suitable for epitope expression, the transcriptional unit will express the Affimer polypeptide and anything else encoded in the construct. The transcription unit includes a transcriptional control sequence, which is transcriptionally linked with a cellular immune response element coding sequence. Transcriptional control sequence may include promoter/enhancer sequences such as cytomegalovirus (CMV) promoter/enhancer sequences, such as described above. However, those skilled in the art will recognize that a variety of other promoter sequences suitable for expression in mammalian cells, including human patient cells, are known and can similarly be used in the constructs disclosed herein. The level of expression of the Affimer polypeptide will depend on the associated promoter and the presence and activation of an associated enhancer element.

In certain embodiments, the Encoded Affimer sequence (encoding the desired Affimer polypeptide) can be cloned into an expression plasmid which contains the regulatory elements for transcription, translation, RNA stability and replication (i.e., including a transcriptional control sequence). Such expression plasmids are well known in the art and one of ordinary skill would be capable of designing an appropriate expression construct for producing a recombinant Affimer polypeptide in vivo.

Minicircle

Minicircle (mcDNA) -based antibody gene transfer can also be adapted for delivery of Encoded Affimers to tissues in vivo. Under certain circumstances, plasmid DNA used for non-viral gene delivery can cause unacceptable inflammatory responses. Where this happens, immunotoxic responses are largely due to the presence of unmethylated CpG motifs and their associated stimulatory sequences on plasmids following bacterial propagation of plasmid DNA. Simple

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methylation of DNA in vitro may be enough to reduce an inflammatory response, but can result in reduced gene expression. The removal of CpG islands by cloning out, or elimination of non-essential sequences has been a successful technique for reducing inflammatory responses. Yew et al. 2000 "Reduced inflammatory response to plasmid DNA vectors by elimination and inhibition of immunostimulatory CpG motifs" Mol Ther 1(3), 255-62.

Since bacterial DNA contains on average 4 times more CpG islands than mammalian DNA, a good solution is to eliminate entirely the bacterial control regions, such as the origin of replication and antibiotic resistance genes, from gene delivery vectors during the process of plasmid production. Thus, the "parent" plasmid is recombined into a "minicircle" which generally comprises the gene to be delivered (in this case, the Encoded Affimer coding sequence) and suitable control regions for its expression, and a miniplasmid which generally comprises the remainder of the parent plasmid.

Removal of bacterial sequences needs to be efficient, using the smallest possible excision site, whilst creating supercoiled DNA minicircles which consist solely of gene expression elements under appropriate--preferably mammalian--control regions. Some techniques for minicircle production use bacterial phage lamda (λ) integrase mediated recombination to produce minicircle DNA. See, for example, Darquet, et al. 1997 Gene Ther 4(12): 1341-9; Darquet et al. 1999 Gene Ther 6(2): 209-18; and Kreiss, et al. 1998 Appl Micbiol Biotechnol 49(5):560-7).

Therefore, embodiments of nucleic acid constructs described herein may be processed in the form of minicircle DNA. Minicircle DNA pertains to small (2-4 kb) circular plasmid derivatives that have been freed from all prokaryotic vector parts. Since minicircle DNA vectors contain no bacterial DNA sequences, they are less likely to be perceived as foreign and destroyed. As a result, these vectors can be expressed for longer periods of time compared to certain conventional plasmids. The smaller size of minicircles also extends their cloning capacity and facilitates their delivery into cells. Kits for producing minicircle DNA are known in the art and are commercially available (System Biosciences, Inc., Palo Alto, Calif.). Information on minicircle DNA is provided in Dietz et al., Vector Engineering and Delivery Molecular Therapy (2013); 21 8, 1526-1535 and Hou et al., Molecular Therapy—Methods & Clinical Development, Article number: 14062 (2015) doi:10.1038/mtm.2014.62. More information on Minicircles is provided in Chen Z Y, He C Y, Ehrhardt A, Kay M A. Mol Ther. 2003 September; 8(3):495-500 and Minicircle DNA vectors achieve sustained expression reflected by active chromatin and transcriptional level. Gracey Maniar L E, Maniar J M, Chen Z Y, Lu J, Fire A Z, Kay M A. Mol Ther. 2013 January; 21(1):131-8

As a nonlimiting example, a minicircle DNA vector may be produced as follows. An expression cassette, which comprises the Encoded Affimer coding sequence along with regulatory elements for its expression, is flanked by attachment sites for a recombinase. A sequence encoding the recombinase is located outside of the expression cassette and includes elements for inducible expression (such as, for example, an inducible promoter). Upon induction of recombinase expression, the vector DNA is recombined, resulting in two distinct circular DNA molecules. One of the circular DNA molecules is relatively small, forming a minicircle that comprises the expression cassette for the Encoded Affimer; this minicircle DNA vector is devoid of any bacterial DNA sequences. The second circular DNA sequence contains the

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remaining vector sequence, including the bacterial sequences and the sequence encoding the recombinase. The minicircle DNA containing the Encoded Affimer sequence can then be separately isolated and purified. In certain embodiments, a minicircle DNA vector may be produced using plasmids similar to pBAD.Φ.C31.hFIX and pBAD.Φ.C31.RHB. See, e.g., Chen et al. (2003) Mol. Ther. 8:495-500.

Exemplary recombinases that may be used for creating a minicircle DNA vector include, but are not limited to, Streptomyces bacteriophage Φ31 integrase, Cre recombinase, and the λ integrase/DNA topoisomerase IV complex. Each of these recombinases catalyzes recombination between distinct sites. For example, Φ31 integrase catalyzes recombination between corresponding attP and attB sites, Cre recombinase catalyzes recombination between loxP sites, and the λ integrase/DNA topoisomerase IV complex catalyzes recombination between bacteriophage λ attP and attB sites. In certain embodiments, such as, for example, with Φ31 integrase or with λ integrase in the absence of the λ is protein, the recombinase mediates an irreversible reaction to yield a unique population of circular products and thus high yields. In other embodiments, such as, for example, with Cre recombinase or with λ integrase in the presence of the λ is protein, the recombinase mediates a reversible reaction to yield a mixture of circular products and thus lower yields. The reversible reaction by Cre recombinase can be manipulated by employing mutant loxP71 and loxP66 sites, which recombine with high efficiency to yield a functionally impaired P71/66 site on the minicircle molecule and a wild-type loxP site on the minicircle molecule, thereby shifting the equilibrium towards the production of the minicircle DNA product.

Published US Application 20170342424 also describes a system making use of a parent plasmid which is exposed to an enzyme which causes recombination at recombination sites, thereby forming a (i) minicircle including the Encoded Affimer sequence and (ii) a miniplasmid comprising the remainder of the parent plasmid. One recombination site is modified at the 5' end such that its reaction with the enzyme is less efficient than the wild type site, and the other recombination site is modified at the 3' end such that its reaction with the enzyme is less efficient than the wild type site, and the other recombination site is modified at the 3' end such that its reaction with the enzyme is less efficient than the wild type site, both modified sites being located in the minicircle after recombination. This favors the formation of minicircle.

c. RNA-mediated Encoded Affimer Gene Transfer

Exemplary nucleic acids or polynucleotides for the encoded Affimer polypeptides of the present invention include, but are not limited to, ribonucleic acids (RNAs), deoxyribonucleic acids (DNAs), threose nucleic acids (TNAs), glycol nucleic acids (GNAs), peptide nucleic acids (PNAs), locked nucleic acids (LNAs, including LNA having a β- D-ribo configuration, a-LNA having an a-L-ribo configuration (a diastereomer of LNA), 2'-amino-LNA having a 2 '-amino functionalization, and 2'-amino- a-LNA having a 2'-amino functionalization), ethylene nucleic acids (ENA), cyclohexenyl nucleic acids (CeNA) or hybrids or combinations thereof.

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mRNA presents an emerging platform for antibody gene transfer that can be adapted by those skilled in the art for delivery of Encoded Affimer constructs of the present invention. Although current results differ considerably, in certain instances the mRNA constructs appear to be able to rival viral vectors in terms of generated serum mAb titers. Levels were in therapeutically relevant ranges within hours after mRNA administration, a marked shift in speed compared to DNA. The use of lipid nanoparticles (LNP) for mRNA transfection, rather than the physical methods typically required for DNA, can provide significant advantages in certain embodiments towards application range.

In their 1990 study, Wolff et al. (1990, supra) found that, in addition to pDNA, intramuscular injection of in vitro transcribed (IVT) mRNA also led to local expression of the encoded protein. mRNA was not pursued as actively as DNA at that time because of its low stability. Progress over the past years allowed mRNA to catch up with DNA and viral vectors as a tool for gene transfer. Reviewed in Sahin et al. (2014) "mRNA-based therapeutics: developing a new class of drugs" Nat Rev Drug Discov. 13(10):759–80. Conceptually, there are several differences with these expression platforms. mRNA does not need to enter into the nucleus to be functional. Once it reaches the cytoplasm, mRNA is translated instantly. mRNA-based therapeutics are expressed more transiently compared to DNA- or viral vector-mediated gene transfer, and do not pose the risk of insertional mutagenesis in the host genome. mRNA production is relatively simple and inexpensive. In terms of administration, mRNA uptake can be enhanced using electroporation. Broderick et al. 2017 "Enhanced delivery of DNA or RNA vaccines by electroporation" Methods Mol Biol. 2017;1499:193–200. Most focus, however, has gone to non-physical transfection methods. Indeed, a variety of mRNA complexing formulations have been developed, including lipid nanoparticles (LNP), which have proven to be safe and very efficient mRNA carriers for administration in a variety of tissues and i.v. Pardi et al. 2015 "Expression kinetics of nucleoside-modified mRNA delivered in lipid nanoparticles to mice by various routes" J Control Release 217:345–51. In line with this progress, IVT mRNA has reached the stage of clinical evaluation.

Beissert et al. WO2017162266 "RNA Replicon for Versatile and Efficient Gene Expression" describes agents and methods suitable for efficient expression of Affimers of the present invention, such as suitable for immunotherapeutic treatment for the prevention and therapy of tumors. For instance, the Affimer polypeptide coding sequence can be provided as an RNA replicon comprising a 5' replication recognition sequence such as from an alphavirus 5' replication recognition sequence. In certain embodiments, the RNA replicon comprises a (modified) 5' replication recognition sequence and an open reading frame encoding the Affimer polypeptide, in particular located downstream from the 5' replication recognition sequence such as that the 5' replication recognition sequence and the open reading frame do not overlap, e.g. the 5' replication recognition sequence does not contain a functional initiation codon and preferably does not contain any initiation codon. Most preferably, the initiation codon of the open reading frame encoding the Affimer polypeptide is in the 5'→ 3' direction of the RNA replicon.

In certain embodiments, to prevent immune activation, modified nucleosides can be incorporated into the in vitro–transcribed mRNA. In certain embodiments, the IVT RNA can be

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5' capped, such an m7G5'ppp5'G2´-O-Met-capped IVT. Efficient translation of the modified mRNA can be ensured by removing double-stranded RNA. Moreover, the 5' and 3' UTRs and the poly(A) tail can be optimized for improved intracellular stability and translational efficiency. See, for example, Stadler et al. (2017) Nature Medicine 23:815–817 and Kariko et al. WO/2017/036889 "Method for Reducing Immunogenicity of RNA".

In certain embodiments, the mRNA that encodes the Affimer polypeptide may include at least one chemical modification described herein. As a non-limiting example, the chemical modification may be 1 -methylpseudouridine, 5-methylcytosine or 1 -methylpseudouridine and 5-methylcytosine. In one embodiment, linear polynucleotides encoding one or more Affimer polypeptides of the present invention which are made using only in vitro transcription (IVT) enzymatic synthesis methods are referred to as "IVT polynucleotides." Methods of making IVT polynucleotides are known in the art and are described in PCT Application WO2013/151666, the contents of which are incorporated herein by reference in their entirety.

In another embodiment, the polynucleotides that encode the Affimer polypeptide of the present invention have portions or regions which differ in size and/or chemical modification pattern, chemical modification position, chemical modification percent or chemical modification population and combinations of the foregoing are known as "chimeric polynucleotides." A "chimera" according to the present invention is an entity having two or more incongruous or heterogeneous parts or regions. As used herein a "part" or "region" of a polynucleotide is defined as any portion of the polynucleotide which is less than the entire length of the polynucleotide. Such constructs are taught in for example PCT Application WO2015/034928.

In yet another embodiment, the polynucleotides of the present invention that are circular are known as "circular polynucleotides" or "circP." As used herein, "circular polynucleotides" or "circP" means a single stranded circular polynucleotide which acts substantially like, and has the properties of, an RNA. The term "circular" is also meant to encompass any secondary or tertiary configuration of the circP. Such constructs are taught in for example PCT Application WO2015/034925 and WO2015/034928, the contents of each of which are incorporated herein by reference in their entirety.

Exemplary mRNA (and other polynucleotides) that can be used to encode Affimer polypeptides of the present invention include those which can be adapted from the specifications and figures of, for example, PCT Publications WO2017/049275, WO2016/118724, WO2016/118725, WO2016/011226, WO2015/196128, WO/2015/196130, WO/2015/196118, WO/2015/089511, with WO2015/105926 (the later titled "Polynucleotides for the In Vivo Production Of Antibodies"), each of which is incorporated by reference herein.

Electroporation, as described below, is one exemplary method for introducing mRNA or other polynucleotides into a cell.

Lipid-containing nanoparticle compositions have proven effective as transport vehicles into cells and/or intracellular compartments for a variety of RNAs (and related polynucleotides described herein). These compositions generally include one or more "cationic" and/or ionizable lipids, phospholipids including polyunsaturated lipids, structural lipids (e.g., sterols),

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and lipids containing polyethylene glycol (PEG lipids). Cationic and/or ionizable lipids include, for example, amine-containing lipids that can be readily protonated.

d. Delivery of Encoded Affimer Constructs into Target Cells

The introduction into host cell of the gene delivery system can be performed through various methods known to those skilled in the art.

Where the present gene delivery system is constructed on the basis of viral vector construction, delivery can be performed as conventional infection methods known in the art.

Physical methods to enhance delivery both viral and non-viral Encoded Affimer constructs include electroporation (Neumann, E. et al., EMBO J., 1:841(1982); and Tur-Kaspa et al., Mol. Cell Biol., 6:716-718(1986)), gene bombardment (Yang et al., Proc. Natl. Acad. Sci., 87:9568-9572 (1990) where DNA is loaded onto (e.g., gold) particles and forced to achieve penetration of the DNA into the cells, sonoporation, magnetofection, hydrodynamic delivery and the like, all of which are known to those of skill in the art.

Electroporation

In the past several years, there has been a great advance in the plasmid DNA delivery technology that is utilized for in vivo production of proteins. This included codon optimization for expression in human cells, RNA optimization to improve mRNA stability as well as more efficient translation at the ribosomal level, the addition of specific leader sequences to enhance translation efficiency, the creation of synthetic inserts to further enhance production in vivo and the use of improved adaptive electroporation (EP) delivery protocols to improve in vivo delivery. EP assists in the delivery of plasmid DNA by generating an electrical field that allows the DNA to pass into the cell more efficiently. In vivo electroporation is a gene delivery technique that has been used successfully for efficient delivery of plasmid DNA to many different tissues. Kim et al. "Gene therapy using plasmid DNA-encoded anti-HER2 antibody for cancers that overexpress HER2" (2016) Cancer Gene Ther. 23(10): 341–347 teaches a vector and electroporation system for intramuscular injection and in vivo electroporation of the plasmids that results in high and sustained antibody expression in sera; the plasmid and electroporation system of Kim et al. can be readily adapted for the in vivo delivery of a plasmid for expressing an encoded Affimer of the present invention.

Accordingly, in certain particular embodiments of the present disclosure, the Encoded Affimer construct is introduced into target cells via electroporation.

Administration of the composition via electroporation may be accomplished using electroporation devices that can be configured to deliver to a desired tissue of a mammal, a pulse of energy effective to cause reversible pores to form in cell membranes, and preferable the pulse of energy is a constant current similar to a pre-set current input by a user. The electroporation device may comprise an electroporation component and an electrode assembly or handle assembly. The electroporation component may include and incorporate one or more of the various elements of the electroporation devices, including: controller,

current waveform generator, impedance tester, waveform logger, input element, status reporting element, communication port, memory component, power source, and power switch. The electroporation may be accomplished using an in vivo electroporation device, for example CELLECTRA EP system (VGX Pharmaceuticals, Blue Bell, Pa.) or Elgen electroporator (Genetronics, San Diego, Calif.) to facilitate transfection of cells by the plasmid.

The electroporation component may function as one element of the electroporation devices, and the other elements are separate elements (or components) in communication with the electroporation component. The electroporation component may function as more than one element of the electroporation devices, which may be in communication with still other elements of the electroporation devices separate from the electroporation component. The elements of the electroporation devices existing as parts of one electromechanical or mechanical device may not limited as the elements can function as one device or as separate elements in communication with one another. The electroporation component may be capable of delivering the pulse of energy that produces the constant current in the desired tissue, and includes a feedback mechanism. The electrode assembly may include an electrode array having a plurality of electrodes in a spatial arrangement, wherein the electrode assembly receives the pulse of energy from the electroporation component and delivers same to the desired tissue through the electrodes. At least one of the plurality of electrodes is neutral during delivery of the pulse of energy and measures impedance in the desired tissue and communicates the impedance to the electroporation component. The feedback mechanism may receive the measured impedance and can adjust the pulse of energy delivered by the electroporation component to maintain the constant current.

A plurality of electrodes may deliver the pulse of energy in a decentralized pattern. The plurality of electrodes may deliver the pulse of energy in the decentralized pattern through the control of the electrodes under a programmed sequence, and the programmed sequence is input by a user to the electroporation component. The programmed sequence may comprise a plurality of pulses delivered in sequence, wherein each pulse of the plurality of pulses is delivered by at least two active electrodes with one neutral electrode that measures impedance, and wherein a subsequent pulse of the plurality of pulses is delivered by a different one of at least two active electrodes with one neutral electrode that measures impedance.

The feedback mechanism may be performed by either hardware or software. The feedback mechanism may be performed by an analogue closed-loop circuit. The feedback occurs every 50 µs, 20 µs, 10 µs or 1 µs, but is preferably a real-time feedback or instantaneous (i.e., substantially instantaneous as determined by available techniques for determining response time). The neutral electrode may measure the impedance in the desired tissue and communicates the impedance to the feedback mechanism, and the feedback mechanism responds to the impedance and adjusts the pulse of energy to maintain the constant current at a value similar to the pre-set current. The feedback mechanism may maintain the constant current continuously and instantaneously during the delivery of the pulse of energy.

Examples of electroporation devices and electroporation methods that may facilitate delivery of the Encoded Affimer constructs of the present invention, include those described in U.S.

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Pat. Nos. 7,245,963; 6,302,874; 5,676,646; 6,241,701; 6,233,482; 6,216,034; 6,208,893; 6,192,270; 6,181,964; 6,150,148; 6,120,493; 6,096,020; 6,068,650; and 5,702,359, the contents of which are incorporated herein by reference in their entirety. The electroporation may be carried out via a minimally invasive device.

In certain embodiments, the electroporation is carried using a minimally invasive electroporation device ("MID"). The device may comprise a hollow needle, DNA cassette, and fluid delivery means, wherein the device is adapted to actuate the fluid delivery means in use so as to concurrently (for example, automatically) inject the Encoded Affimer nucleic acid construct into body tissue during insertion of the needle into the body tissue. This has the advantage that the ability to inject the DNA and associated fluid gradually while the needle is being inserted leads to a more even distribution of the fluid through the body tissue. The pain experienced during injection may be reduced due to the distribution of the DNA being injected over a larger area.

The MID may inject the Encoded Affimer nucleic acid construct into tissue without the use of a needle. The MID may inject the Encoded Affimer nucleic acid construct as a small stream or jet with such force that the nucleic acid pierces the surface of the tissue and enters the underlying tissue and/or muscle. The force behind the small stream or jet may be provided by expansion of a compressed gas, such as carbon dioxide through a micro-orifice within a fraction of a second. Examples of minimally invasive electroporation devices, and methods of using them, are described in published U.S. Patent Application No. 20080234655; U.S. Pat. No. 6,520,950; U.S. Pat. No. 7,171,264; U.S. Pat. No. 6,208,893; U.S. Pat. No. 6,009,347; U.S. Pat. No. 6,120,493; U.S. Pat. No. 7,245,963; U.S. Pat. No. 7,328,064; and U.S. Pat. No. 6,763,264, the contents of each of which are herein incorporated by reference.

The MID may comprise an injector that creates a high-speed jet of liquid that painlessly pierces the tissue. Such needle-free injectors are commercially available. Examples of needle-free injectors that can be utilized herein include those described in U.S. Pat. Nos. 3,805,783; 4,447,223; 5,505,697; and 4,342,310, the contents of each of which are herein incorporated by reference.

A desired Encoded Affimer nucleic acid construct in a form suitable for direct or indirect electrotransport may be introduced (e.g., injected) using a needle-free injector into the tissue to be treated, usually by contacting the tissue surface with the injector so as to actuate delivery of a jet of the agent, with sufficient force to cause penetration of the nucleic acid into the tissue. For example, if the tissue to be treated is a mucosa, skin or muscle, the agent is projected towards the mucosal or skin surface with sufficient force to cause the agent to penetrate through the stratum corneum and into dermal layers, or into underlying tissue and muscle, respectively. Needle-free injectors are well suited to deliver Encoded Affimer nucleic acid construct to all types of tissues, including into tumors (intratumoral delivery).

The MID may have needle electrodes that electroporate the tissue. By pulsing between multiple pairs of electrodes in a multiple electrode array, for example set up in rectangular or square patterns, provides improved results over that of pulsing between a pair of electrodes. Disclosed, for example, in U.S. Pat. No. 5,702,359 entitled "Needle Electrodes for Mediated

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Delivery of Drugs and Genes" is an array of needles wherein a plurality of pairs of needles may be pulsed during the therapeutic treatment. In that application, which is incorporated herein by reference as though fully set forth, needles were disposed in a circular array, but have connectors and switching apparatus enabling a pulsing between opposing pairs of needle electrodes. A pair of needle electrodes for delivering the Encoded Affimer nucleic acid construct to cells may be used. Such a device and system is described in U.S. Pat. No. 6,763,264, the contents of which are herein incorporated by reference. Alternatively, a single needle device may be used that allows injection of the DNA and electroporation with a single needle resembling a normal injection needle and applies pulses of lower voltage than those delivered by presently used devices, thus reducing the electrical sensation experienced by the patient.

The MID may comprise one or more electrode arrays. The arrays may comprise two or more needles of the same diameter or different diameters. The needles may be evenly or unevenly spaced apart. The needles may be between 0.005 inches and 0.03 inches, between 0.01 inches and 0.025 inches; or between 0.015 inches and 0.020 inches. The needle may be 0.0175 inches in diameter. The needles may be 0.5 mm, 1.0 mm, 1.5 mm, 2.0 mm, 2.5 mm, 3.0 mm, 3.5 mm, 4.0 mm, or more spaced apart.

The MID may consist of a pulse generator and a two or more-needle vaccine injectors that deliver the Encoded Affimer nucleic acid construct and electroporation pulses in a single step. The pulse generator may allow for flexible programming of pulse and injection parameters via a flash card operated personal computer, as well as comprehensive recording and storage of electroporation and patient data. The pulse generator may deliver a variety of volt pulses during short periods of time. For example, the pulse generator may deliver three 15 volt pulses of 100 ms in duration. An example of such a MID is the Elgen 1000 system by Inovio Biomedical Corporation, which is described in U.S. Pat. No. 7,328,064, the contents of which are herein incorporated by reference.

The MID may be a CELLECTRA (Inovio Pharmaceuticals, Plymouth Meeting, Pa.) device and system, which is a modular electrode system, that facilitates the introduction of a macromolecule, such as an Encoded Affimer nucleic acid construct, into cells of a selected tissue in a body. The modular electrode system may comprise a plurality of needle electrodes; a hypodermic needle; an electrical connector that provides a conductive link from a programmable constant-current pulse controller to the plurality of needle electrodes; and a power source. An operator can grasp the plurality of needle electrodes that are mounted on a support structure and firmly insert them into the selected tissue in a body or plant. The nucleic acid is then delivered via the hypodermic needle into the selected tissue. The programmable constant-current pulse controller is activated and constant-current electrical pulse is applied to the plurality of needle electrodes. The applied constant-current electrical pulse facilitates the introduction of the nucleic acid into the cell between the plurality of electrodes. Cell death due to overheating of cells is minimized by limiting the power dissipation in the tissue by virtue of constant-current pulses. The Cellectra device and system is described in U.S. Pat. No. 7,245,963, the contents of which are herein incorporated by reference.

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The MID may be an Elgen 1000 system (Inovio Pharmaceuticals). The Elgen 1000 system may comprise device that provides a hollow needle; and fluid delivery means, wherein the apparatus is adapted to actuate the fluid delivery means in use so as to concurrently (for example automatically) inject fluid, the described Encoded Affimer nucleic acid construct herein, into body tissue during insertion of the needle into the said body tissue. The advantage is the ability to inject the fluid gradually while the needle is being inserted leads to a more even distribution of the fluid through the body tissue. It is also believed that the pain experienced during injection is reduced due to the distribution of the volume of fluid being injected over a larger area.

In addition, the automatic injection of fluid facilitates automatic monitoring and registration of an actual dose of fluid injected. This data can be stored by a control unit for documentation purposes if desired.

It will be appreciated that the rate of injection could be either linear or non-linear and that the injection may be carried out after the needles have been inserted through the skin of the subject to be treated and while they are inserted further into the body tissue.

Suitable tissues into which fluid may be injected by the apparatus of the present invention include tumor tissue, skin and other epithelial tissues, liver tissue and muscle tissue, merely as examples.

The apparatus further comprises needle insertion means for guiding insertion of the needle into the body tissue. The rate of fluid injection is controlled by the rate of needle insertion. This has the advantage that both the needle insertion and injection of fluid can be controlled such that the rate of insertion can be matched to the rate of injection as desired. It also makes the apparatus easier for a user to operate. If desired means for automatically inserting the needle into body tissue could be provided.

A user could choose when to commence injection of fluid. Ideally however, injection is commenced when the tip of the needle has reached the target tissue and the apparatus may include means for sensing when the needle has been inserted to a sufficient depth for injection of the fluid to commence. This means that injection of fluid can be prompted to commence automatically when the needle has reached a desired depth (which will normally be the depth at which muscle tissue begins). The depth at which muscle tissue begins could for example be taken to be a pre-set needle insertion depth such as a value of 4 mm which would be deemed sufficient for the needle to get through the skin layer.

The sensing means may comprise an ultrasound probe. The sensing means may comprise a means for sensing a change in impedance or resistance. In this case, the means may not as such record the depth of the needle in the body tissue but will rather be adapted to sense a change in impedance or resistance as the needle moves from a different type of body tissue into muscle. Either of these alternatives provides a relatively accurate and simple to operate means of sensing that injection may commence. The depth of insertion of the needle can further be recorded if desired and could be used to control injection of fluid such that the volume of fluid to be injected is determined as the depth of needle insertion is being recorded.

The apparatus may further comprise: a base for supporting the needle; and a housing for receiving the base therein, wherein the base is moveable relative to the housing such that the needle is retracted within the housing when the base is in a first rearward position relative to the housing and the needle extends out of the housing when the base is in a second forward position within the housing. This is advantageous for a user as the housing can be lined up on the skin of a patient, and the needles can then be inserted into the patient's skin by moving the housing relative to the base.

As stated above, it is desirable to achieve a controlled rate of fluid injection such that the fluid is evenly distributed over the length of the needle as it is inserted into the skin. The fluid delivery means may comprise piston driving means adapted to inject fluid at a controlled rate. The piston driving means could for example be activated by a servo motor. However, the piston driving means may be actuated by the base being moved in the axial direction relative to the housing. It will be appreciated that alternative means for fluid delivery could be provided. Thus, for example, a closed container which can be squeezed for fluid delivery at a controlled or non-controlled rate could be provided in the place of a syringe and piston system.

The apparatus described above could be used for any type of injection. It is however envisaged to be particularly useful in the field of electroporation and so it may further comprise means for applying a voltage to the needle. This allows the needle to be used not only for injection but also as an electrode during, electroporation. This is particularly advantageous as it means that the electric field is applied to the same area as the injected fluid. There has traditionally been a problem with electroporation in that it is very difficult to accurately align an electrode with previously injected fluid and so users have tended to inject a larger volume of fluid than is required over a larger area and to apply an electric field over a higher area to attempt to guarantee an overlap between the injected substance and the electric field. Using the present invention, both the volume of fluid injected and the size of electric field applied may be reduced while achieving a good fit between the electric field and the fluid.

U.S. Pat. No. 7,245,963 by Draghia-Akli, et al. describes modular electrode systems and their use for facilitating the introduction of a biomolecule into cells of a selected tissue in a body or plant. The modular electrode systems may comprise a plurality of needle electrodes; a hypodermic needle; an electrical connector that provides a conductive link from a programmable constant-current pulse controller to the plurality of needle electrodes; and a power source. An operator can grasp the plurality of needle electrodes that are mounted on a support structure and firmly insert them into the selected tissue in a body or plant. The biomolecules are then delivered via the hypodermic needle into the selected tissue. The programmable constant-current pulse controller is activated and constant-current electrical pulse is applied to the plurality of needle electrodes. The applied constant-current electrical pulse facilitates the introduction of the biomolecule into the cell between the plurality of electrodes. The entire content of U.S. Pat. No. 7,245,963 is hereby incorporated by reference.

U.S. Patent Pub. 2005/0052630 submitted by Smith, et al. describes an electroporation device which may be used to effectively facilitate the introduction of a biomolecule into cells of a selected tissue in a body or plant. The electroporation device comprises an electro-kinetic

device ("EKD device") whose operation is specified by software or firmware. The EKD device produces a series of programmable constant-current pulse patterns between electrodes in an array based on user control and input of the pulse parameters, and allows the storage and acquisition of current waveform data. The electroporation device also comprises a replaceable electrode disk having an array of needle electrodes, a central injection channel for an injection needle, and a removable guide disk. The entire content of U.S. Patent Pub. 2005/0052630 is hereby incorporated by reference.

The electrode arrays and methods described in U.S. Pat. No. 7,245,963 and U.S. Patent Pub. 2005/0052630 may be adapted for deep penetration into not only tissues such as muscle, but also other tissues or organs. Because of the configuration of the electrode array, the injection needle (to deliver the biomolecule of choice) is also inserted completely into the target organ, and the injection is administered perpendicular to the target issue, in the area that is pre-delineated by the electrodes. The electrodes described in U.S. Pat. No. 7,245,963 and U.S. Patent Pub. 2005/005263 are preferably 20 mm long and 21 gauge.

Use of in vivo electroporation enhances plasmid DNA uptake in tumor tissue, resulting in expression within the tumor, and delivers plasmids to muscle tissue, resulting in systemic expression of secreted proteins, such as cytokines (see, e.g., US8026223). Additional exemplary techniques, vectors and devices for electroporating Affimer polypeptide transgenes into cells in vivo include PCT Publications WO/2017/106795, WO/2016/161201, WO/2016/154473, WO/2016/112359 and WO/2014/066655.

Typically, the electric fields needed for in vivo cell electroporation are generally similar in magnitude to the fields required for cells in vitro. In one embodiment, the magnitude of the electric field ranges from approximately, 10 V/cm to about 1500 V/cm, preferably from about 300 V/cm to 1500 V/cm and preferably from about 1000 V/cm to 1500 V/cm. Alternatively, lower field strengths (from about 10 V/cm to 100 V/cm, and more preferably from about 25 V/cm to 75 V/cm) the pulse length is long. For example, when the nominal electric field is about 25-75 V/cm, if is preferred that the pulse length is about 10 msec.

The pulse length can be about 10 s to about 100 ms. There can be any desired number of pulses, typically one to 100 pulses per second. The delay between pulse sets can be any desired time, such as one second. The waveform, electric field strength and pulse duration may also depend upon the type of cells and the type of molecules that are to enter the cells via electroporation.

Also encompassed are electroporation devices incorporating electrochemical impedance spectroscopy ("EIS"). Such devices provide real-time information on in vivo, in particular, intratumoral electroporation efficiency, allowing for the optimization of conditions. Examples of electroporation devices incorporating EIS can be found, e.g., in WO2016/161201 , which is hereby incorporated by reference.

Uptake of the Encoded Affimer nucleic acid constructs of the present invention may also be enhanced by plasma electroporation also termed avalanche transfection. Briefly, microsecond discharges create cavitation microbubbles at electrode surface. The mechanical force created by the collapsing microbubbles combined with the magnetic field serve to increase transport

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efficiency across the cell membrane as compared with the diffusion mediated transport associated with conventional electroporation. The technique of plasma electroporation is described in United States Patent Nos. 7,923,251 and 8,283,171. This technique may also be employed in vivo for the transformation of cells. Chaiberg, et al (2006) Investigative Ophthalmology & Visual Science 47:4083-4090; Chaiberg, et al United States Patent No 8, 101 169 Issued January 24, 2012.

Other alternative electroporation technologies are also contemplated. In vivo nucleic acid delivery can also be performed using cold plasma. Plasma is one of the four fundamental states of matter, the others being solid, liquid, and gas. Plasma is an electrically neutral medium of unbound positive and negative particles (i.e. the overall charge of a plasma is roughly zero). A plasma can be created by heating a gas or subjecting it to a strong electromagnetic field, applied with a laser or microwave generator. This decreases or increases the number of electrons, creating positive or negative charged particles called ions (Luo, et al. (1998) Phys. Plasma 5:2868-2870) and is accompanied by the dissociation of molecular bonds, if present.

Cold plasmas (i.e., non-thermal plasmas) are produced by the delivery of pulsed high voltage signals to a suitable electrode. Cold plasma devices may take the form of a gas jet device or a dielectric barrier discharge (DBD) device. Cold temperature plasmas have attracted a great deal of enthusiasm and interest by virtue of their provision of plasmas at relatively low gas temperatures. The provision of plasmas at such a temperature is of interest to a variety of applications, including wound healing, anti-bacterial processes, various other medical therapies and sterilization. As noted earlier, cold plasmas (i.e., non-thermal plasmas) are produced by the delivery of pulsed high voltage signals to a suitable electrode. Cold plasma devices may take the form of a gas jet device, a dielectric barrier discharge (DBD) device or multi-frequency harmonic-rich power supply.

Dielectric barrier discharge device, relies on a different process to generate the cold plasma. A dielectric barrier discharge (DBD) device contains at least one conductive electrode covered by a dielectric layer. The electrical return path is formed by the ground that can be provided by the target substrate undergoing the cold plasma treatment or by providing an in-built ground for the electrode. Energy for the dielectric barrier discharge device can be provided by a high voltage power supply, such as that mentioned above. More generally, energy is input to the dielectric barrier discharge device in the form of pulsed DC electrical voltage to form the plasma discharge. By virtue of the dielectric layer, the discharge is separated from the conductive electrode and electrode etching and gas heating is reduced. The pulsed DC electrical voltage can be varied in amplitude and frequency to achieve varying regimes of operation. Any device incorporating such a principle of cold plasma generation (e.g., a DBD electrode device) falls within the scope of various embodiments of the present invention.

Cold plasma has been employed to transfect cells with foreign nucleic acids. In particular, transfection of tumor cells (see, e.g., Connolly, et al. (2012) Human Vaccines & Immune-therapeutics 8: 1729-1733; and Connolly et al (2015) Bioelectrochemistry 103: 15-21) .

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In certain illustrative embodiments, the transgene construct encoding the Affimer polypeptide of the present invention is delivered using an electroporation device comprising: an applicator; a plurality of electrodes extending from the applicator, the electrodes being associated with a cover area; a power supply in electrical communication with the electrodes, the power supply configured to generate one or more electroporating signals to cells within the cover area; and a guide member coupled to the electrodes, wherein the guide member is configured to adjust the cover area of the electrodes. At least a portion of the electrodes can be positioned within the applicator in a conical arrangement. The one or more electroporating signals may be each associated with an electric field. The device may further comprise a potentiometer coupled to the power supply and electrodes. The potentiometer may be configured to maintain the electric field substantially within a predetermined range.

The one or more electroporating signals may be each associated with an electric field. The device may further comprise a potentiometer coupled to the power supply and the electrodes. The potentiometer may be configured to maintain the electric field within a predetermined range so as to substantially prevent permanent damage in the cells within the cover area and /or substantially minimize pain. For instance, potentiometer may be configured to maintain the electric field to about 1300 V/cm.

The power supply may provide a first electrical signal to a first electrode and a second electrical signal to a second electrode. The first and second electrical signals may combine to produce a wave having a beat frequency. The first and second electrical signals may each have at least one of a unipolar waveform and a bipolar waveform. The first electrical signal may have a first frequency and a first amplitude. The second electrical signal may have a second frequency and a second amplitude. The first frequency may be different from or the same as the second frequency. The first amplitude may be different from or the same as the second amplitude.

In certain embodiments, the present invention provides a method for treating a subject having a tumor, the method comprising: injecting the tumor with an effective dose of plasmid coding for a Affimer polypeptide; and administering electroporation therapy to the tumor. In certain embodiments, the electroporation therapy further comprises the administration of at least one voltage pulse of about 200 V/cm to about 1500 V/cm over a pulse width of about 100 microseconds to about 20 milliseconds.

In certain embodiments, the plasmid (or a second electroporated plasmid) further encodes at least one immunostimuiatory cytokine, such as selected from the group encoding IL-12, IL-15, and a combination of IL-12 and IL-15.

Transfection Enhancing Formulations

Encoded Affimer nucleic acid constructs can also be encapsulated in liposomes, preferably cationic liposomes (Wong, T. K. et al., Gene, 10:87(1980); Nicolau and Sene, Biochim. Biophys. Acta, 721:185-190 (1982); and Nicolau et al., Methods Enzymol., 149:157-176 (1987)) or polymersomes (synthetic liposomes) which can interact with the cell membrane and fuse or undergo endocytosis to effect nucleic acid transfer into the cell. The DNA also can be formed

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into complexes with polymers (polyplexes) or with dendrimers which can directly release their load into the cytoplasm of a cell.

Illustrative carriers useful in this regard include microparticles of poly(lactide-co-glycolide), polyacrylate, latex, starch, cellulose, dextran and the like. Other illustrative carriers include supramolecular biovectors, which comprise a non-liquid hydrophilic core (e.g., a cross-linked polysaccharide or oligosaccharide) and, optionally, an external layer comprising an amphiphilic compound, such as a phospholipid (see e.g., U.S. Pat. No. 5,151,254 and PCT applications WO 94/20078, WO/94/23701 and WO 96/06638). The amount of active agent contained within a sustained release formulation depends upon the site of implantation, the rate and expected duration of release and the nature of the condition to be treated or prevented.

Biodegradable microspheres (e.g., polylactate polyglycolate) may be employed as carriers for compositions. Suitable biodegradable microspheres are disclosed, for example, in U.S. Pat. Nos. 4,897,268; 5,075,109; 5,928,647; 5,811,128; 5,820,883; 5,853,763; 5,814,344, 5,407,609 and 5,942,252. Modified hepatitis B core protein carrier systems such as described in WO/99 40934, and references cited therein, will also be useful for many applications. Another illustrative carrier/delivery system employs a carrier comprising particulate-protein complexes, such as those described in U.S. Pat. No. 5,928,647, which can have the added benefit when used intratumorally to deliver the coding sequence for an Affimer

Biodegradable polymeric nanoparticles facilitate non-viral nucleic acid transfer to cells. Small (approximately 200 nm), positively charged (approximately 10 mV) particles are formed by the self-assembly of cationic, hydrolytically degradable poly(beta-amino esters) and plasmid DNA.

Polynucleotides may also be administered to cells by direct microinjection, temporary cell permeabilizations (e.g., co-administration of repressor and/or activator with a cell permeabilizing agent), fusion to membrane translocating peptides, and the like.

Lipid-mediated nucleic acid delivery and expression of foreign nucleic acids, including mRNA, in vitro and in vivo has been very successful. Lipid based non-viral formulations provide an alternative to viral gene therapies. Current in vivo lipid delivery methods use subcutaneous, intradermal, intratumoral, or intracranial injection. Advances in lipid formulations have improved the efficiency of gene transfer in vivo (see PCT Application WO 98/07408). For instance, a lipid formulation composed of an equimolar ratio of l,2-bis(oleoyloxy)-3-(trimethyl ammonio)propane (DOTAP) and cholesterol can significantly enhances systemic in vivo gene transfer. The DOTAP:cholesterol lipid formulation forms unique structure termed a "sandwich liposome". This formulation is reported to "sandwich" DNA between an invaginated bi-layer or 'vase' structure. Beneficial characteristics of these lipid structures include a positive p, colloidal stabilization by cholesterol, two-dimensional nucleic acid packing and increased serum stability.

Cationic liposome technology is based on the ability of amphipathic lipids, possessing a positively charged head group and a hydrophobic lipid tail, to bind to negatively charged DNA or RNA and form particles that generally enter cells by endocytosis. Some cationic liposomes also contain a neutral co-lipid, thought to enhance liposome uptake by mammalian cells. Similarly, other polycations, such as poly-L-lysine and polyethylene-imine, complex with

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nucleic acids via charge interaction and aid in the condensation of DNA or RNA into nanoparticles, which are then substrates for endosome-mediated uptake. Several of these cationic-nucleic acid complex technologies have been developed as potential clinical products, including complexes with plasmid DNA (pDNA), oligodeoxynucleotides, and various forms of synthetic RNA, and be used as part of the delivery system for the Encoded Affimer nucleic acid constructs of the present invention.

The Encoded Affimer nucleic acid constructs disclosed herein may be associated with polycationic molecules that serve to enhance uptake into cells. Complexing the nucleic acid construct with polycationic molecules also helps in packaging the construct such their size is reduced, which is believed to assist with cellular uptake. Once in the endosome, the complex dissociates due to the lower pH, and the polycationic molecules can disrupt the endosome's membrane to facilitate DNA escape into the cytoplasm before it can be degraded. Preliminary data shows that the nucleic acid construct embodiments had enhanced uptake into SCs over DCs when complexed with the polycationic molecules polylysine or polyethyleneimine.

One example of polycationic molecules useful for complexing with nucleic acid constructs includes cell penetrating peptides (CPP), examples include polylysine (described above), polyarginine and Tat peptides. Cell penetrating peptides (CPP) are small peptides which can bind to DNA and, once released, penetrate cell membranes to facilitate escape of the DNA from the endosome to the cytoplasm. Another example of a CPP pertains to a 27 residue chimeric peptide, termed MPG, was shown some time ago to bind ss- and ds-oligonucleotides in a stable manner, resulting in a non-covalent complex that protected the nucleic acids from degradation by DNase and effectively delivered oligonucleotides to cells in vitro (Mahapatro A, et al., J Nanobiotechnol, 2011, 9:55). The complex formed small particles of approximately 150 nm to 1 um when different peptide:DNA ratios were examined, and the 10:1 and 5:1 ratios (150 nm and 1 um respectively). Another CPP pertains to a modified tetrapeptide [tetralysine containing guanidinocarbonylpyrrole (GCP) groups (TL-GCP)], which was reported to bind with high affinity to a 6.2 kb plasmid DNA resulting in a positive charged aggregate of 700-900 nm Li et al., Agnew Chem Int Ed Enl 2015; 54(10):2941-4). RNA can also be complexed by such polycationic molecules for in vivo delivery.

Other examples of polycationic molecules that may be complexed with the nucleic acid constructs described herein include polycationic polymers commercially available as JETPRIME® and In Vivo JET (Polypus-transfection, S.A., Illkirch, France).

In certain embodiments, the present invention contemplates a method of delivering an mRNA (or other polynucleotide)f encoding a Affimer polypeptide to a patient's cells by administering a nanoparticle composition comprising (i) a lipid component comprising a compound of formula (I), a phospholipid, a structural lipid, and a PEG lipid; and (ii) an mRNA (or other polynucleotide)f, said administering comprising contacting said mammalian cell with said nanoparticle composition, whereby said mRNA (or other polynucleotide)f is delivered to said cell.

In exemplary embodiments, the PEG lipid is selected from the group consisting of a PEG-modified phosphatidylethanolamine, a PEG-modified phosphatide acid, a PEG-modified

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ceramide, a PEG-modified dialkylamine, a PEG-modified diacylglycerol and a PEG-modified dialkylglycerol. In exemplary embodiments, the structural lipid is selected from the group consisting of cholesterol, fecosterol, sitosterol, ergosterol, campesterol, stigmasterol, brassicasterol, tomatidine, ursolic acid, and alpha-tocopherol. In certain preferred embodiments, the structural lipid is cholesterol.

In exemplary embodiments, the phospholipid includes a moiety selected from the group consisting of phosphatidyl choline, phosphatidyl ethanolamine, phosphatidyl glycerol, phosphatidyl serine, phosphatidic acid, 2-lysophosphatidyl choline, and a sphingomyelin. In certain embodiments, the phospholipid includes one or more fatty acid moieties selected from the group consisting of lauric acid, myristic acid, myristoleic acid, palmitic acid, palmitoleic acid, stearic acid, oleic acid, linoleic acid, alpha-linolenic acid, erucic acid, arachidic acid, arachidonic acid, phytanoic acid, eicosapentaenoic acid, behenic acid, docosapentaenoic acid, and docosahexaenoic acid. In certain preferred embodiments, the phospholipid is selected from the group consisting of 1 ,2-dilinoleoyl-sn-glycero-3-phosphocholine (DLPC), 1 ,2-dimyristoyl-sn-glycero-phosphocholine (DMPC), 1 ,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), 1 ,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), 1 ,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), 1 ,2-diundecanoyl-sn-glycero-phosphocholine (DUPC), 1 -palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), 1 ,2-di-0-octadecenyl-sn-glycero-3-phosphocholine (1 8:0 Diether PC), 1 -oleoyl-2-cholesterylhemisuccinoyl-sn-glycero-3-phosphocholine (OChemsPC), 1 -hexadecyl-sn-glycero-3-phosphocholine (C16 Lyso PC), 1 ,2-dilinolenoyl-sn-glycero-3-phosphocholine, 1 ,2-diarachidonoyl-sn-glycero-3-phosphocholine, 1 ,2-didocosahexaenoyl-sn-glycero-3-phosphocholine,1 ,2-dioleoyl-sn-glycero-3-phosphoethanola mine (DOPE), 1 ,2-diphytanoyl-sn-glycero-3-phosphoethanolamine (ME 1 6.0 PE), 1 ,2-distearoyl-sn-glycero-3-phosphoethanolamine, 1 ,2-dilinoleoyl-sn-glycero-3-phosphoethanolamine, 1 ,2-dilinolenoyl-sn-glycero-3-phosphoethanolamine, 1 ,2-diarachidonoyl-sn-glycero-3-phosphoethanolamine, 1 ,2-didocosahexaenoyl-sn-glycero-3-phosphoethanolamine, 1 ,2-dioleoyl-sn-glycero-3-phospho-rac-(1 -glycerol) sodium salt (DOPG), and sphingomyelin In certain preferred embodiments, the phospholipid is DOPE or DSPC.

To further illustrate, the phospholipid can be DOPE and said the component can comprise about 35 mol % to about 45 mol % said compound, about 1 0 mol % to about 20 mol % DOPE, about 38.5 mol % to about 48.5 mol % structural lipid, and about 1 .5 mol % PEG lipid. The lipid component can be about 40 mol % said compound, about 15 mol % phospholipid, about 43.5 mol % structural lipid, and about 1 .5 mol % PEG lipid.

In certain embodiments, the wt/wt ratio of lipid component to Affimer polypeptide encoding mRNA (or other polynucleotide) is from about 5:1 to about 50:1 and more preferably about 10:1 to about 40:1

In certain embodiments, the mean size of said nanoparticle composition is from about 50 nm to about 150 nm, and even more preferably is from about 80 nm to about 120 nm.

In certain embodiments, the polydispersity index of said nanoparticle composition is from about 0 to about 0.18, more preferably from about 0.13 to about 0.17.

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In certain embodiments, the nanoparticle composition has a zeta potential of about -10 to about +20 mV.

In certain embodiments, the nanoparticle composition further comprises a cationic and/or ionizable lipid selected from the group consisting of 3-(didodecylamino)-N1 ,N 1 ,4-tridodecyl-1 -piperazineethanamine (KL1 0), 14,25-ditridecyl-1 5, 1 8,21 ,24-tetraaza-octatriacontane (KL25), 1,2-dilinoleyloxy-N,N-dimethylaminopropane (DLin-DMA), 2,2-dilinoleyl-4-dimethylaminomethyl-[1 ,3]-dioxolane (DLin-K-DMA), heptatriaconta-6, 9,28,31 -tetraen-1 9-yl 4-(dimethylamino)butanoate (DLin-MC3-DMA), 2,2-dilinoleyl-4-(2-dimethylaminoethyl)-[1 ,3]-dioxolane (DLin-KC2-DMA), 1 ,2-dioleyloxy-N,N-dimethylaminopropane (DODMA), and (2R)-2-({8-[(3P)-cholest-5-en-3-yloxy]octyl}oxy)-N,N-dimethyl-3-[(9Z,1 2Z)-octadeca-9, 12-dien-1 -yl oxy]propan-1 -amine (Octyl-CLinDMA (2R)).

The invention is now described by way of example. These examples are illustrative and are not intended to be construed as limiting the invention, which is defined by the scope of the claims.

Some data in these examples has been produced with scaffolds having a C-Terminal G6H tag (i.e. GHHHHHH (SEQ ID NO: 104) as an addition at the C-terminal end of the polypeptide). This is standard in the art for ease of manipulation/detection to carry out the experiments shown, for example by using standard 'off-the-shelf' electra vector system® cloning vectors from ATUM, Newark, California, USA. Especially suitably are the pMOTHER and pDAUGHTER plasmids, or vectors derived from them. Of course a skilled operator may easily use a parallel cloning system based on the same principles, or a different system based on operator choices.

V. Pharmaceutical Compositions

The present invention also provides pharmaceutical compositions comprising an Affimer polypeptide described herein and a pharmaceutically acceptable vehicle. In some embodiments, the pharmaceutical compositions find use in immunotherapy. In some embodiments, the pharmaceutical compositions find use in immuno-oncology. In some embodiments, the compositions find use in inhibiting tumor growth. In some embodiments, the pharmaceutical compositions find use in inhibiting tumor growth in a subject (e.g., a human patient). In some embodiments, the compositions find use in treating cancer. In some embodiments, the pharmaceutical compositions find use in treating cancer in a subject (e.g., a human patient).

Formulations are prepared for storage and use by combining a purified Affimer polypeptide of the present invention with a pharmaceutically acceptable vehicle (e.g., a carrier or excipient). Those of skill in the art generally consider pharmaceutically acceptable carriers, excipients, and/or stabilizers to be inactive ingredients of a formulation or pharmaceutical composition.

In some embodiments, an Affimer polypeptide described herein is lyophilized and/or stored in a lyophilized form. In some embodiments, a formulation comprising an Affimer polypeptide described herein is lyophilized.

Suitable pharmaceutically acceptable vehicles include, but are not limited to, nontoxic buffers such as phosphate, citrate, and other organic acids; salts such as sodium chloride; antioxidants

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including ascorbic acid and methionine; preservatives such as octadecyldimethylbenzyl ammonium chloride, hexamethonium chloride, benzalkonium chloride, benzethonium chloride, phenol, butyl or benzyl alcohol, alkyl parabens, such as methyl or propyl paraben, catechol, resorcinol, cyclohexanol, 3-pentanol, and m-cresol; low molecular weight polypeptides (e.g., less than about 10 amino acid residues); proteins such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, histidine, arginine, or lysine; carbohydrates such as monosaccharides, disaccharides, glucose, mannose, or dextrins; chelating agents such as EDTA; sugars such as sucrose, mannitol, trehalose or sorbitol; salt-forming counter-ions such as sodium; metal complexes such as Zn-protein complexes; and non-ionic surfactants such as TWEEN or polyethylene glycol (PEG). (Remington: The Science and Practice of Pharmacy, 22.sup.nd Edition, 2012, Pharmaceutical Press, London.).

The pharmaceutical compositions of the present invention can be administered in any number of ways for either local or systemic treatment. Administration can be topical by epidermal or transdermal patches, ointments, lotions, creams, gels, drops, suppositories, sprays, liquids and powders; pulmonary by inhalation or insufflation of powders or aerosols, including by nebulizer, intratracheal, and intranasal; oral; or parenteral including intravenous, intraarterial, intratumoral, subcutaneous, intraperitoneal, intramuscular (e.g., injection or infusion), or intracranial (e.g., intrathecal or intraventricular).

The therapeutic formulation can be in unit dosage form. Such formulations include tablets, pills, capsules, powders, granules, solutions or suspensions in water or non-aqueous media, or suppositories. In solid compositions such as tablets the principal active ingredient is mixed with a pharmaceutical carrier. Conventional tableting ingredients include corn starch, lactose, sucrose, sorbitol, talc, stearic acid, magnesium stearate, dicalcium phosphate or gums, and diluents (e.g., water). These can be used to form a solid preformulation composition containing a homogeneous mixture of a compound of the present invention, or a non-toxic pharmaceutically acceptable salt thereof. The solid preformulation composition is then subdivided into unit dosage forms of a type described above. The tablets, pills, etc. of the formulation or composition can be coated or otherwise compounded to provide a dosage form affording the advantage of prolonged action. For example, the tablet or pill can comprise an inner composition covered by an outer component. Furthermore, the two components can be separated by an enteric layer that serves to resist disintegration and permits the inner component to pass intact through the stomach or to be delayed in release. A variety of materials can be used for such enteric layers or coatings, such materials include a number of polymeric acids and mixtures of polymeric acids with such materials as shellac, cetyl alcohol and cellulose acetate.

The Affimer polypeptides described herein can also be entrapped in microcapsules. Such microcapsules are prepared, for example, by coacervation techniques or by interfacial polymerization, for example, hydroxymethylcellulose or gelatin-microcapsules and poly-(methylmethacylate) microcapsules, respectively, in colloidal drug delivery systems (for example, liposomes, albumin microspheres, microemulsions, nanoparticles and nanocapsules)

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or in macroemulsions as described in Remington: The Science and Practice of Pharmacy, 22nd Edition, 2012, Pharmaceutical Press, London.

In certain embodiments, pharmaceutical formulations include an Affimer polypeptide of the present invention complexed with liposomes. Methods to produce liposomes are known to those of skill in the art. For example, some liposomes can be generated by reverse phase evaporation with a lipid composition comprising phosphatidylcholine, cholesterol, and PEG-derivatized phosphatidylethanolamine (PEG-PE). Liposomes can be extruded through filters of defined pore size to yield liposomes with the desired diameter.

In certain embodiments, sustained-release preparations comprising Affimer polypeptides described herein can be produced. Suitable examples of sustained-release preparations include semi-permeable matrices of solid hydrophobic polymers containing an Affimer polypeptide, where the matrices are in the form of shaped articles (e.g., films or microcapsules). Examples of sustained-release matrices include polyesters, hydrogels such as poly(2-hydroxyethyl-methacrylate) or poly(vinyl alcohol), polylactides, copolymers of L-glutamic acid and 7 ethyl-L-glutamate, non-degradable ethylene-vinyl acetate, degradable lactic acid-glycolic acid copolymers such as the LUPRON DEPOT.TM. (injectable microspheres composed of lactic acid-glycolic acid copolymer and leuprolide acetate), sucrose acetate isobutyrate, and poly-D-(-)-3-hydroxybutyric acid.

EXAMPLES

Example 1 - Mutations Can Be Combined Independently To Modulate Stability

The following mutations were made relative to SEQ ID NO: 1:

Mutations	Stability (change in Tm compared to SEQ ID NO: 1)
hSteA Y35W T51L N32G	+9.9 °C
hSteA Y35W Q42E M65I	+10.8 °C
hSteA Y35W Q42E M65I E29K K30E E33K	+14.8 °C
hSteA Y35W ^{59}AG-NK N32G	+8.6 °C
hSteA Y35W N32G M65I	+11.8 °C

These all also increased the Tm of hSteA Y35W.

This shows that the order of mutation does not matter. In other words, this demonstrates that the mutations taught herein to modulate stability (e.g. Tm) can be made independently or combined independently.

It is an advantage of the invention that particular mutations taught herein to modulate stability do not depend on each other to achieve their individual effects.

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Example 2: Heterologous Peptide Insertions

test heterologous peptide GGSGGSGGS inserted in to L2 and L4

3 different positions for each loop (9 combinations)

Thermal stability measured on Optim 2

3r2 measured in absence of denaturant but the stabilities were over range

3r2 measured again in presence of 1 M GuHCl to bringing Tm within range

3t4 measured in presence of SYPRO Orange as no intrinsic tryptophan fluorescence in this polypeptide

In summary various options illustrated are shown below:

SQT (SEQ ID NO:24 of WO 2009/136182)

MIPRGLSEAKPATPEIQEIVDKVKPQLEEKTNETYGKLEAVQYKTQVL[A¦¦ST]NYY IKVRAGDNKYMHLKVFNG[P¦~~PGQN~~¦ADR]VLTGYQVDKNKDDELTGF

3r2 (SEQ ID NO: 19)

MIPGGLSEAKPATPEIQEIVDKVKPQLEKETGKTWGKLEAVEYKTQVD[A¦~~G~~¦L]NYY IKVRVN-GKYIHLKVFKS[L¦~~PGQN~~¦EDL]VLTGYQVDKNKDDELTGF

3t4 (SEQ ID NO: 23)

MIPGGLSEAKPATPEIQEIVDKVKPQLEEKTGETYGKLEAVEYKTQVD[A¦~~G~~¦T]NYY IKVRAGDNKYIHLKVFKS[L¦~~PGQN~~¦EDL]VLTGYQVDKNKDDELTGF

3r2 (SEQ ID NO: 19)/t4 (SEQ ID NO: 23)

...QVD[A¦GGSGGSGGS¦L]NYY...FKS[L¦GGSGGSGGS¦EDL]VLT... 0

3r2 (SEQ ID NO: 19)/t4 (SEQ ID NO: 23)

...QVD[¦GGSGGSGGS¦GL]NYY...FKS[¦GGSGGSGGS¦NEDL]VLT... -1

3r2 (SEQ ID NO: 19)/t4 (SEQ ID NO: 23)

...QVD[AG¦GGSGGSGGS¦]NYY...FKS[LP¦GGSGGSGGS¦DL]VLT... +1

Results are shown in Figure 1.

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This demonstrates that inserting heterologous peptides into loops 3r2 advantageously does not adversely affect stability.

For L2 Tm, 48-L2-50 > 49-L2-51 > 50-L2-52

For L4 Tm, 73-L4-78 is usually most stable, except in combination with 50-L2-52 where it is by far the least stable

The most stable combination is 48-L2-50 with 73-L4-78

3t4 did not give good data, even in the presence of SYPRO, except for one variant which was clearly much less stable than the rest. This is the same combination as for 3r2.

Thus, most suitably the insertions/loops for both 3r2 and 3t4 should be as follows:

L2 inserted between residues D48 and G50, deleting residue A49

L4 inserted between residues L73 and E78, deleting residues P74, G75, Q76 and N77 (using hSteA numbering)

Example 3: Exemplary Scaffold Proteins

Exemplary scaffold proteins are demonstrated.

In this experiment, Tm is measured by DSC (r/t) and Optim 2 (3r).

Exemplary Scaffolds for Research Applications:

3r1 – hSteA Y35W N32G V48D M65I Q42E T51L (A59V ΔD61) (E29K K30E E33K) (SEQ ID NO: 18)

3r2 – hSteA Y35W N32G V48D M65I Q42E T51L (A59V G60N ΔD61 N62G) (E29K K30E E33K) (SEQ ID NO: 19)

Exemplary Scaffolds for Therapeutic Applications:

SEQ ID NO: 20	3t1 – hSteA	N32G V48D
SEQ ID NO: 21	3t2 – hSteA	N32G V48D M65I
SEQ ID NO: 22	3t3 – hSteA	N32G V48D M65I T51L
SEQ ID NO: 23	3t4 – hSteA	N32G V48D M65I Q42E
SEQ ID NO: 24	3t5 – hSteA	N32G V48D M65I Q42E T51L

Figure 2 shows data for Tm as a measure of thermal stability.

Figure 3 shows Tm measured at different pH values.

Figure 4 shows CD spectra.

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We generated DSC data for exemplary scaffolds as above and observed the following Tm values:

3t1 = 85.6 °C

3t2 = 89.2 °C

3t3 = 91.6 °C

3t4 = 92.4 °C

3t5 = 94.7 °C

Thus we demonstrate that exemplary scaffold proteins show improved stability.

Example 4: Structural Integrity of Scaffold Proteins

Scaffold proteins of the invention advantageously maintain their structural integrity when complete with target peptide insertions. To demonstrate this, we measured the near and far UV CD spectra for the 3r2 and 3t4 scaffolds with heterologous peptide insertions. In this example, the heterologous peptide insertions are 9mer insertions having the sequence (GGS)3 i.e. GGSGGSGGS (SEQ ID NO: 92).

The near UV CD spectra report on the environment of the aromatic side chains. Peaks, positive or negative, in a near UV CD spectrum indicate that the environment around the aromatic residues is fixed. This is interpreted as a protein having a fixed tertiary structure. Adding the heterologous peptide (GGS)3 to loops 2 and 4 results in near UV CD spectra with the same shape as the "empty" scaffolds, albeit with different magnitudes, indicating that the addition of heterologous peptides has not disrupted the tertiary structure of the scaffold proteins.

The far UV CD spectra report on gross secondary structure. The alpha-helix, beta-strand and random coil all have distinctly different far UV CD spectra. Adding the heterologous peptide (GGS)3 to loops 2 and 4 results in far UV CD spectra with the same shape as the "empty" scaffolds, albeit with different magnitudes, indicating that the addition of heterologous peptides has not disrupted the gross secondary structure of the scaffold proteins.

Referring to figure 5, far UV CD spectra of 3r2 (solid line) and 3r2$(GGS)_3$ (dashed line) showing mean residue ellipticity ($[\theta]_{MRE}$). 3r2 is an "empty" scaffold, whose loops do not contain heterologous peptides. 3r2$(GGS)_3$ contains the heterologous peptide sequence GGSGGSGGS (SEQ ID NO: 92) in place of A49 (loop 2) (i.e. 48-<heterologous peptide>-50) and in place of P74-G75-Q76-N77 (loop 4) (i.e. 73-<heterologous peptide>-78).

Referring to figure 6: Far UV CD spectra of 3t4 (solid line) and 3t4$(GGS)_3$ (dashed line) showing mean residue ellipticity ($[\theta]_{MRE}$). 3t4 is an "empty" scaffold, whose loops do not contain heterologous peptides. 3t4$(GGS)_3$ contains the heterologous peptide sequence GGSGGSGGS (SEQ ID NO: 92) in place of A49 (loop 2) and in place of P74-G75-Q76-N77 (loop 4).

Referring to figure 7: Near UV CD spectra of 3r2 (solid line) and 3r2$(GGS)_3$ (dashed line) showing molar ellipticity ($[\theta]$). 3r2 is an "empty" scaffold, whose loops do not contain

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heterologous peptides. 3r2$(GGS)_3$ contains the heterologous peptide sequence GGSGGSGGS (SEQ ID NO: 92) in place of A49 (loop 2) and in place of P74-G75-Q76-N77 (loop 4).

Referring to figure 8: Near UV CD spectra of 3t4 (solid line) and 3t4$(GGS)_3$ (dashed line) showing molar ellipticity ([θ]). 3t4 is an "empty" scaffold, whose loops do not contain heterologous peptides. 3t4$(GGS)_3$ contains the heterologous peptide sequence GGSGGSGGS (SEQ ID NO: 92) in place of A49 (loop 2) and in place of P74-G75-Q76-N77 (loop 4).

Thus it can be seen that over the wavelengths measured, the shape of the spectra for the scaffolds bearing heterologous peptide insertions are essentially no different from the scaffolds without insertions. This shows the structure has been maintained with heterologous peptide insertions. This is shown for different exemplary scaffolds of the invention.

Example 5: Immunogenicity

The immunogenicity of polypeptides according to the present invention was modelled.

%rank is the %rank of predicted affinity compared with a set of 200000 random natural human peptides, e.g. a value of 1% means the 15mer peptide is predicted to bind as strongly as the top 1% of the 200000 random peptides. Any rank below 10% (i.e. 10%-100%) is not considered immunogenic. These calculations were carried out using the NetMHCIIpan (v3.1) server. Forty-one MHC alleles were tested in total for each sequence. This plot shows the result for one allele (the DRB1 1502 allele) as a representative example.

Referring to Figure 9, it can concluded that the mutations taught herein produce only small movements up or down on immunogenicity. The plots are largely similar to hSteA. No large immunogenic region has been introduced. No large changes/deviations from wild type have been introduced.

Example 6: Serum Stability

Stability such as serum stability was tested.

Each protein was incubated in human serum (Sigma) at 37 °C for up to 16 days. Samples were withdrawn at days 0, 1, 2, 4, 8 and 16 and kept frozen until measurement. All samples were analysed using the Wes simple western system (Protein Simple) to detect the presence of monomer and any other species present. Detection was *via* an anti-(His6) monoclonal. Over the course of this experiment, SQT showed a steady decrease in the area of the monomer peak (Figure 6a), whereas the other proteins remained steady. The decrease in the amount of SQT monomer was matched by an increase in SQT dimer (Figure 6b). This is expected as SQT has V48L and so is still able to dimerise.

There was no evidence of any material appearing at lower molecular weights, for example from protease digestion.

The same results were seen in buffer (rather than serum).

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Thus it is demonstrated that polypeptides according to the present invention are stable and do not significantly degrade in human serum over the time course shown, or indeed in standard storage buffer.

Example 7: Biological Neutrality (Papain binding)

Human stefin A is an inhibitor of the cysteine protease papain. Human stefin A, SQT and variants of hSteA referred to as 3r2 and 3t4 were tested in a papain activity assay. 3r2 and 3t4 were also tested with heterologous peptides (GGSGGSGGS) (SEQ ID NO: 92) inserted between residues 48-50 and 73-78 (hSteA residue numbering). Activated papain was incubated with a dilution series of each variant before substrate (N-Carbobenzyloxy-Phe-Arg-7-amido-4-methylcoumarin) was added. The fluorescence of each reaction was measured (excitation at 380 nm, emission at 460 nm) for 5 minutes after substrate emission. Data are presented in Figure 11. Although hSteA is a potent inhibitor of papain, none of the variants tested show significant inhibition of papain for the concentrations tested. This demonstrates that biological neutrality has been achieved in exemplary scaffold proteins of the invention.

Example 8: Exemplary Library Build

1. **Vector preparation**

a. SapI digest

- Reaction mixture:

60 µL SapI (10 U/µL)

7.5 µL MfeI-HF (20 U/µL)

150 µg vector (contains a truncated gene III; library will be scaffold-linker-gene III)

150 µL CutSmart buffer (10x)

add H20 to 1500 µL

- Incubate at 37 ºC for 1 h (500 µL aliquots).
- Check success of digest on agarose gel.

b. Phenol/chloroform extraction

- Add one volume of phenol/chloroform (Sigma; 77617) to one volume of your sample (larger volumes are easier to handle; 500 µL are ideal) in a 1.5 mL tube, make sure the tube is fully closed and mix thoroughly by inverting.
- Spin sample at 13,000 x g for 5 min (at room temperature).
- Transfer the upper layer carefully into a fresh tube (initially 400 µL, then the rest).

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- Add one volume of chloroform (Sigma; C0549) to the sample, make sure the tube is fully closed and mix thoroughly by inverting.
- Spin sample at 13,000 x g for 5 min (at room temperature).
- Transfer the upper layer carefully into a fresh 2 mL tube (initially 400 µL, then the rest).
- The DNA is now extracted.

c. Ethanol precipitation

- Add 0.1x volume of 3 M Sodium Acetate (pH 5.2; ThermoFisher; R1181), 1 µL glycogen (Roche; 10901393001) and 2.5x volume of pre-chilled (-20 ºC) absolute ethanol to the phenol/chloroform extracted sample; mix thoroughly by inverting.
- Incubate the samples at -80 ºC for at least 2 h (overnight would be better).
- Spin precipitated DNA in a pre-cooled centrifuge at 16,000 x g / 4 ºC for 15 min.
- Remove supernatant by pipetting and wash pellet by adding 400 µL pre-chilled 70% (v/v) ethanol opposite of the pellet.
- Spin again at 16,000 x g / 4 ºC for 5 min.
- Remove as much ethanol as possible by carefully pipetting.
- Leave tubes open for pellets to dry at room temperature.
- Resuspend DNA in molecular grade water (typically 100 µL).

d. Purification with Chromaspin TE-1000 columns

- Further purification of digested vector DNA via Chromaspin TE-1000 columns in order to remove digestion fragments smaller than 300 bp. Purified vector DNA will be in the flow through. This is not a concentration step. 70-100 µL DNA can be applied with a maximum concentration of 1 mg/mL.
- Mix the slurry of the columns by inverting (Takara Clontech; 636079).
- Snap off the end of the column and place it in a collection tube.
- Remove the lid of the column and place it together with the collection tube in a 15 mL falcon tube.
- Spin at 700 x g / 4 ºC for 5 min.
- Transfer the column into a new collection tube and back into a 15 mL falcon tube.
- Apply the sample by carefully pipetting it on top of the slurry.
- Spin at 700 x g / 4 ºC for 5 min.
- The purified vector DNA is now in the flow through and can be transferred into a 1.5 mL tube for storage at -20 ºC.

2. <u>Insert preparation</u>

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a. SapI digest

- Reaction mixture:

  15 µg library DNA (biotinylated)
  75 µL CutSmart buffer (10x)
  30 µL SapI (10 U/µL)
  add H_2O to 750 µL

- Split in 3x 250 µL aliquots.
- Incubate at 37 ºC for 1 h (Eppendorf Thermomixer) and subsequently heat inactivate at 65 ºC/20 min (heat block).

b. Beads purification I (Streptavidin beads)

- Prepare washing buffer (2x):
  10 mM Tris-HCl (pH 7.5)
  1 mM EDTA
  2 M NaCl
- Mix 50 µL M280 streptavidin beads (LifeTechnologies 11205D) with 1 mL washing buffer (1x). Mix by vortexing for ≥ 5 s.
- Immobilise beads on a magnetic rack for 1 min.
- Remove supernatant and add again 1 mL of washing buffer (1x).
- Immobilise beads on a magnetic rack for 1 min.
- Remove supernatant and resuspend beads in 250 µL washing buffer (2x).
- Add 250 µL digest to washed beads and incubate on a roller mixer at room temperature for 15 min.
- Immobilise beads on a magnetic rack for 2-3 min.
- Transfer supernatant into a new tube (contains digested library insert).

c. Beads purification II (AMPure beads)

- Transfer supernatant into a new tube (contains digested library insert).
- Add 600 µL AMPure beads to 250 µL digested and streptavidin-purified insert. Incubate on a roller mixer for 10 min.
- Immobilise beads on a magnet for 2-3 min. Discard supernatant.
- Wash beads with 1.1 mL 70% (v/v) ethanol.
- Immobilise beads on a magnet for 2-3 min. Discard supernatant.
- Wash beads again with 1.1 mL 70% (v/v) ethanol.
- Immobilise beads on a magnet for 2-3 min. Discard supernatant.
- Air-dry the beads for 10 min.

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- Resuspend beads in 50 µL water for 2 min.
- Immobilise beads on a magnet for 2-3 min and transfer supernatant into a new tube.
- Resuspend beads again in 50 µL water for 2 min.
- Immobilise beads on a magnet for 2-3 min and transfer supernatant into the same tube as the previous one.
- Check success of digest and purification on an analytical agarose gel.

3. <u>Test ligations and initial QC</u>

a. Ligations

Testing different vector:insert ratios

- Ligation mixture (include negative control without insert):

100 ng digested vector

X ng digested library insert (X varies with ligation ratio)

0.5 µL T4 DNA ligase (2000 U/µL)

2 µL T4 DNA ligase buffer (10x)

Ad H_2O to 20 µL

- Incubate ligation mixture at room temperature for 30 min. Inactivate ligase at 65 ºC for 10 min.

b. Electroporations

- Place electroporation cuvettes (2 mm) in their bags, 1.5 mL Eppendorf tubes and sterile water on ice to pre-chill.
- Place aliquots of electrocompetent TG1 cells on ice to thaw.
- Add 60 µL ice-cold sterile water to 60 µL TG1 cells.
- Aliquot 2 µL ligation mixture into pre-chilled 1.5 mL tubes.
- Add 25 µL of diluted TG1 cells to each tube and mix by flicking.
- Transfer cell/DNA mixture into pre-chilled cuvette, place cuvette into electroporator and pulse cells (25 µF/200 ohm/2500 V).
- Add immediately 975 µL Recovery Medium, pipette up and down 3x and transfer cell suspension into new 1.5 mL tubes.
- Incubate cells at 37 ºC / 1000 rpm for 1 h.
- Dilute cells 10^{-2} and 10^{-3} in 2YT and plate 50 µL on 2YT agar plates supplemented with 25 µg/mL chloramphenicol and 2% glucose. Plates are incubated at 30 ºC for 16 h.
- Count cfu the next day.

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c. Sequencing

- Choose best vector:insert ratio and pick colonies from counting plates (192 in total) into 1 mL 2YT supplemented with 25 µg/mL chloramphenicol and 2% glucose
- Incubate at 37 ºC / 800 rpm / 35% humidity overnight.
- Isolate phagemid DNA and sequence with the M13-RP primer

4. <u>Bulk ligation</u>

a. Ligation

- Ligation mixture (2x 3.5 mL):

15-20 µg SapI-digested pALSphm-dummyT2
X µg SapI-digested library insert (X depends on the determined optimal ligation ratio)
70 µL T4 DNA ligase (2000 U/µL)
350 µL T4 DNA ligase buffer (10x)
add nuclease-free water to 3.5 mL
⇨ split into 14x 500 µL aliquots

- Incubate at 16 ºC overnight.
- Inactivate ligase at 65 ºC for 10 min.

b. Phenol/chloroform extraction

- **See 1.b**

c. Re-buffering with Amicon filter units

- Add extracted DNA to Amicon filter unit (50 K). Total volume should be 500 µL. It can be added up to 500 µL with nuclease-free water if necessary.
- Spin column at 3,000 x g for 10-15 min until the liquid volume in the upper column is down to 30-50 µL.
- Discard flow-through and top up the concentrated sample with 400 µL nuclease/free water. Mix water and sample and spin again at 3,000 x g for 10-15 min until the liquid volume in the upper column is down to 30-50 µL.
- Re-buffer the sample with a total of 8x400 µL nuclease-free water.
- After the last spinning step carefully transfer the column upside down into a fresh collection tube. Spin at 1,000 x g for 2 min and transfer the eluted DNA from the collection tube into a DNA-LoBind tube.
- Measure DNA concentration.

5. <u>Big scale transformations</u>

a. Shooting the library

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- Defrost the Recovery Medium at 37 ºC and keep it pre-warmed.
- Prepare a disposable 125 mL flask with 18-19 mL 2YT media and pre-warm it at 37 ºC (19 mL for 1x transformation, 18 mL for 2x transformations)
- Place the cuvettes (2 mm) in their bags on ice.
- Pre-chill the nuclease-free water on ice.
- Defrost the bulk ligation and keep it on ice.
- Defrost the TG1 cells on ice for about 10 min.
- Add 60 µL pre-chilled nuclease-free water to the cells and 10 µL of the ligation mixture. Mix by flicking.
- Transfer the diluted cells/ligation mixture into a pre-chilled cuvette and make sure the cells are at the bottom of the cuvette by gently tapping it onto the bench.
- Place the cuvette in the electroporator and apply a pulse (25 µF / 200 ohm / 2500 V).
- No later than 10s after the pulse add 870 µL Recovery Medium and pipette gently up and down 3 times.
- Transfer the transformed cells into the prepared 125 mL flask and incubate them at 37 ºC / 220 rpm for 1h. (Two consecutive transformations can be pooled in one flask).
- Transfer culture into a 50 mL tube and spin at 3,220 x g for 5 min. Discard supernatant and resuspend cell pellet in 1 mL 2YT. Remove 10 µL for serial dilutions ($10^{-2/-4/-5/-6/-7}$) and plate the remaining cells on two Bioassay dishes with 2YT agar (25 µg/mL chloramphenicol, 2% glucose) per transformation. Make a note of the volume plated.
- Plate 20 µL of the $10^{-5/-6/-7}$ dilutions onto small agar plates (2YT agar with 25 µg/mL chloramphenicol, 2% glucose).
- Incubate Bioassay dishes and agar plates at 30 ºC overnight.
- Repeat transformations until sufficient transformed cells have been collected (~10x than aimed for library size). Bioassay dishes can be sealed with parafilm and stored at 4 ºC until all transformations have been finished.

b. QC by Sanger sequencing

- Repeat steps of 3.c

c. Pooling transformed cells and making glycerols

- Prepare 2YT media supplemented with 15% (v/v) glycerol (final concentration).
- Add 10-15 mL 2YT/glycerol mixture to one Bioassay dish and scrape off colonies.
- Once all colonies have been scraped off transfer cell suspension onto next tray and continue scraping off colonies. If suspension gets very viscous it can be topped up with fresh media. However, the final volume shouldn't exceed 14 mL.

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- Once colonies of all transformations (coming from the same batch) have been pooled, transfer cell suspension into a 15 mL falcon tube.
- Measure OD_{600} of each tube and combine cells suspensions depending on diversity calculated from counting plates for each transformation batch.
- Aliquots are made of containing cells 15x fold over-representing library diversity and stored at -80 ºC.

d. Testing viability of glycerols

- Defrost one aliquot of pooled library glycerols.
- Make serial dilutions of pooled glycerol stocks in 2YT (10^{-6}/10^{-7}).
- Plate 20 µL of each dilution on LB agar plates supplemented with 25 µg/mL chloramphenicol and 2% glucose (duplicates!). Incubate plates at 37 ºC overnight.
- Cfu numbers indicate actual concentration of viable cells per mL.

6. <u>Phage propagation</u>

a. Calculation of glycerol stock needed

- Based on previous calculations regarding the viability of the glycerols, calculate how many glycerols you will need to cover 10x library diversity

b. Phage cultures

- Inoculate 6x 500 ml pre-warmed 2xYT containing 25 µg/ml chloramphenicol and 5% glucose with glycerols to OD_{600} = 0.08-0.1 in 2 litre disposable flasks to allow good aeration.
- Grow at 37ºC with shaking at 250 rpm until OD_{600} is 0.5 (75-90 min).
- *Option: if calculated amount of cells can't be accommodated in six flasks with an OD_{600} = 0.08-0.1 then prepare some pre-cultures (same media and sterile flasks), let cultures grow for 1 h and use those pre-cultures to inoculate main six culture flasks*
- Transfer cultures into conical 500 mL centrifuge pots (max. 500 mL) and add $2x10^{12}$ M13K07 helper phage per 500 mL culture. Mix well.
- Incubate without shaking in a 37 ºC water bath for 60 min.
- Spin at 3,300 xg, 20 °C for 15 min. Check and re-spin if necessary. Resuspend the pellet of each centrifuge tube in 2xYT containing 25 µg/ml chloramphenicol and 50 µg/ml kanamycin (and 0.1% glucose). Use 500ml per 250 mL of original culture.
- Transfer culture to disposable 2 L flasks (max. 500 mL per flask). Flasks from pre-infection step can be re-used.
- Incubate shaking (170 rpm) at 25 ºC overnight.

c. Phage purification

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- Transfer PEG-NaCl aliquots (and 0.5 L or 0.25 L centrifuge pots if available) to the cold room to pre-cool overnight.
- The overnight culture is centrifuged at 3,300 xg / 4 ºC for 30 min. The supernatant is recovered and 100 mL pre-chilled PEG/NaCl is added per 400 mL of supernatant. It is advisable to pre-alliquot the PEG/NaCl the day before and leave it in the cold room overnight as this will ensure the tubes and PEG is chilled and will aid the recovery of phage.
- The mixture is incubated on ice in the cold room for 2-3 h. Do not incubate with PEG overnight. This will result in larger pellets, but will be mostly due to host DNA and bacterial debris.
- The mixture is centrifuged at 3,300 xg / 4 ºC for 30 min and the supernatant is discarded.
- The resulting pellets are each resuspended in PBS (8 mL per 400 mL SN + 100 mL PEG/NaCl). Once the pellet has been fully resuspended, transfer the phage solution into 50 mL falcon tubes and spin again at 11,600 xg / 4 ºC for 10 min.
- Transfer supernatant into new 50 mL tubes and add pre-chilled PEG/NaCl (2 mL per 8 mL PBS). The solution is mixed well by inversion and left on ice for 1 h.
- Mixture is centrifuged at 3,300 xg / 4 ºC for 30 min and the supernatant discarded.
- The pellet is resuspended in PBS (5 mL per 8 mL PBS used after first precipitation), and centrifuged at 11,600 xg for 10 min to remove any remaining bacterial debris.
- The supernatant is pooled (not filtered). Glycerol is added to the phage solution (final concentration 15 %) and mixed thoroughly.
- The phage stocks are now prepared and should be stored in PBS / 15 % glycerol at 4 ºC (not longer than a couple of days until the titre and display levels are determined) and then at -80 ºC in working size aliquots (each phage aliquot should have at least 10x over-representation of the library). Use protein LoBind tubes.
- It is advisable to determine the titre of the phage by both optical density and *E.coli* infection . Display levels should be determined by western blotting.

d. Contamination/Infectivity test

- Set up a 5 ml culture (2YT medium supplemented 12 µg/ml tetracycline) of ER2738 *E.coli* cells from a single colony in a 14 cm vented tube.
- Incubate overnight in an orbital incubator at 37°C, 220 rpm.
- Measure the OD600 of a 10^{-1} dilution of the overnight ER2738 culture.
- If the OD_{600} is <5 this may indicate that they will not be suitable for infection. Discard the overnight culture and prepare a fresh overnight culture.
- Prepare a 20 mL culture at OD_{600nm} = 0.18 — 0.20 by diluting the ON in 2YT supplemented with 12 µg/mL tetracycline. Check the OD_{600nm}.

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- Incubate at 37ºC, 220 rpm for 45-90mins, until the cells reach an OD_{600} range of 0.50-0.70.
- During this incubation prepare the following:
  - **Top agar control** (contamination check):
    - Set a water bath to 45ºC and melt top agar in a microwave.
    - Aliquot 4 x 3 ml melted top agar into 14 cm vented tubes and place in the water bath to cool for at least 30 mins prior to use.
    - Prepare phage samples by serial dilution in PBS and place on ice:

1E10 ph/mL

1E9 ph/mL

1E8 ph/mL

    - During the second half of the incubation, add 10 µl phage to a 2 ml microfuge tube.
    - Prepare another 2 ml microfuge tube with 10 µL PBS (this is the negative control).
    - Leave the tubes at room temperature until required.
  - **Amplified phage control**:
    - Prepare phage samples by serial dilution and place on ice:

2E9 ph/mL

2E8 ph/mL

2E7 ph/mL

2E6 ph/mL

2E5 ph/mL

    - During the second half of the incubation, add 10 µl of diluted purified phage to 1.5 mL tubes.
    - Leave the tubes at room temperature until required.
- Once the ER2738 cells reach OD_{600} = 0.50-0.70 add 1 mL cells to each prepared 1.5 and 2 mL tube. Mix all tubes by inversion.
- Immediately incubate all tubes at 37ºC for 15 mins without shaking.
- After the 15 min incubation:
  - **Amplified phage control**: Transfer the 3x 2 ml microfuge tubes to a tube shaker at 37ºC, 1000 rpm and incubate for a further hour.
  - **Top agar control** (eluted phage): Add 300 µl of the cell/phage mixture to the 14 cm vented tubes prepared with top agar. Mix gently by swirling the tube and pour immediately onto LB-agar plates supplemented with 12 µg/ml tetracycline and tilt the

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plate so that the top agar solution covers the plate. Leave the plates agar-down in a dark place to allow top agar to set.

- **Amplified phage control:** Plate 50 µl from each dilution onto LB-agar plates supplemented with 100 µg/ml carbenicillin or 25 µg/mL chloramphenicol or 50 µg/mL kanamycin.
- Incubate all plates agar-up at 37ºC overnight in a static incubator.
- Calculate infective phage [pfu/ml].

7. <u>Display level (anti-gIIIp western blot)</u>

a. SDS-PAGE

- Prepare 1E11 phage in 6.5 µL PBS and add 2.5 µL of 4x Loading dye and 1 µL 10x reducing agent. Prepare duplicate samples.
- Incubate at 70 °C for 10 min.
- Transfer the phage prep into the wells of a 12-well BOLT Bis-Tris gel.
- Run the gel at 200 V for 22 min.

b. Blotting

- Wet a filter paper (from blue bag) with MilliQ water.
- Peel off foil from the NC Mini/Regular Stacks and place stack with the plastic tray in the iBlot2 device.
- Lift the copper/gel layer off the stack and place it onto the removed foil.
- Remove and discard the thin plastic covering the nitrocellulose membrane of the stack.
- Open the cassette holding the SDS gel, remove wells and bottom edge and place it on to the NC membrane (ideally without repositioning). Any bubbles can be removed by carefully streaking over the gel with wetted gloves.
- Place previously wetted filter paper on top of the gel and remove any bubbles by rolling across the filter paper with a roller.
- Place copper/gel layer back on to the filter paper (gel side facing down) and use roller again.
- Take a filter paper with an attached electrode from the NC Mini stacks box and place it on top of the stack with the electrode covering the electronics in the iBlot2 device.
- Close the lid of the iBlot2 device and wait for "Start the last run" lighting up on the display. Reposition filter paper with electrode if necessary.
- Choose in "templates" the P0 programme (takes 7 min).

c. Detection of gIIIp

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- Transfer the blotted nitrocellulose membrane into a 50 mL falcon tube and add 5 mL of 3 % (w/v) skim milk in TBS pH 7.5 to it.
- Incubate on a mixing roller at RT for 1 h.
- Discard the blocking solution and wash the membrane by adding 5 mL TBS-T (0.05 %).
- Mix on a mixing roller at RT for 5 min. Repeat 2 more times.
- Primary antibody: Add 5 mL of a 1:1000 dilution of anti-gIIIp antibody in 1 % (w/v) skim milk in TBS pH 7.5.
- Incubate on a mixing roller at RT for 1 h.
- Wash membrane 3x with TBS-T as before.
- Secondary antibody: Add 5 mL of a 1:2000 dilution of HRP-coupled anti-mouse antibody in 1 % (w/v) skim milk in TBS pH 7.5.
- Incubate on a mixing roller at RT for 1 h.
- Wash membrane 3x with TBS-T as before.
- Detect bound antibodies with 1.5 mL of a chemiluminescence substrate (e.g. ECL). Leave solution on the membrane for 1 min, then tilt the tray and catch any excessive ECL solution with a paper towel.
- Image the membrane.
- Save the resulting image, quantify the bands and evaluate display levels.

Example 9 - Further Exemplary Scaffold Proteins

The invention embraces Stefin A scaffolds with mutations as described above with a certain defined sequence identity with reference to human Stefin A (SEQ ID NO: 1) as mentioned in the claims. Here we provide examples of useful scaffolds according to the present invention illustrating functionality at different levels of sequence identity.

In this example, the polypeptides of the invention comprise amino acid sequence derived from canine SteA.

Mutations relative to canine wild type sequence are underlined (mutations relative to SEQ ID NO: 1 can be identified by sequence alignment).

cSteA3r2: SEQ ID NO: 93

MMPGGLTEAKPATPEVQEIANEVKPQLEKETGKTWQEFEAVEYKTQVDAGLNYYIKVRVN-GKYIHLKIFKGLPGQNPTLTLTGYQTDKSKDDELTGF

cSteA3r2 with $(GGS)_3$ heterologous peptide insertions (boxed):SEQ ID NO: 94

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MMPGGLTEAKPATPEVQEIANEVKPQLEKETGKTWQEFEAVEYKTQVDGGSGGSGGSGLNYYIK
VRVN-GKYIHLKIFKGLGGSGGSGGSPTLTLTGYQTDKSKDDELTGF

These have been successfully expressed.

Exemplary Tm measurements are as follows:

cSteA 3r2 Tm = 79.2 °C

cSteA 3r2 with $(GGS)_3$ in loops 2 and 4 Tm = 83.6 °C

This demonstrates that heterologous peptides can be inserted in the positions as taught with reference to hSteA.

Further exemplary sequences based on cSteA (changes from wild type cSteA are underlined) include:

cSteAtA SEQ ID NO: 95

MIPGGLTEAK PATPEVQEIA NEVKPQLEEK TGETYQEFEA VEYKTQVDAG
INYYIKVRVG DNSYIHLKIF KGLPGQNPTL TLTGYQTDKS KDDELTGF

cSteAtA comprises a minimal set of mutations which have the advantages of preventing possible ambiguous initiation of translation (M2I – reverts to hSteA sequence), removing a glycosylation site (N32G) and minimising domain swap dimerization (V48D – already described). These mutations are useful and may be made individually.

cSteAtB SEQ ID NO: 96

MIPGGLTEAK PATPEVQEIA NEVKPQLEEK TGETYQEFEA VEYKTQVDAG
INYYIKVRVG DNSYIHLKIF KSLPGQNEDL TLTGYQTDKS KDDELTGF

cSteAtB includes the minimal mutations and further mutations to advantageously make the "entry" and "exit" of Loop 4 the same as hSteA (G72S, P78E and T79D – all reverting to hSteA sequence)

cSteAtC SEQ ID NO: 97

MIPGGLTEAK PATPEVQEIV NEVKPQLEEK TGETYQELEA VEYKTQVDAG
INYYIKVRVG DNSYIHLKIF KGLPGQNPTL TLTGYQTDKS KDDELTGF

cSteAtC includes the minimal mutations and further mutations relative to wild type cSteA to "revert" residues back to the human sequence (which residues were found to be destabilising in hSteA (A20V, F38L – both reverting to hSteA sequence)). This has the advantage of rendering the polypeptide based on cSteA more stable.

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cSteAtD SEQ ID NO: 98

MIPGGLTEAK PATPEVQEIV NEVKPQLEEK TGETYQELEA VEYKTQVDAG
INYYIKVRVG DNSYIHLKIF KSLPGQNEDL TLTGYQTDKS KDDELTGF

cSteAtD combines includes the minimal mutations and mutations to make the "entry" and "exit" of Loop 4 the same as hSteA (G72S, P78E and T79D – all reverting to hSteA sequence)

cSteAt1AL SEQ ID NO: 99

MIPGGLTEAK PATPEVQEIA NEVKPQLEEK TGETYQEFEA VEYKTQVDGGSGGSGGSG
INYYIKVRVG DNSYIHLKIF KGLGGSGGSGGSPTL TLTGYQTDKS KDDELTGF

As cSteAtA but including GGSGGSGGS (SEQ ID NO: 92) heterologous peptide insertions (boxed) - one in place of residue 48 and one in place of residues 74 - 77

cSteAt2BL SEQ ID NO: 100

MIPGGLTEAK PATPEVQEIA NEVKPQLEEK TGETYQEFEA VEYKTQVDGGSGGSGGSG
INYYIKVRVG DNSYIHLKIF KSLGGSGGSGGSEDL TLTGYQTDKS KDDELTGF

As cSteAtB but including GGSGGSGGS (SEQ ID NO: 92) heterologous peptide insertions (boxed) - one in place of residue 48 and one in place of residues 74 - 77

cSteAt3CL (SEQ ID NO: 101)

MIPGGLTEAK PATPEVQEIV NEVKPQLEEK TGETYQELEA VEYKTQVDGGSGGSGGSG
INYYIKVRVG DNSYIHLKIF KGLGGSGGSGGSPTL TLTGYQTDKS KDDELTGF

As cSteAtC but including GGSGGSGGS (SEQ ID NO: 92) heterologous peptide insertions (boxed) - one in place of residue 48 and one in place of residues 74 - 77

cSteAt4DL (SEQ ID NO: 102)

MIPGGLTEAK PATPEVQEIV NEVKPQLEEK TGETYQELEA VEYKTQVDGGSGGSGGSG
INYYIKVRVG DNSYIHLKIF KSLGGSGGSGGSEDL TLTGYQTDKS KDDELTGF

As cSteAtD but including GGSGGSGGS (SEQ ID NO: 92) heterologous peptide insertions (boxed) - one in place of residue 48 and one in place of residues 74 - 77

Additional Exemplary Scaffolds

Wild type cSteA already contains the beneficial residues E42 and I65 and I51 (T51L is most prefrerred, but T51I is equally as good). The minimal mutant cSteAA (above) is equivalent to 3t5 derived from hSteA sequence.

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Here we demonstrate an equivalent to hSteA 3r1 using the canine sequence, which is given below (cSteAr1).

cSteAr1 (SEQ ID NO: 103)
MMPGGLTEAKPATPEVQEIANEVKPQLEKETGKTWQEFEAVEYKTQVDAGLNYYIKVRVG-
NKYIHLKIFKGLPGQNPTLTLTGYQTDKSKDDELTGF

cSteA 3r2 is already presented above (SEQ ID NO: 93).

Example 10: Increased Tm's

Polypeptides having mutation(s) were made as described herein.

In this example, polypeptides were based on the human Ste A sequence (SEQ ID NO: 1) with mutations as described; data relative to wild type human SteA are shown below.

Table of Increasing Stability Substitutions

Mutation	**Effect on Tm (°C)**
E29M	+0.7
E29K K30E E33K	+3.9
N32G	+3.5
T34K	+3.0
T34R	+2.6
T34V	+2.2
Q42E	+3.2
T45V	+1.8
T45I	+1.0
T51I	+4.8
T51L	+4.6
T51V	+4.4
T51F	+1.0
A59V	+3.9
A59I	+3.4
A59L	+1.1
A59L G60N D61G N32K	+0.8

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A59V G60 D61N N62K	+1.0
A59 G60N G61G N62K	+1.3
A59 G60N ΔD61 N62G	+2.0
A59 G60 ΔD61 N62	+2.2
A59L G60N ΔD61 N62G	+3.1
A59V G60N D61G N32K	+3.4
A59I G60N ΔD61 N62G	+3.6
A59I G60N D61G N62K	+3.6
A59V G60N ΔD61 N62G	+3.9
A59V G60 ΔD61 N62	+4.7
K63R	+1.7
M65I	+5.5
M65V	+3.9
L67I	+1.0
N90T	+1.4

Example 11: Decreased Tm's

Polypeptides having mutation(s) were made as described herein.

In this example, polypeptides were based on the human Ste A sequence (SEQ ID NO: 1) with mutations as described; data relative to wild type human SteA are shown below.

Table of Decreasing Stability Substitutions

Mutation	**Effect on Tm (°C)**
A12I	-6.5
A12V	-4.5
I16L	-5.7
V20A	-10.6
V20I	-9.1
V20L	-5.2
V20I L38A	-16.7
V20L L38A	-15.3
V20L L38V	-11.0

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V20I L38V	-10.4
Q26E	-3.2
T31K	-7.4
N32D	-3.8
N32H	-1.6
T34D	-3.7
T34P	-1.9
L38A	-12.8
L38V	-7.8
L38F	-7.2
A40I	-8.0
A40V	-2.8
Q42D	-2.7
V48E	-6.7
V48D	-6.2
V48G	-4.8
V48A	-2.5
V48L	-2.0
G50S	-7.5
T51A	-2.4
Y54D T83D Q86E	-21.0
A59 G60P ΔD61 N62P	-9.7
A59 G60P D61P N62K	-6.9
A59 G60P ΔD61 N62G	-3.8
A59 G60P D61G N62K	-1.4
A59 G60 D61N N62K	-1.1
T83D Q86E	-4.8

Example 12: Demonstrations of various 3r2 binders and various type 4 (canine) binders

Type 3r2 library

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Figures 12 -14 show that the outputs of selections against human epidermal growth factor receptor 2 (Her2), the humanized monoclonal therapeutic antibody Trastuzumab, and human programmed death-ligand 1 (PD-L1), tested in an "iQue assay". These data show that Affimer reagents can bind tightly and specifically to targets, without cross-reactivity to related and unrelated proteins. These data also show that the results from Affimer reagents are repeatable, as loop sequences are often represented multiple times in an iQue assay, giving similar results.

The Intellicyt iQue Screener is a flow-cytometry based, multiplexed bead assay where the target proteins and relevant controls are immobilised on fluorescently indexed beads. The Affimer reagent is incubated with the beads and binds to the target protein, the complex is then detected with an anti-HA Alexa488 labelled antibody. For screening, iQue assays are used to select the best Affimer reagents based on their affinity and selectivity towards the desired target. Ideally, Affimer reagents will show a strong positive signal to the bead coated in their target, but not to any of the other beads.

In figures 12 - 14, Affimer Working ID is an internal identifier; heterologous peptides are present (inserted) in the Loop 2 and Loop 4 positions. Amino acid sequence is determined from DNA sequencing data. The targets tested in the iQue assay were EGFR, mIgG2b, hIgGG1 Fc, hPD-L2, Her2 (R&D), hPD-L1, Trastuzumab, hIgG, Her2 (Sino), Her 3, mPD-L1, Avastin, Humira, Rituximab, mPD-L2, Her 4 and a no target control. FL1-A is the measured signal. QSHxx, where xx is a number, refers to a specific type of fluorescently indexed bead.

Figure 12 shows output of an iQue assay for binders to Her2.

Figure 13 shows output of an iQue assay for binders to Trastuzumab.

Figure 14 shows output of an iQue assay for binders to human PD-L1.

Figures 15 and 16 show typical multi-point ELISA data, where an Affimer reagent is coated on to a 96 well plate and is used as a capture reagent. These data show that binders identified in either a phage ELISA (on plates, but with the Affimer reagent attached to a phage) or an iQue assay (on beads, but a purified Affimer reagent) can detect target in a typical ELISA application, that the signal is titratable, and, particularly for PD-L1, a low concentration of target can be detected.

Here, Affimer reagents identified to PD-L1 (Figure 15) and Her2 (Figure 16) were coated on ELISA plates at a fixed concentration. After washing and blocking, the appropriate biotinylated targets are titrated and left to bind to the coated Affimer reagents. After a further wash, streptavidin-HRP is used to detect the presence of captured target with the use of an appropriate substrate. Both anti-PD-L1 Affimer reagents bind biotinylated PD-L1 at low

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concentrations and biotinylated PD-L1 does not bind to the ELISA plate (no coat controls; each clone was on a separate ELISA plate, and each contained its own no coat control). The anti-Her2 Affimer reagents do not perform as well as the anti-PD-L1 Affimer reagents, with lower overall signals, but they do demonstrate a titratable response. Here, biotinylated Her2 does interact slightly with the ELISA plate at the highest concentration tested.

Figure 15 shows ELISA data for two different anti-PD-L1 Affimer reagents (clones A8 and G8).

Figure 16 shows ELISA data for two different anti-Her2 Affimer reagents (clones C4 and E10).

Type 4 library (canine scaffold)

The type 4 library, based on the putative canine homologue of Stefin A, has been used to select Affimer reagents against canine PD-L1, specifically those that will inhibit the interaction between canine PD-1 can canine PD-L1. The output of an iQue assay for these reagents (Figure 17; description of iQue Screener as above) shows that we are also able to use this canine scaffold to find specific binding reagents. Here, the top 6 Affimer reagents are showing cross-reactivity to human Fc, the canine PD-L1 used in the selection process is a canine PD-L1 / human Fc fusion protein. Rather than actually binding to canine PD-L1, they are in reality very good binding reagents to the human Fc domain. The use of a scaffold based on a non-human protein such as a canine protein is useful for producing non-human (bio)therapeutics such as canine (bio)therapeutics, which should be much less immunogenic and therefore better tolerated than a therapeutic derived from a different species.

Figure 17 shows output of an iQue assay for binders to canine PD-L1 by canine Stefin A-based Affimer reagents. In Figure 17, the targets tested in the iQue assay were canine PD-L1, human VEGFR2, SMA(4A6)+BA-peptide, human Fc, canine PD-1, amine PEG11, mIgG2b, fumonisin and SMA (2A9) + BA-peptide, as well as a no target control.

One industrial use for the canine scaffold is to generate canine therapeutic reagents. Suitably the canine Affimer reagents bind to their target, and suitably do so in such a way as to disrupt the binding of the natural ligand to have a biological effect. In this selection, we wanted to find canine Affimer reagents that prevent canine PD-1 binding to canine PD-L1, which would have the potential for use as check-point inhibitors in cancer treatment. Here, eleven of the anti-canine PD-L1 Affimer reagents were tested for their ability to inhibit the interaction of canine PD-L1 with canine PD-1 in a competition ELISA. The results showed that some canine Affimer reagents were able to bind to canine PD-L1 with approximately the same affinity as canine PD-1, and other with a range of lower affinities.

Next, an ELISA plate was coated with canine PD-1. Separately, biotinylated canine PD-L1 is incubated with a titration of the different Affimer reagents, and canine PD-1 as a control (the

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competition reactions). After incubation, these competition reactions are then incubated with the coated ELISA plate. Where there is no inhibition, the biotinylated PD-L1 will be free to bind the PD-1 on the surface of the plate, and this will give a high signal when detected with a streptavidin-HRP reagent and suitable substrate. If, however, the Affimer reagent binds to canine PD-L1 in such a way as to inhibit binding to PD-1, it will give a low signal. Figure 18 shows the competition ELISA data for the canine Affimer reagents that affinities similar to PD-1, and Figure 19 shows the data for canine Affimer reagents that have lower affinities than PD-1.

Figure 18 shows competition ELISA for canine Affimer reagents that bind to cPD-L1 with similar affinity to canine PD-1. The curves are fits to a 4-parameter logistic model.

Figure 19 shows competition ELISA for canine Affimer reagents that bind to cPD-L1 with lower affinity to canine PD-1. The curves are fits to a 4-parameter logistic model.

All the Affimers in Figures 12 - 14 are 3r2 (SEQ ID NO: 19) except mIgG2b G12 and Zika NS1 C12 - see below.

Mouse IgG2b G12 has the adhiron scaffold ("type 2 scaffold", the consensus of plant stefins so unrelated to the human stefin A. The sequence is as follows:

MSAATGVRAVPGNENSLEIEELARFAVDEHNKKENALLEFVRVVKAKEQXXXXXXXXXTMYYLTLEAKDGG KKKLYEAKVWVKXXXXXXXXXNFKELQEFKPVGDAAAAHHHHHHG (SEQ ID NO: 105)

The canine Affimers have the mutations M2I, N32G and V48D from wildtype cSteA (underlined); the following sequnce:

MIPGGLTEAK PATPEVQEIA NEVKPQLEEK TGETYQEFEA VEYKTQVDAG INYYIKVRVG DNSYIHLKIF KGLPGQNPTL TLTGYQTDKS KDDELTGF (SEQ ID NO: 106)

In this example, the Zika NS1 C12 is also based on the adhiron scaffold:

MSAATGVRAVPGNENSLEIEELARFAVDEHNKKENALLEFVRVVKAKEQXXXXXXXXXTMYYLTLEAKDGG KKKLYEAKVWVKXXXXXXXXXNFKELQEFKPVGDG (SEQ ID NO: 107)

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CLAIMS

1. A polypeptide comprising an amino acid sequence having at least 80% identity to amino acid residues 1 to 11, 13 to 15, 17 to 19, 21 to 25, 27 to 28, 35 to 37, 39, 41, 43 to 44, 46 to 47, 49 to 50, 52 to 53, 55 to 58, 63 to 64, 66, 68 to 82, 84 to 85, and 87 to 98 of SEQ ID NO: 1;

   characterised in that said polypeptide comprises one or more mutations relative to SEQ ID NO: 1 selected from the group consisting of:

   T51L, T51V, M65V, N32G, A59I, E29M, T34V, T34R, T45I, T45V, T51F, A59L, L67I, (E29K, K30E, E33K), (A59L, G60N, D61G, N62K), (A59V, D61N, N62K), (G60N, D61G, N62K), (G60N, ΔD61, N62G), ΔD61, (A59L, G60N, ΔD61, N62G), (A59V, G60N, D61G, N62K), (A59I, G60N, D61G, N62K), (A59I, G60N, ΔD61, N62G), (A59V, G60N, ΔD61, N62G), and (A59V, ΔD61);

   wherein said polypeptide has a Tm higher than the Tm of SEQ ID NO: 1.

2. A polypeptide according to claim 1, wherein said polypeptide has a Tm higher than 89.0ºC

3. A polypeptide comprising an amino acid sequence having at least 80% identity to amino acid residues 1 to 11, 13 to 15, 17 to 19, 21 to 25, 27 to 28, 35 to 37, 39, 41, 43 to 44, 46 to 47, 49 to 50, 52 to 53, 55 to 58, 63 to 64, 66, 68 to 82, 84 to 85, and 87 to 98 of SEQ ID NO: 1;

   characterised in that said polypeptide comprises one or more mutations relative to SEQ ID NO: 1 selected from the group consisting of:

   L38A, V20I, A40I, L38V, A12I, A12V, I16L, V20L, Q26E, T31K, N32D, N32H, T34D, T34P, A40V, Q42D, V48E, V48G, V48A, T51A, (V20I, L38A), (V20L, L38A), (V20I, L38V), (V20L, L38V), (Y54D, T83D, Q86E), (G60P, ΔD61, N62P), (G60P, D61P, N62K), (G60P, ΔD61, N62G), (G60P, D61G, N62K), (D61N, N62K) and (T83D, Q86E);

   wherein said polypeptide has a Tm lower than the Tm of SEQ ID NO: 1.

4. A polypeptide according to any of claims 1 to 3, wherein said polypeptide further comprises at least one heterologous peptide insertion, wherein said heterologous peptide insertion comprises a heterologous peptide inserted at one of the following positions relative to SEQ ID NO: 1:

   a) 47-<heterologous peptide>-55

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b) 46-<heterologous peptide>-54

c) 46-<heterologous peptide>-50

d) 48-<heterologous peptide>-50

e) 49-<heterologous peptide>-51

f) 50-<heterologous peptide>-52

g) 66-<heterologous peptide>-85

h) 67-<heterologous peptide>-84

i) 70-<heterologous peptide>-74

j) 72-<heterologous peptide>-74

k) 71-<heterologous peptide>-73

l) 72-<heterologous peptide>-81

m) 73-<heterologous peptide>-80

n) 79-<heterologous peptide>-81

o) 80-<heterologous peptide>-81

p) 82-<heterologous peptide>-83

q) 72-<heterologous peptide>-77

r) 73-<heterologous peptide>-78

s) 74-<heterologous peptide>-79

t) 4-<heterologous peptide>-5

5. A polypeptide according to claim 4 wherein said polypeptide comprises two heterologous peptide insertions, a first heterologous peptide insertion at any of positions (a) to (f), and a second heterologous peptide insertion at any of positions (g) to (s).

6. A polypeptide according to claim 4 wherein said polypeptide comprises two heterologous peptide insertions, a first heterologous peptide insertion at any of positions (a) to (f), and a second heterologous peptide insertion at position (t).

7. A polypeptide according to claim 4 wherein said polypeptide comprises two heterologous peptide insertions, a first heterologous peptide insertion at any of positions (g) to (s), and a second heterologous peptide insertion at position (t).

8. A polypeptide according to claim 4 wherein said polypeptide comprises three heterologous peptide insertions, a first heterologous peptide insertion at any of positions (a) to (f), and a

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second heterologous peptide insertion at any of positions (g) to (s), and a third heterologous peptide insertion at position (t).

9. A polypeptide comprising an amino acid sequence having at least 80% identity to amino acid residues 1 to 11, 13 to 15, 17 to 19, 21 to 25, 27 to 28, 35 to 37, 39, 41, 43 to 44, 46 to 47, 49 to 50, 52 to 53, 55 to 58, 63 to 64, 66, 68 to 82, 84 to 85, and 87 to 98 of SEQ ID NO: 1; wherein said polypeptide comprises at least one heterologous peptide insertion; characterised in that said polypeptide comprises one or more mutations relative to SEQ ID NO: 1 selected from the group consisting of:

M65I, T51I, T51L, T51V, M65V, A59V, N32G, A59I, L38A, V20A, V20I, A40I, L38V, G50S, L38F, A12I, A12V, I16L, V20L, Q26E, E29M, T31K, N32D, N32H, T34V, T34R, T34K, T34D, T34P, A40V, Q42E, Q42D, T45I, T45V, V48E, V48D, V48G, V48A, V48L, T51F, T51A, A59L, K63R, L67I, N90T, (V20I, L38A), (V20L, L38A), (V20I, L38V), (V20L, L38V), (E29K, K30E, E33K), (Y54D, T83D, Q86E), (A59L, G60N, D61G, N62K), (A59V, D61N, N62K), (G60N, D61G, N62K), (G60N, ΔD61, N62G), ΔD61, (A59L, G60N, ΔD61, N62G), (A59V, G60N, D61G, N62K), (A59I, G60N, D61G, N62K), (A59I, G60N, ΔD61, N62G), (A59V, G60N, ΔD61, N62G), (A59V, ΔD61), (G60P, ΔD61, N62P), (G60P, D61P, N62K), (G60P, ΔD61, N62G), (G60P, D61G, N62K), (D61N, N62K), and (T83D, Q86E);

wherein said heterologous peptide insertion comprises a heterologous peptide inserted at one of the following positions relative to SEQ ID NO: 1:

d) 48-<heterologous peptide>-50,

e) 49-<heterologous peptide>-51,

f) 50-<heterologous peptide>-52,

q) 72-<heterologous peptide>-77,

r) 73-<heterologous peptide>-78; or

s) 74-<heterologous peptide>-79.

10. A polypeptide according to claim 9, wherein said polypeptide comprises two heterologous peptide insertions, a first heterologous peptide insertion at any of positions (d) to (f), and a second heterologous peptide insertion at any of positions (q) to (s).

11. A polypeptide according to claim 9, wherein said one or more mutations relative to SEQ ID NO: 1 is or are selected from the group consisting of:

M65I, T51I, T51L, T51V, M65V, A59V, N32G, A59I, E29M, T34V, T34R, T34K, Q42E, T45I, T45V, T51F, A59L, K63R, L67I, N90T, (E29K, K30E, E33K), (A59L, G60N, D61G, N62K), (A59V, D61N, N62K), (G60N, D61G, N62K), (G60N, ΔD61, N62G), ΔD61, (A59L, G60N, ΔD61, N62G), (A59V, G60N, D61G, N62K), (A59I, G60N, D61G, N62K), (A59I, G60N, ΔD61, N62G), (A59V, G60N, ΔD61, N62G), and (A59V, ΔD61);

preferably wherein said polypeptide has a Tm higher than the Tm of SEQ ID NO: 1.

12. A polypeptide according to claim 9, wherein said one or more mutations relative to SEQ ID NO: 1 is or are selected from the group consisting of:

L38A, V20A, V20I, A40I, L38V, G50S, L38F, A12I, A12V, I16L, V20L, Q26E, T31K, N32D, N32H, T34D, T34P, A40V, Q42D, V48E, V48D, V48G, V48A, V48L, T51A, (V20I, L38A), (V20L, L38A), (V20I, L38V), (V20L, L38V), (Y54D, T83D, Q86E), (G60P, ΔD61, N62P), (G60P, D61P, N62K), (G60P, ΔD61, N62G), (G60P, D61G, N62K), (D61N, N62K) and (T83D, Q86E);

preferably wherein said polypeptide has a Tm lower than the Tm of SEQ ID NO: 1.

13. A polypeptide according to claim 9, wherein said one or more mutations relative to SEQ ID NO: 1 is or are selected from the group consisting of:

T51L, T51V, M65V, N32G, A59I, L38A, V20I, A40I, L38V, A12I, A12V, I16L, V20L, Q26E, E29M, T31K, N32D, N32H, T34V, T34R, T34D, T34P, A40V, Q42D, T45I, T45V, V48E, V48G, V48A, T51F, T51A, A59L, L67I, (V20I, L38A), (V20L, L38A), (V20I, L38V), (V20L, L38V), (E29K, K30E, E33K), (Y54D, T83D, Q86E), (A59L, G60N, D61G, N62K), (A59V, D61N, N62K), (G60N, D61G, N62K), (G60N, ΔD61, N62G), ΔD61, (A59L, G60N, ΔD61, N62G), (A59V, G60N, D61G, N62K), (A59I, G60N, D61G, N62K), (A59I, G60N, ΔD61, N62G), (A59V, G60N, ΔD61, N62G), (A59V, ΔD61), (G60P, ΔD61, N62P), (G60P, D61P, N62K), (G60P, ΔD61, N62G), (G60P, D61G, N62K), (D61N, N62K) and (T83D, Q86E).

14. A polypeptide according to claim 13, wherein said one or more mutations relative to SEQ ID NO: 1 is or are selected from the group consisting of:

T51L, T51V, M65V, N32G, A59I, E29M, T34V, T34R, T45I, T45V, T51F, A59L, L67I, (E29K, K30E, E33K), (A59L, G60N, D61G, N62K), (A59V, D61N, N62K), (G60N, D61G, N62K), (G60N, ΔD61,

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N62G), ΔD61, (A59L, G60N, ΔD61, N62G), (A59V, G60N, D61G, N62K), (A59I, G60N, D61G, N62K), (A59I, G60N, ΔD61, N62G), (A59V, G60N, ΔD61, N62G), and (A59V, ΔD61);

preferably wherein said polypeptide has a Tm higher than the Tm of SEQ ID NO: 1.

15. A polypeptide according to claim 13, wherein said one or more mutations relative to SEQ ID NO: 1 is or are selected from the group consisting of:

L38A, V20I, A40I, L38V, A12I, A12V, I16L, V20L, Q26E, T31K, N32D, N32H, T34D, T34P, A40V, Q42D, V48E, V48G, V48A, T51A, (V20I, L38A), (V20L, L38A), (V20I, L38V), (V20L, L38V), (Y54D, T83D, Q86E), (G60P, ΔD61, N62P), (G60P, D61P, N62K), (G60P, ΔD61, N62G), (G60P, D61G, N62K), (D61N, N62K) and (T83D, Q86E);

preferably wherein said polypeptide has a Tm lower than the Tm of SEQ ID NO: 1.

16. A polypeptide according to any of claims 11 or 14 wherein said polypeptide has a Tm higher than the Tm of SEQ ID NO: 1.

17. A polypeptide according to any of claims 12 or 15 wherein said polypeptide has a Tm lower than the Tm of SEQ ID NO: 1.

18. A polypeptide according to any preceding claim wherein said polypeptide further comprises one or more mutations relative to SEQ ID NO: 1 selected from the group consisting of: G4R, G4W, V48D, V48E, G50 S, Y35W, Y43W, Y53W, Y54W, Y64W, F70W, Y85W, F98W, (K71N S72G L73P), or (E78A L80R).

19. A polypeptide according to any preceding claim comprising five or fewer mutations relative to SEQ ID NO: 1.

20. A polypeptide according to claim 19 wherein said five or fewer mutations are selected from the group consisting of Y35W, N32G, V48D, M65I, Q42E and T51L.

21. A polypeptide according to claim 19 wherein said five or fewer mutations are selected from the group consisting of N32G, V48D, M65I, Q42E and T51L.

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22. A polypeptide according to claim 19 having each of the mutations in one of the following groups:

    i) N32G V48D

    ii) N32G V48D M65I

    iii) N32G V48D M65I T51L

    iv) N32G V48D M65I Q42E

    v) N32G V48D M65I Q42E T51L

23. A polypeptide according to claim 22 having each of the mutations iv) N32G, V48D, M65I and Q42E.

24. A polypeptide according to any of claims 1 to 18 having each of the mutations in one of the following groups:

    a) Y35W N32G V48D M65I Q42E T51L (A59V ΔD61) (E29K K30E E33K)

    b) Y35W N32G V48D M65I Q42E T51L (A59V G60N ΔD61 N62G) (E29K K30E E33K)

25. A polypeptide according to claim 24 having each of the mutations b) Y35W N32G V48D M65I Q42E T51L (A59V G60N ΔD61 N62G) (E29K K30E E33K).

26. A polypeptide according to any preceding claim wherein said heterologous peptide is 6 to 36 amino acids in length.

27. A fusion protein comprising:
    a. a polypeptide according to any preceding claim; and
    b. one or more additional amino acid sequences selected from the group consisting of: secretion signal sequences, peptide linker sequences, affinity tags, transmembrane domains, cell surface retention sequence, substrate recognition sequences for post-translational modifications, multimerization domains to create multimeric structures of the protein aggregating through protein-protein interactions, half-life extending polypeptide moieties, polypeptide sequences for altering tissue localization and antigen binding site of an antibody, one or more additional polypeptides according to any preceding claim binding to the same or different targets, and one or more additional Affimer polypeptide sequences binding to the same or different targets.

28. A fusion protein according to claim 27, comprising one or more half-life extending polypeptide moieties selected from the group consisting of an Fc domain or portion

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thereof, an albumin protein or portion thereof, an albumin-binding polypeptide moiety, transferrin or portion thereof, a transferrin-binding polypeptide moiety, fibronectin or portion thereof, or a fibronectin-binding polypeptide moiety.

29. The polypeptide of claim 28, wherein the Fc domain or a portion thereof retains FcN binding.

30. The polypeptide of claim 28, wherein the Fc domain or a portion thereof is from IgA, IgD, IgE, IgG, and IgM or a subclass (isotype) thereof such as IgG1, IgG2, IgG3, IgG4, IgA1 or IgA2.

31. The polypeptide of claim 28, wherein the Fc domain or a portion thereof retains effector function selected from C1q binding, complement dependent cytotoxicity (CDC), antibody-dependent cell-mediated cytotoxicity (ADCC); phagocytosis; down regulation of B cell receptor, or a combination thereof.

32. The polypeptide of claim 20, wherein the half-life extending polypeptide moiety increases the serum half-life of the protein by at least 5-fold relative to its absence from the protein.

33. A polypeptide according to any preceding claim which is an affimer polypeptide.

34. A nucleic acid comprising nucleotide sequence encoding a polypeptide according to any of claims 1 to 33.

35. A vector comprising a nucleic acid according to claim 34.

36. A library comprising a population of polypeptides according to any of claims 1 to 33, wherein at least two individual polypeptides within said population comprise different heterologous peptide insertions.

37. A library comprising a population of nucleic acids, said nucleic acids comprising nucleotide sequences encoding a population of polypeptides according to claim 36.

38. A host cell comprising a polypeptide according to any of claims 1 to 33, a nucleic acid according to claim 34, or a library according to claim 36 or 37.

39. A polypeptide according to any of claims 1 to 33 for use in medicine.

40. A polypeptide according to any of claims 1 to 33 for use in the manufacture of a medicament.

41. A method for identifying a peptide capable of binding a structure of interest, said method comprising:

(i) providing a polypeptide according to any of claims 1 to 33 comprising a heterologous peptide insertion;

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(ii) contacting said polypeptide with said structure of interest; and

(iii) monitoring the association between the polypeptide and the structure of interest;

wherein association of the polypeptide with the structure of interest identifies the peptide as a candidate peptide capable of binding said structure.

42. Use of a polypeptide according to any of claims 1 to 33 as a scaffold protein.

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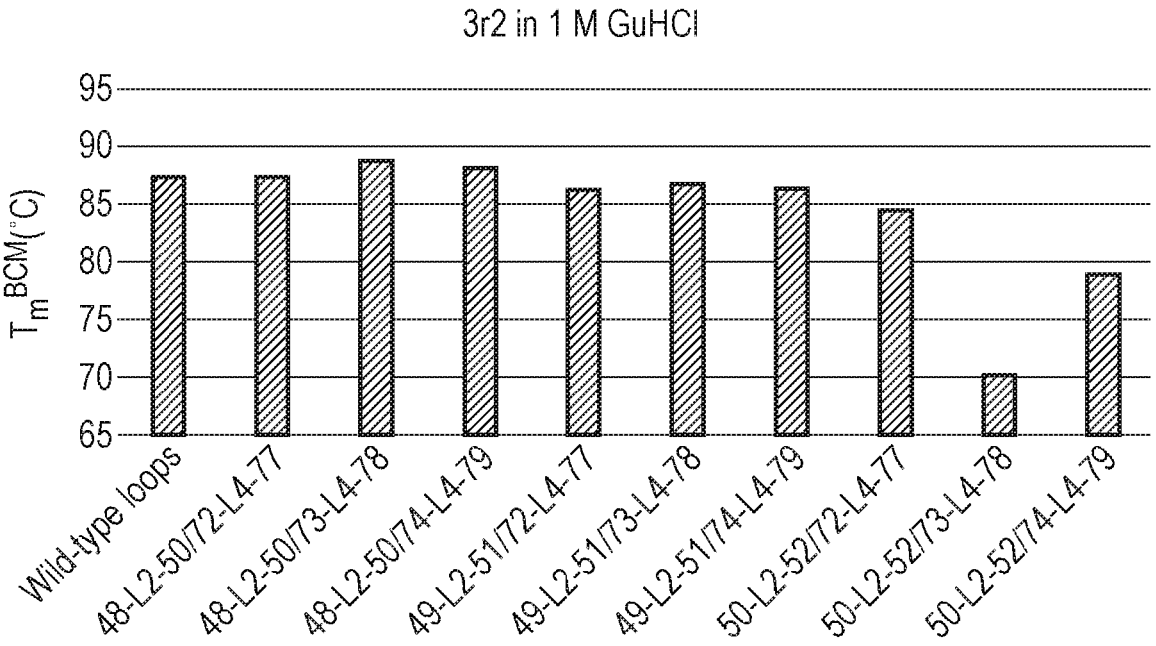


FIG. 1

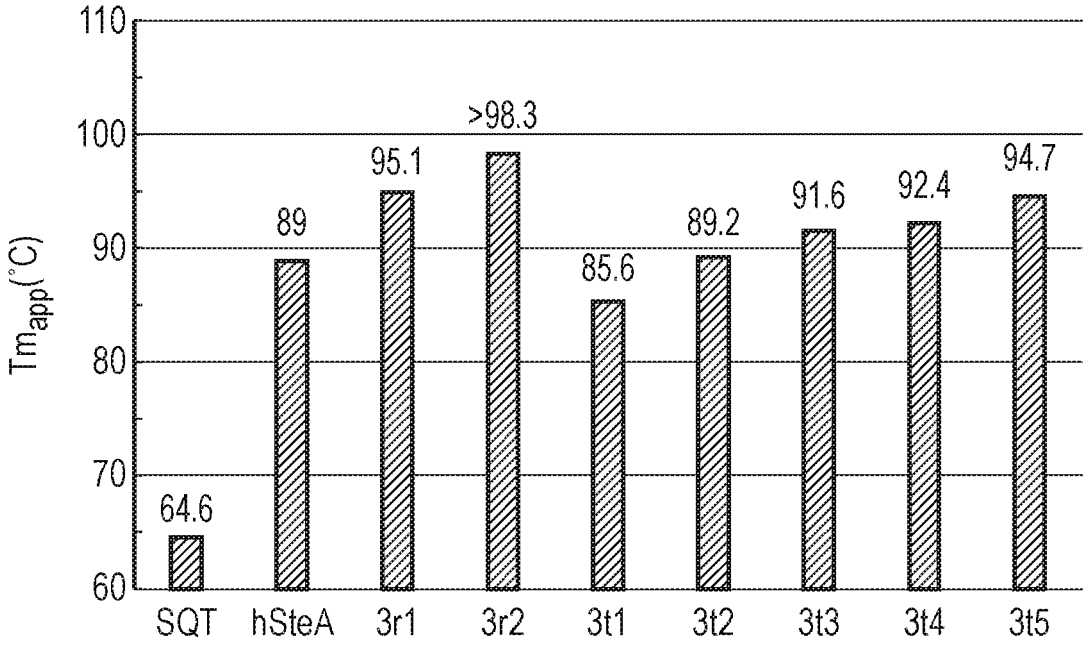


FIG. 2

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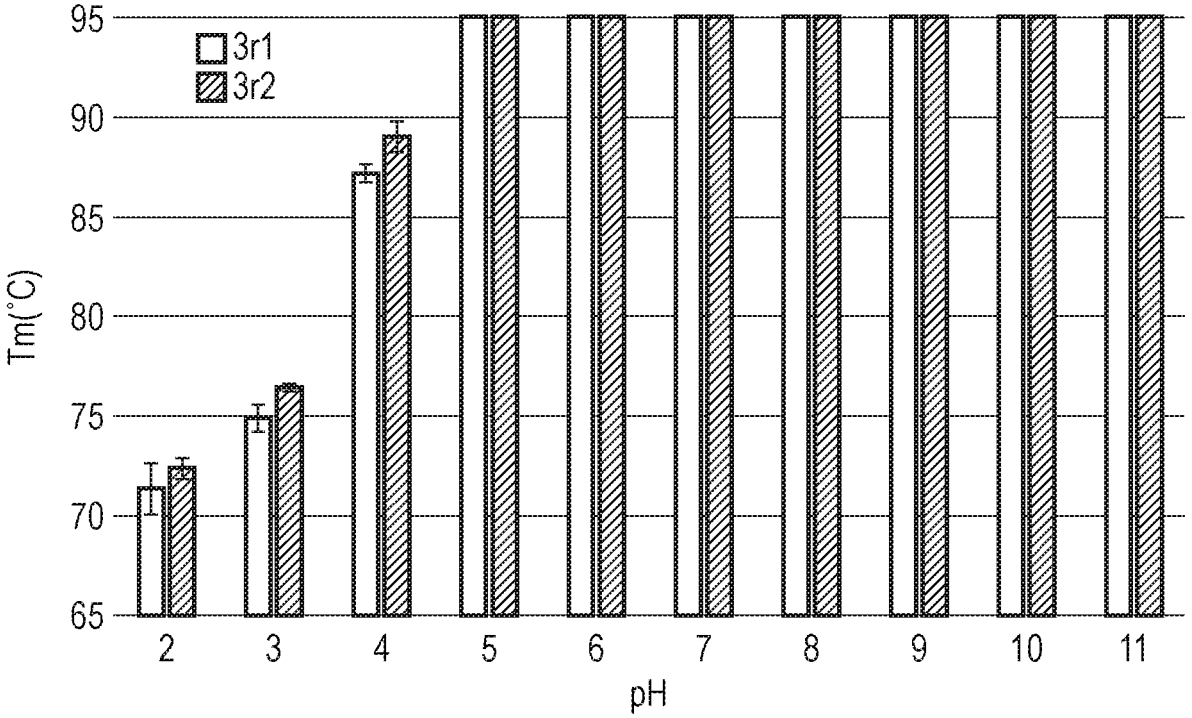


FIG. 3

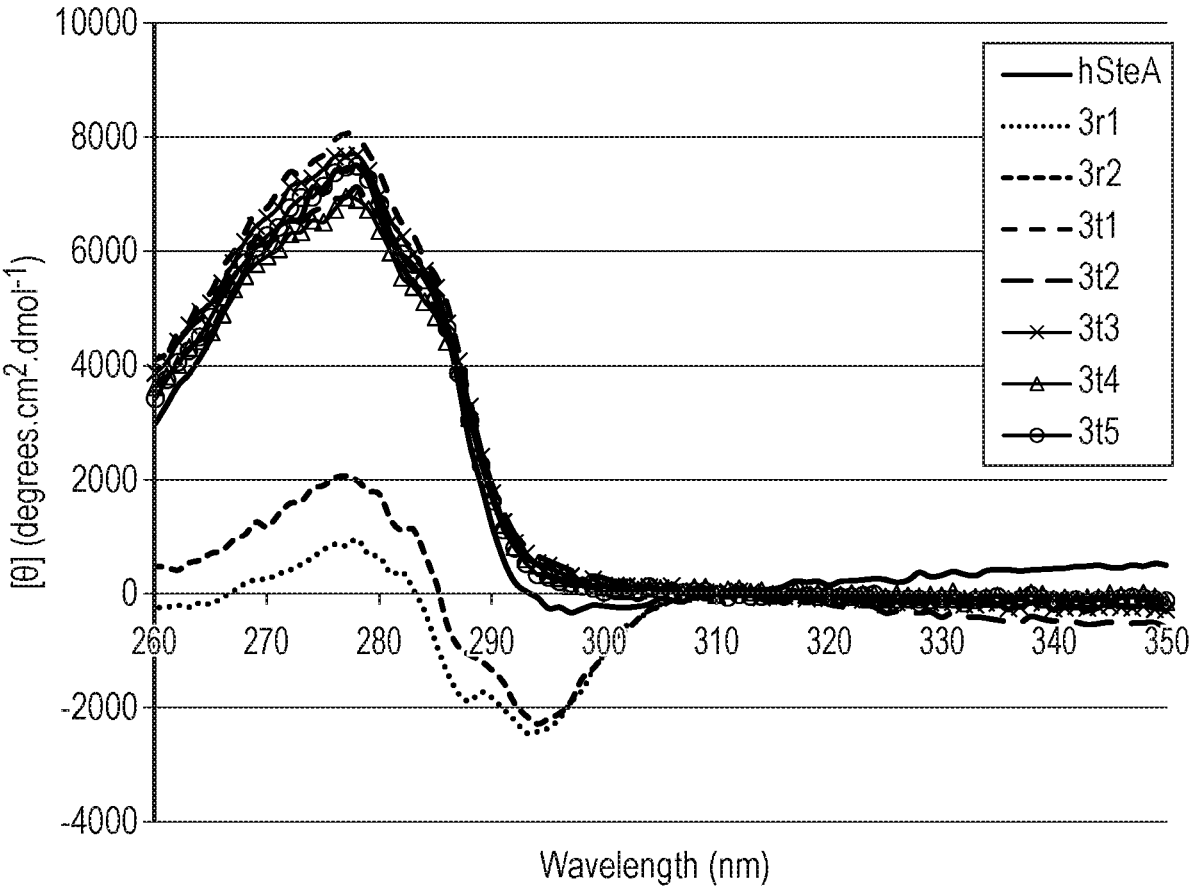


FIG. 4a

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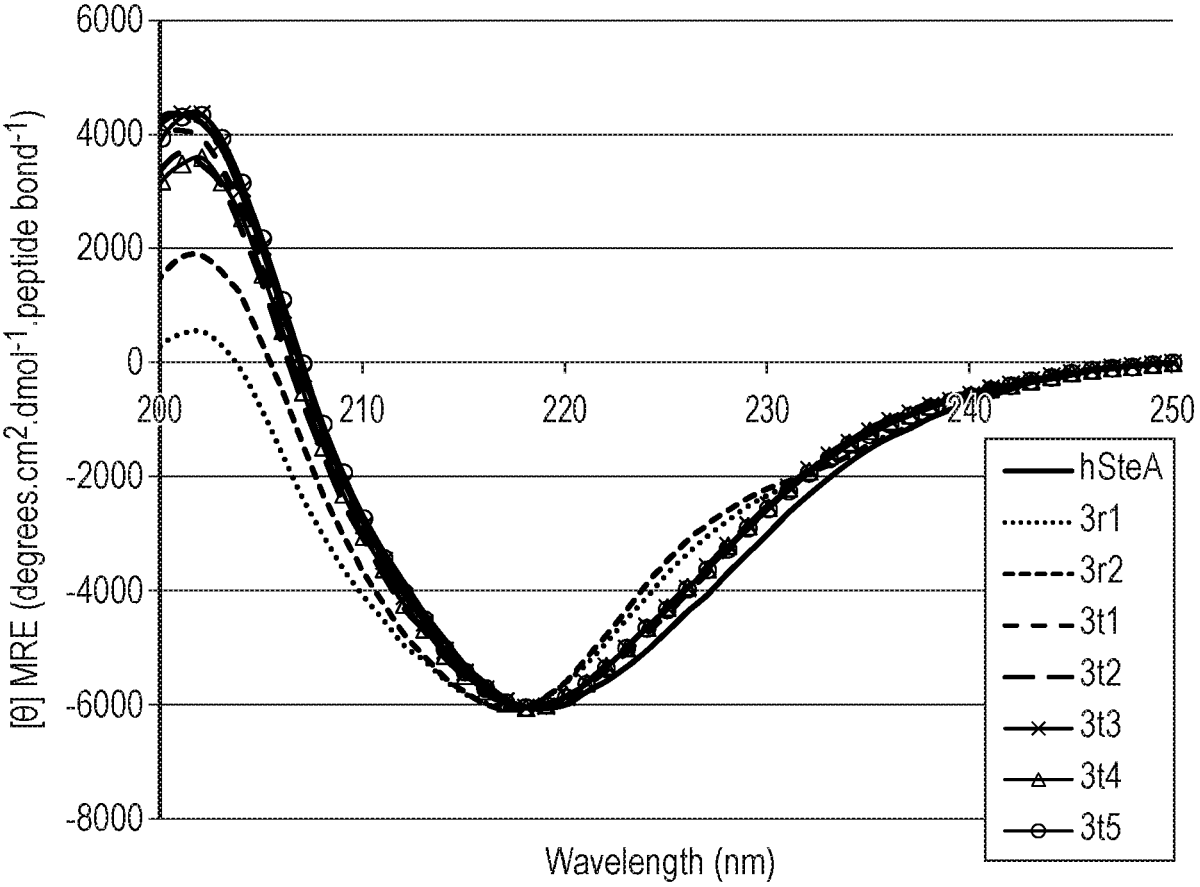


FIG. 4b

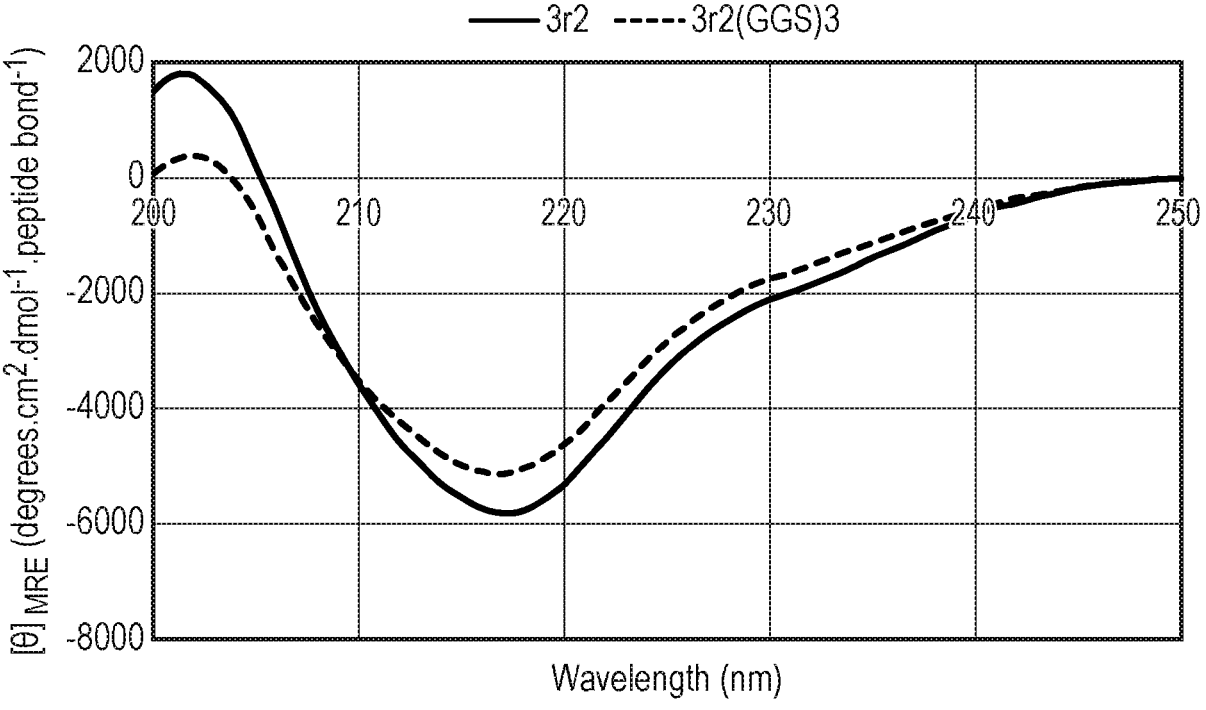


FIG. 5

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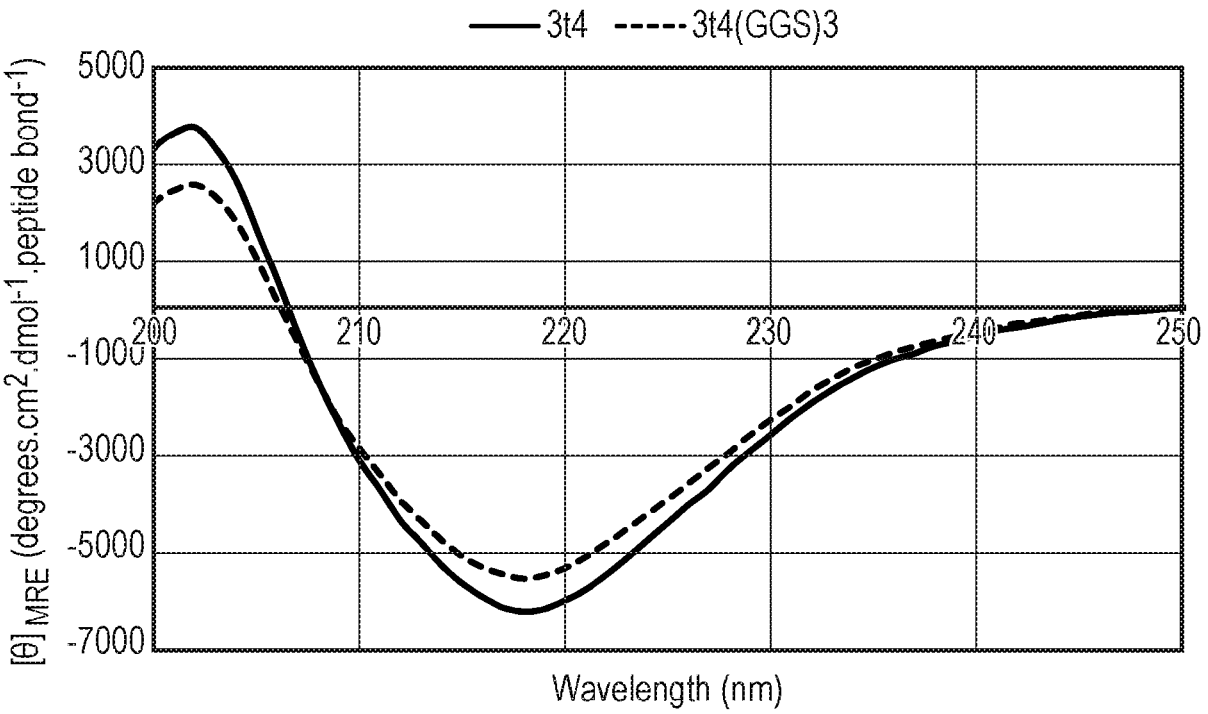


FIG. 6

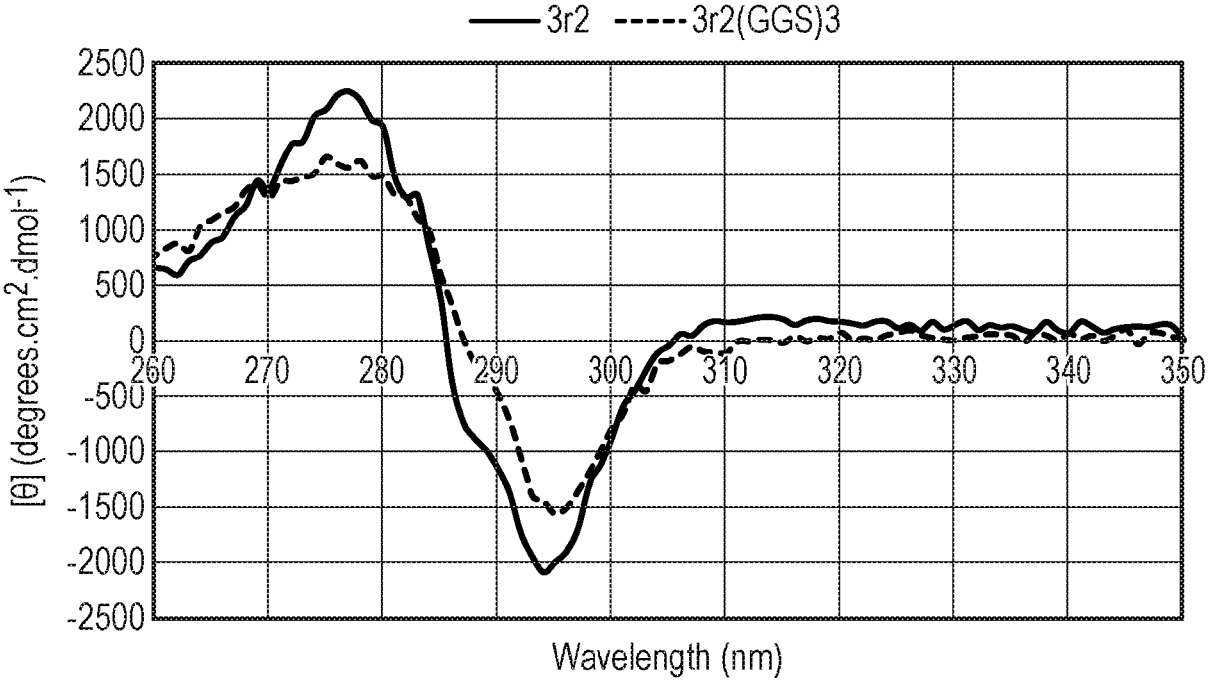


FIG. 7

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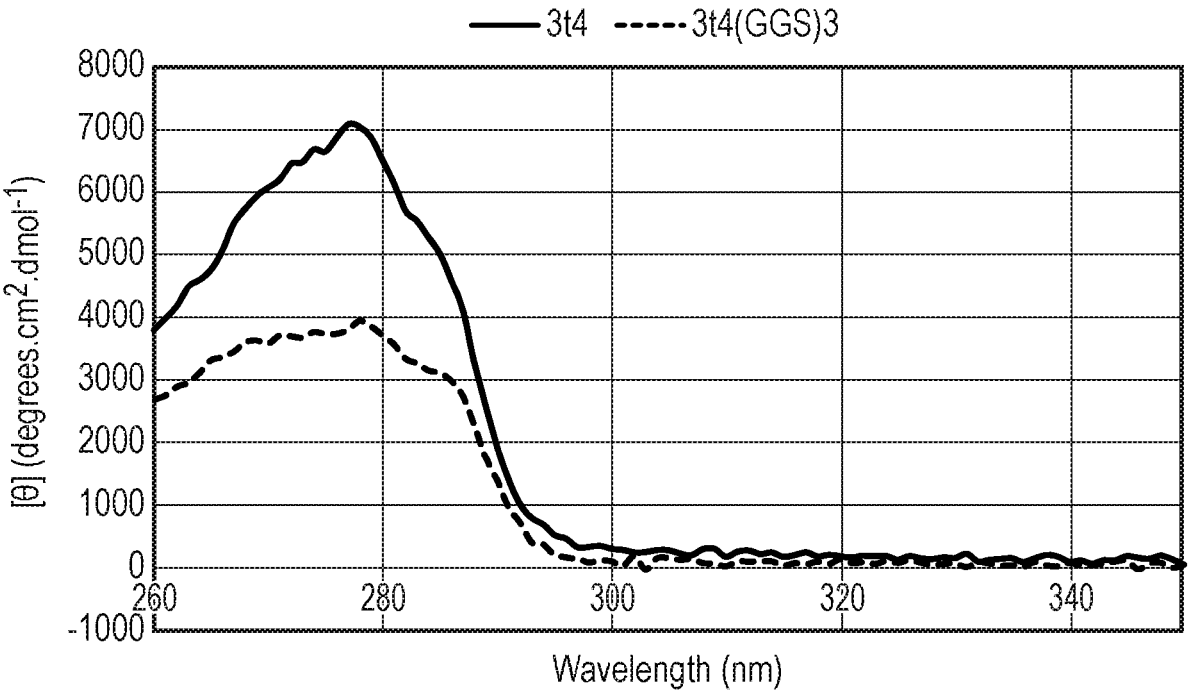


FIG. 8

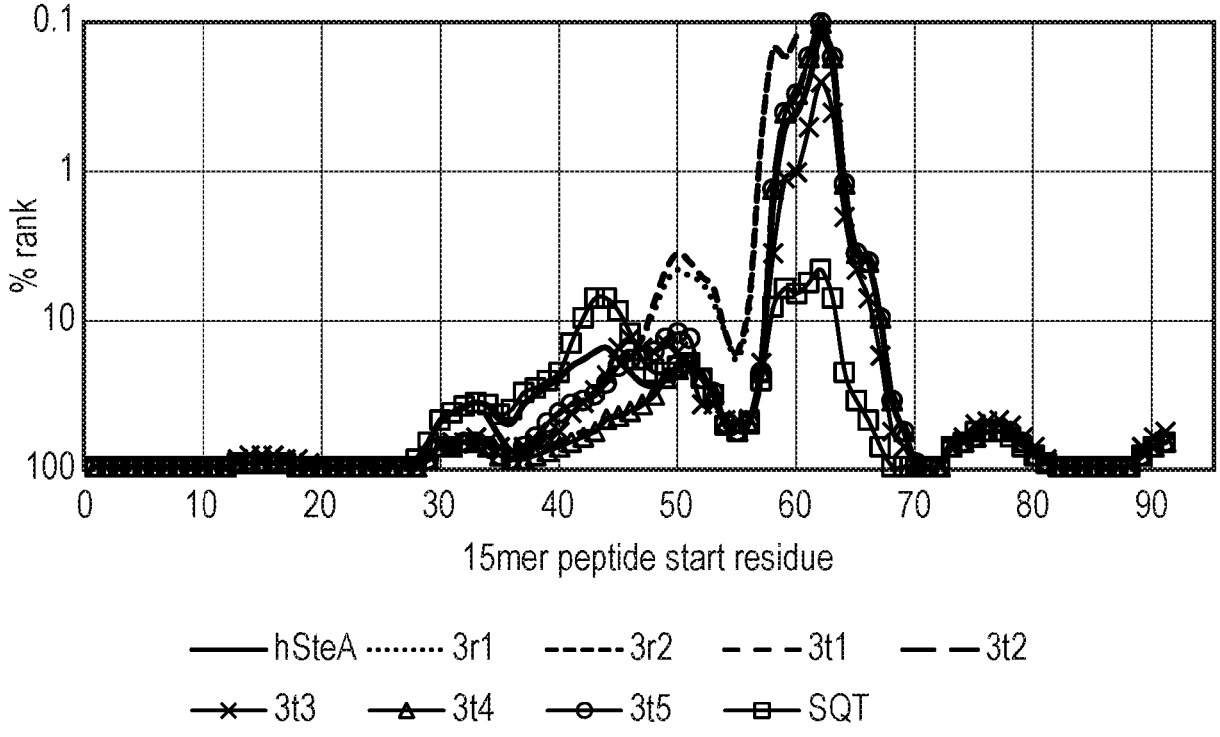


FIG. 9

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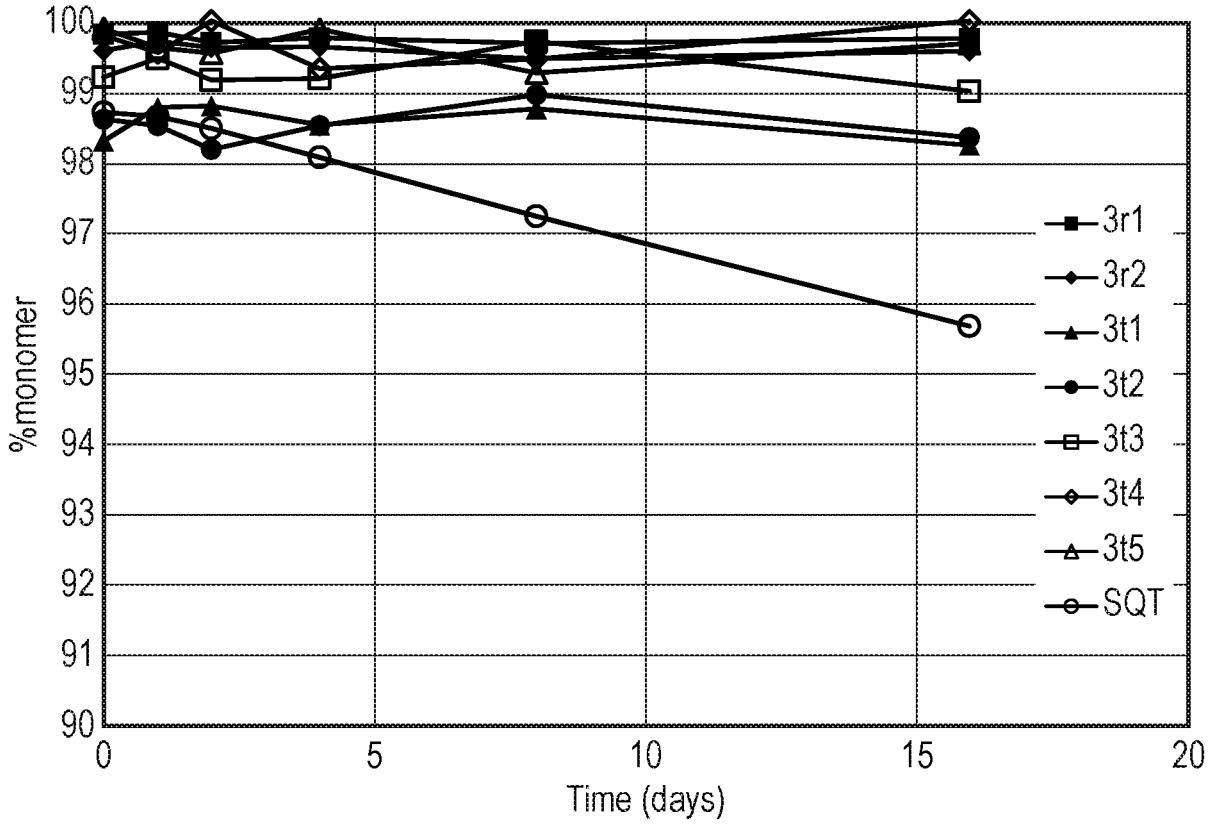


FIG. 10a

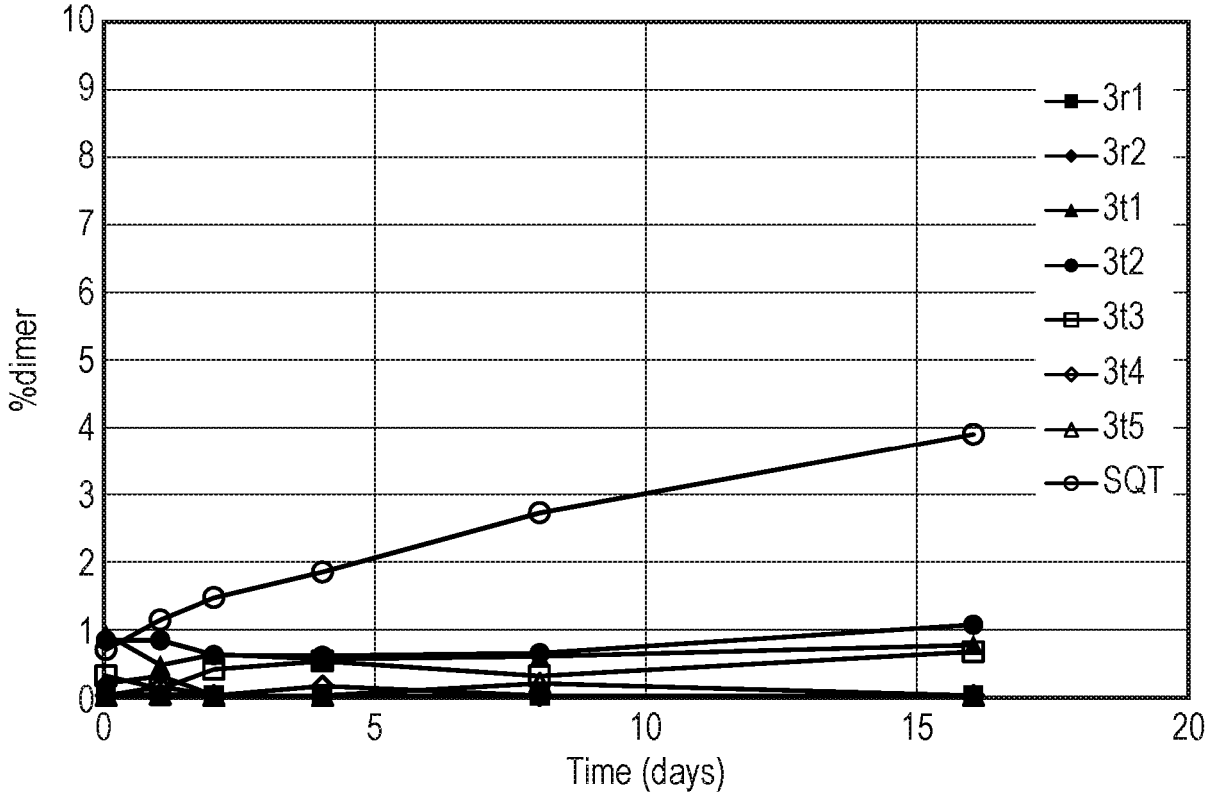


FIG. 10b

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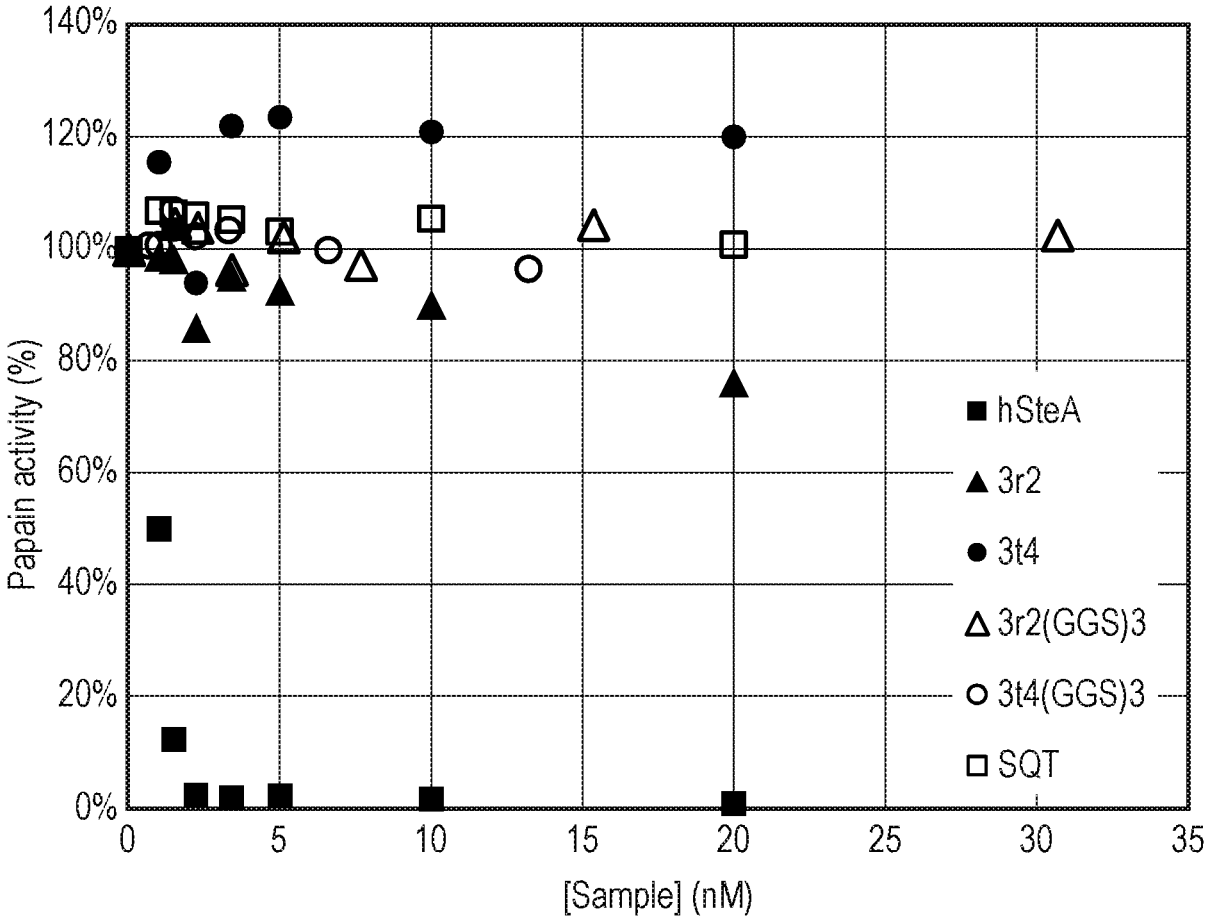


FIG. 11

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Affimer Working ID	EGFR (FL1-A of QSH01)	mIgG2b (FL1-A of QSH03)	hIgG1 Fc (FL1-A of QSH05)
PBS (BLANK)	1774	1167	1230
PBS (BLANK)	1734	1232	950
PBS (BLANK)	1705.5	1156	1062.5
NEGATIVE - - HA (NEGATIVE)	1768.5	1265.5	974
NEGATIVE - - HA (NEGATIVE)	1667.5	1132	1025
NEGATIVE - - HA (NEGATIVE)	1666	1136	1100
NEGATIVE - - HA (NEGATIVE)	1637.5	1027.5	1228.5
MIGG2B G12 - HA (POSITIVE)	1813	158466	1095
MIGG2B G12 - HA (POSITIVE)	1772	155424.5	1094
MIGG2B G12 - HA (POSITIVE)	1679	118498.5	1003
MIGG2B G12 - HA (POSITIVE)	1668	134053	1162
00451_644628	1722	1235	963
00451_644620	1764	1110	1154
00451_644599	1817	1224	1055
00451_644624	1717	1081.5	1144
00451_644654	1642	1055	1154
00451_644622	1543	1213	1052
00451_644633	1785	1141	1062
00451_644617	1750	1087	998
00451_644605	1736	1183	1051.5
00451_644671	1536	1052	1219
00451_644607	1725	1188.5	1117.5
00451_644691	1779	1127.5	1130
00451_644614	1768	1216	1087
00451_644634	1718	1142	1128.5
00451_644657	1627	1093	1097
00451_644644	1687.5	1174	1070
00451_644696	1771	1101.5	1141
00451_644670	1620	1030	1086
00451_644668	1689	1104.5	1115
00451_644588	1726	1167	1267
00451_644695	1796.5	1221	1232
00451_644656	1766	1134	1022





FIG. 12

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hPD-L2 (FL1-A of QSH06)	Her2 (R&D) (FL1-A of QSH08)	hPD-L1 (FL1-A of QSH10)	Trastuzumab (FL1-A of QSH11)	hIgG (FL1-A of QSH13)
1303	866	1413	1543	1076
1223.5	808	1220.5	1376.5	995.5
1240	882	1200	1529	904.5
1243	822	1287	1359	1076
1064	790	1121	1252	976.5
1220	801	1471.5	1551.5	1033
1105	718	1024.5	1472	1058
1140	868	980.5	1469	1084.5
1240	1053	1452.5	1446.5	1215.5
1181	866	1087.5	1448	1037
1264	1011	1297	1529	1091
1276	2452.5	1164	1458	977.5
1284	2398	1282	1400	1044
1280.5	2129.5	1132	1495	1000
1152	1962	1146	1373	931
1134	1864.5	1080	1222	1003
1208.5	1027.5	1265	1551	1019
1235	930	1098	1255.5	1089.5
1323.5	867.5	1108	1327	979
1170	950.5	1178.5	1359	1084
1057	931	1022	1319	1009
1275	785	1052	1103	962
1165	893	1057	1470	954
1103	868	1249	1470.5	1208
1310.5	858	1327	1477	1038
1117	759	1115	1280.5	1101
1217	864	1383.5	1453	1067
1260	820	1197.5	1255	1043
1066	865.5	1205.5	1381	989
1248	729	1186.5	1341.5	1074
1167	849	1210	1451	1074
1280.5	968.5	1323.5	1411	1089
1286	965.5	1499	1437	1060

(A) (2) (B)

FIG. 12 (Continued)

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Her2 (Sino) (FL1-A of QSH15)	Her3 (FL1-A of QSH16)	mPD-L1 (FL1-A of QSH18)	No target ctr (FL1-A of QSH22)	Avastin (FL1-A of QSH23)
1046	1411	1164.5	1623	1213
748.5	1303	1181	1373	1149
926	1292	1177	1491	1143
678	1343	1202	1465	1027.5
717.5	1408	1178	1381	1154
874	1410.5	1182.5	1559	1019
879	1208	1104.5	1392.5	1091
872	1268	1058.5	1221	1170
940	1370	1002.5	1506	1140.5
973	1431.5	1041	1408	1158.5
1003	1423	1229.5	1402	987
102773.5	1435	1272.5	1442	1092
91635	1435	1087.5	1485	1221.5
86041	1274.5	1122	1544.5	1278
84281	1361.5	1057	1342	1223
82266	1225	1117	1372	1095
14155	1206.5	1155	1388	1173
13150.5	1292	1017	1359	1244
3728	1408	1105	1336	1103
3138	1330	1108	1493	1029
1550	1217.5	1114	1352.5	1100
1547.5	1400	1076	1513	1315.5
1518	1361	1013	1497.5	1363.5
1434.5	1275.5	1225	1479.5	1131.5
1399.5	1370	1167	1435.5	1062
1356.5	1308	1114	1372	1208
1348	1237.5	1057	1493	1123.5
1348	1324	1121	1502	1163.5
1340	1205.5	1035	1332	941.5
1337	1315.5	1187	1516.5	1155.5
1334	1430.5	1160	1588	1189
1324	1528	1218.5	1350.5	1108
1308	1305	1141	1594	1183

(B) (3) (C)

FIG. 12 (Continued)

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Humira (FL1-A of QSH25)	Rituximab (FL1-A of QSH26)	mPD-L2 (FL1-A of QSH27)	Her4 (FL1-A of QSH28)
990	2200	1242	1476.5
969	2059.5	1185.5	1397
926.5	1876.5	1181	1421
947.5	2073	1147.5	1254
863	1885	1119	1303
1014	2079	1074.5	1461
906	2005	1044	1375
853	2026.5	971	1311
996	2145	1177	1415
1039.5	2147	1149	1365
966	1998.5	1130	1462.5
963	2099.5	1099.5	1387.5
1108	2152	1001	1521
960	1975	1181	1345
1006	2023.5	1175.5	1404
968	1903	1081	1313.5
922.5	2046	1036	1466
985	1947	1111	1326.5
942	1979	1183	1291
989	2084	1181	1383
903.5	1929	1103	1283
896	2036	1059.5	1513
1052	2096.5	1224	1342
1044	2108	1119	1266.5
906	1984	1181	1406
855	2048.5	1183	1491
984	1971	1114	1332
981	2030	1197	1434
884	2032	996.5	1346
969	1939	1081	1293
1051	2005.5	1189	1418
861	2172	1273	1484.5
1004	2067	1212	1324

←C ④↓

FIG. 12 (Continued)

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1

00451_644719	1785	1130	1158
00451_644689	1663	1222	1057.5
00451_644631	1548	1244.5	1181
00451_644658	1693.5	1183.5	1022
00451_644717	1734	1172	1087
00451_644661	1753.5	1054.5	968.5
00451_644708	1780.5	1206.5	1172.5
00451_644655	1814	1155	1117
00451_644706	1626	993	1284
00451_644629	1763.5	1125	1116
00451_644619	1769.5	1122	1110
00451_644652	1643	1127	1041.5
00451_644609	1750	1179.5	1273
00451_644659	1729	1160.5	1099
00451_644703	1641.5	1061.5	939
00451_644660	1714	1121	1182
00451_644612	1733	1262	1021.5
00451_644667	1706	1261	983
00451_644653	1699	1132	959.5
00451_644645	1716	1139.5	1057.5
00451_644596	1830.5	1235	1103
00451_644643	1571	1009	990
00451_644697	1635.5	1127	1183.5
00451_644598	1690	1266	1121
00451_644707	1717	1269.5	1221
00451_644583	1708	1383.5	1237
00451_644714	1725	1151.5	1200
00451_644642	1693	1171	1150
00451_644621	1590	1153	1073
00451_644711	1615.5	961.5	1088
00451_644636	1644.5	1175	1158
00451_644632	1695	1113.5	1003.5
00451_644582	1830	1154	1173
00451_644722	1654	1179	1196
00451_644584	1736.5	1165	1100

5

D

FIG. 12 (Continued)

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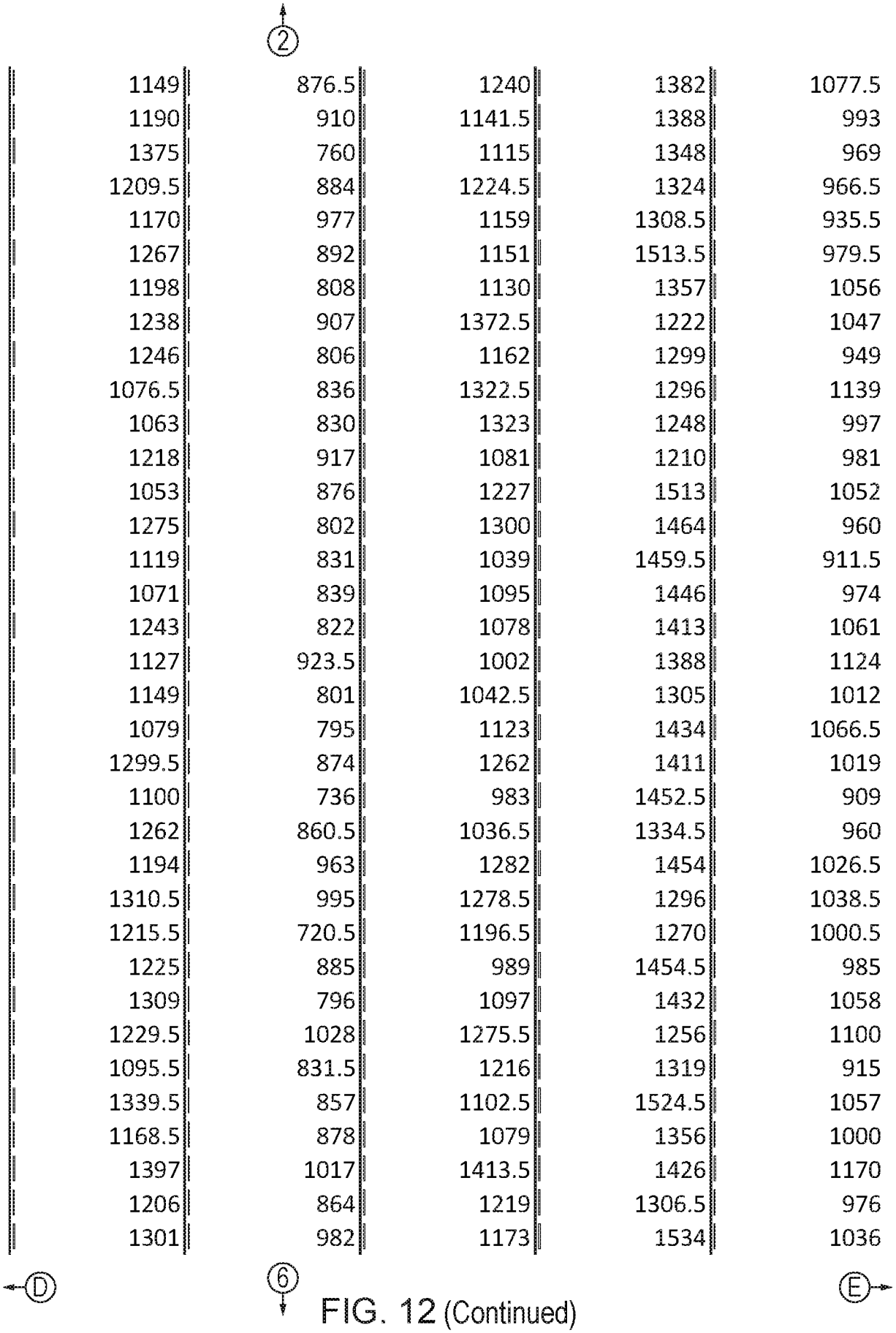


FIG. 12 (Continued)

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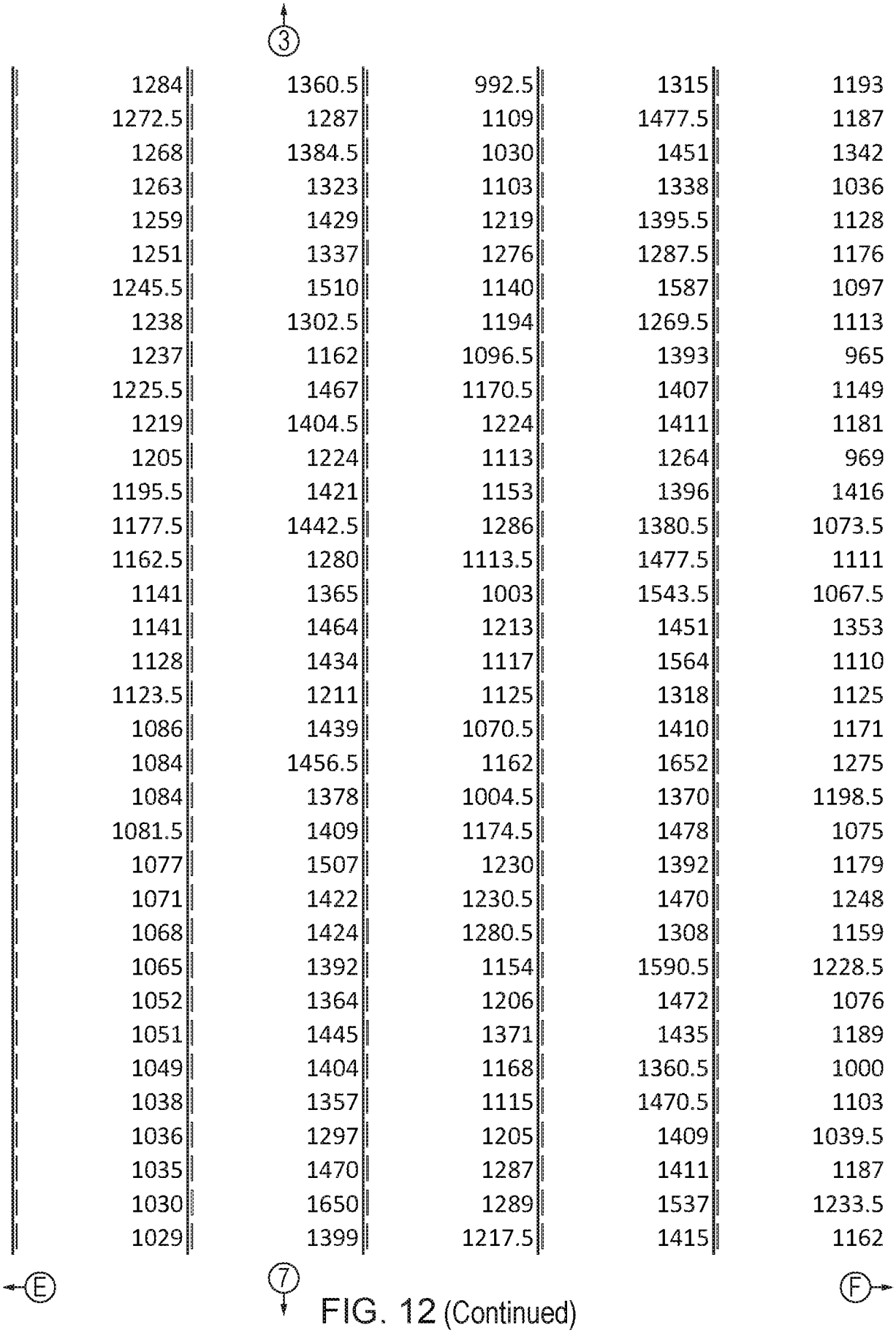
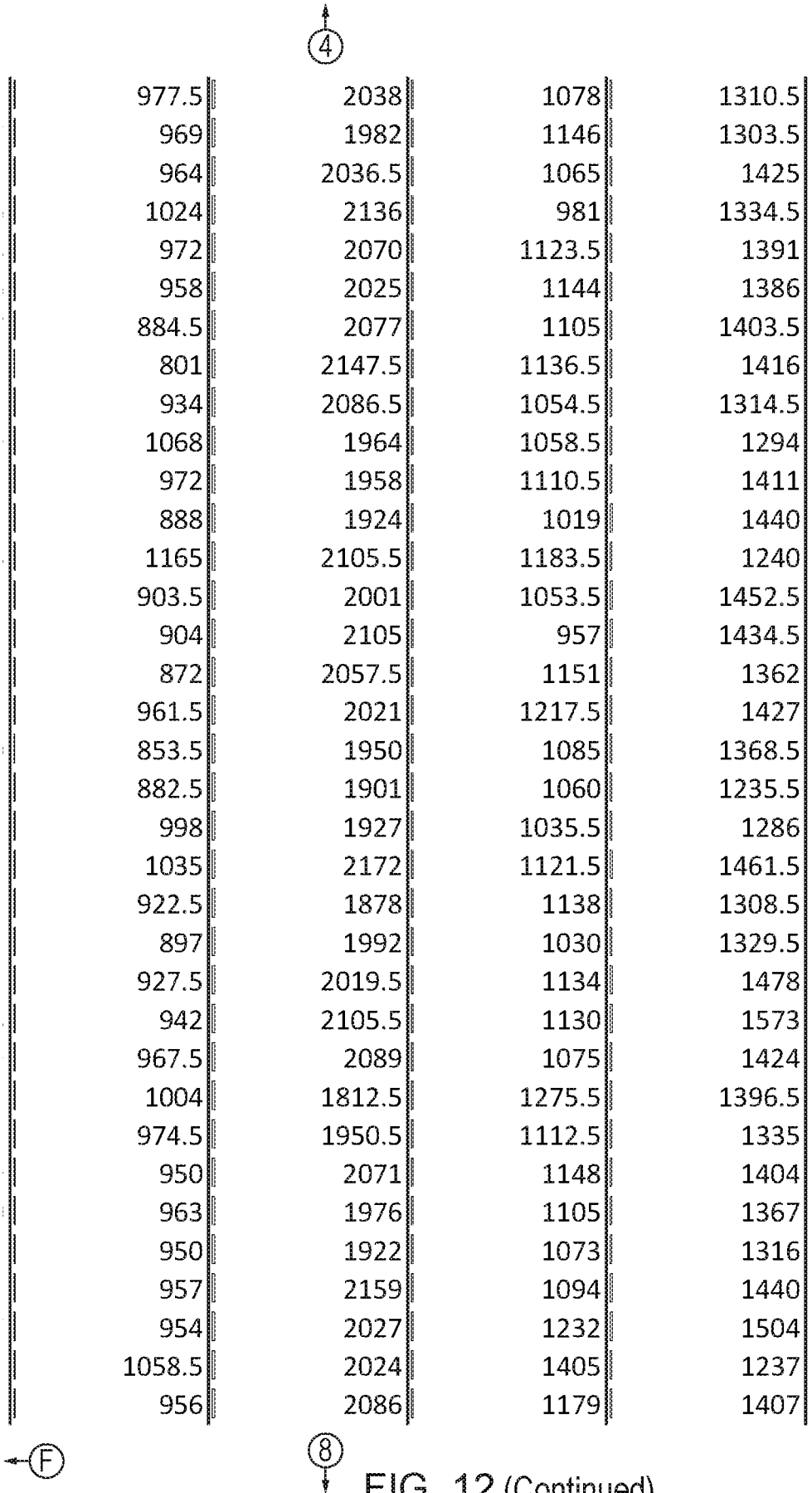


FIG. 12 (Continued)

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977.5	2038	1078	1310.5
969	1982	1146	1303.5
964	2036.5	1065	1425
1024	2136	981	1334.5
972	2070	1123.5	1391
958	2025	1144	1386
884.5	2077	1105	1403.5
801	2147.5	1136.5	1416
934	2086.5	1054.5	1314.5
1068	1964	1058.5	1294
972	1958	1110.5	1411
888	1924	1019	1440
1165	2105.5	1183.5	1240
903.5	2001	1053.5	1452.5
904	2105	957	1434.5
872	2057.5	1151	1362
961.5	2021	1217.5	1427
853.5	1950	1085	1368.5
882.5	1901	1060	1235.5
998	1927	1035.5	1286
1035	2172	1121.5	1461.5
922.5	1878	1138	1308.5
897	1992	1030	1329.5
927.5	2019.5	1134	1478
942	2105.5	1130	1573
967.5	2089	1075	1424
1004	1812.5	1275.5	1396.5
974.5	1950.5	1112.5	1335
950	2071	1148	1404
963	1976	1105	1367
950	1922	1073	1316
957	2159	1094	1440
954	2027	1232	1504
1058.5	2024	1405	1237
956	2086	1179	1407

FIG. 12 (Continued)

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5

00451_644680	1976	1310	1095
00451_644724	1720	1272.5	1246
00451_644716	1761	1069.5	1079
00451_644641	1730	1200	986
00451_644692	1842	1135	1221
00451_644682	1804	1218	1130
00451_644712	1752	1163	1130
00451_644587	1836	1170.5	1309.5
00451_644597	1846	1216	1148
00451_644713	1867.5	1239.5	1191.5
00451_644585	1577	1073	1349.5
00451_644592	1726	1187	1211
00451_644603	1604	1078	1027
00451_644646	1604.5	1109	1104.5
00451_644580	1824	1303	1133
00451_644581	1857	1221	1197
00451_644702	1736	1227	1018
00451_644677	1848	1383	1222
00451_644594	1829	1112	1165.5
00451_644630	1631	1074	1041
00451_644676	1725	1244	1160
00451_644684	1644	1200	1186
00451_644673	1523	1073	958.5
00451_644593	1678	1302	1065
PBS (BLANK)	1939	1294	1143
00451_644611	1749	1232	1210
00451_644613	1709	1172	1058.5
00451_644710	1639	1203	1124
00451_644674	1663.5	1173.5	1017
00451_644726	1670	1108	1202.5
00451_644600	1723	1222	1052
00451_644690	1638.5	1173	1138
00451_644693	1599	1163	1093
00451_644627	1490	1144.5	1243.5
00451_644586	1749	1323	1155.5

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G

FIG. 12 (Continued)

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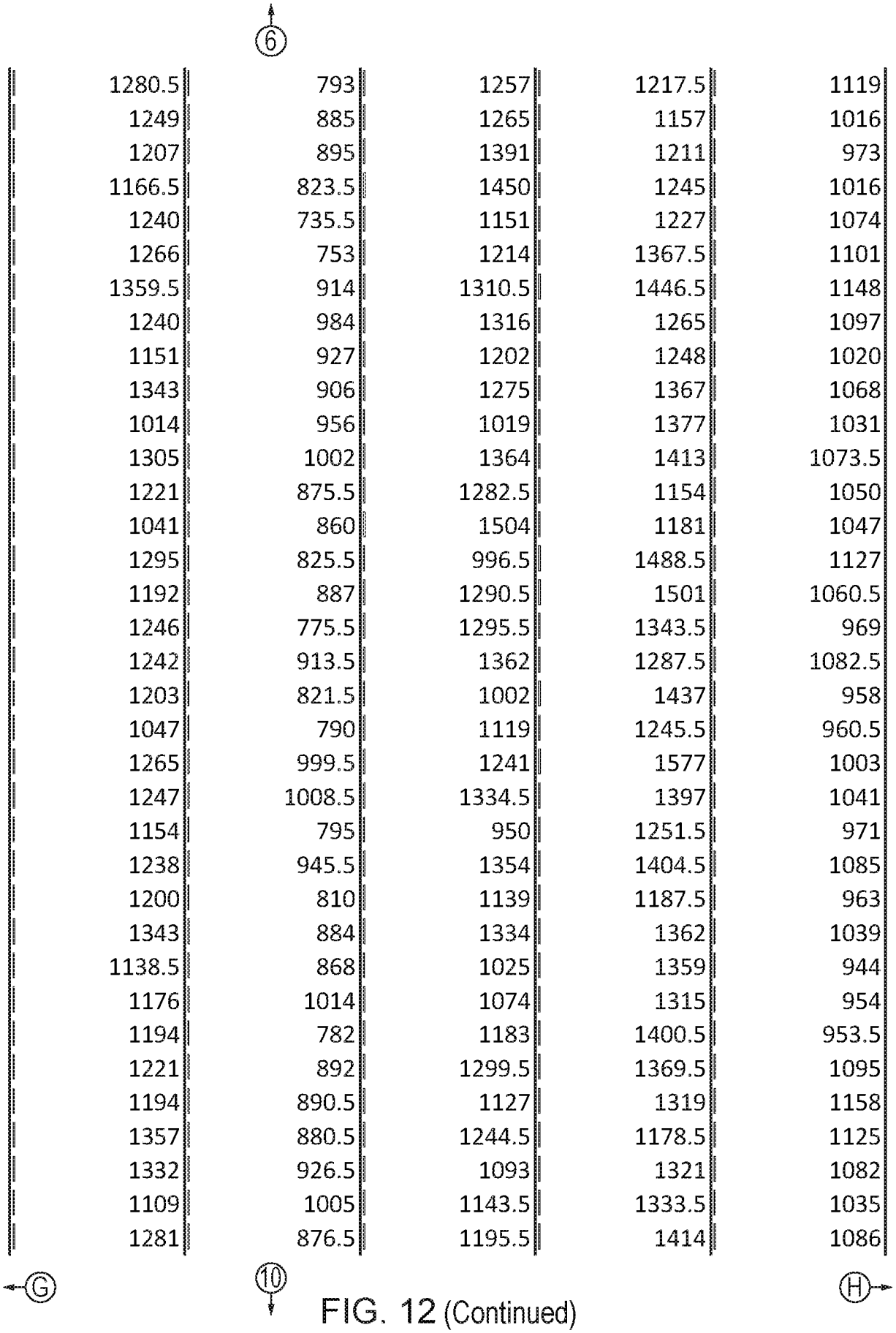


FIG. 12 (Continued)

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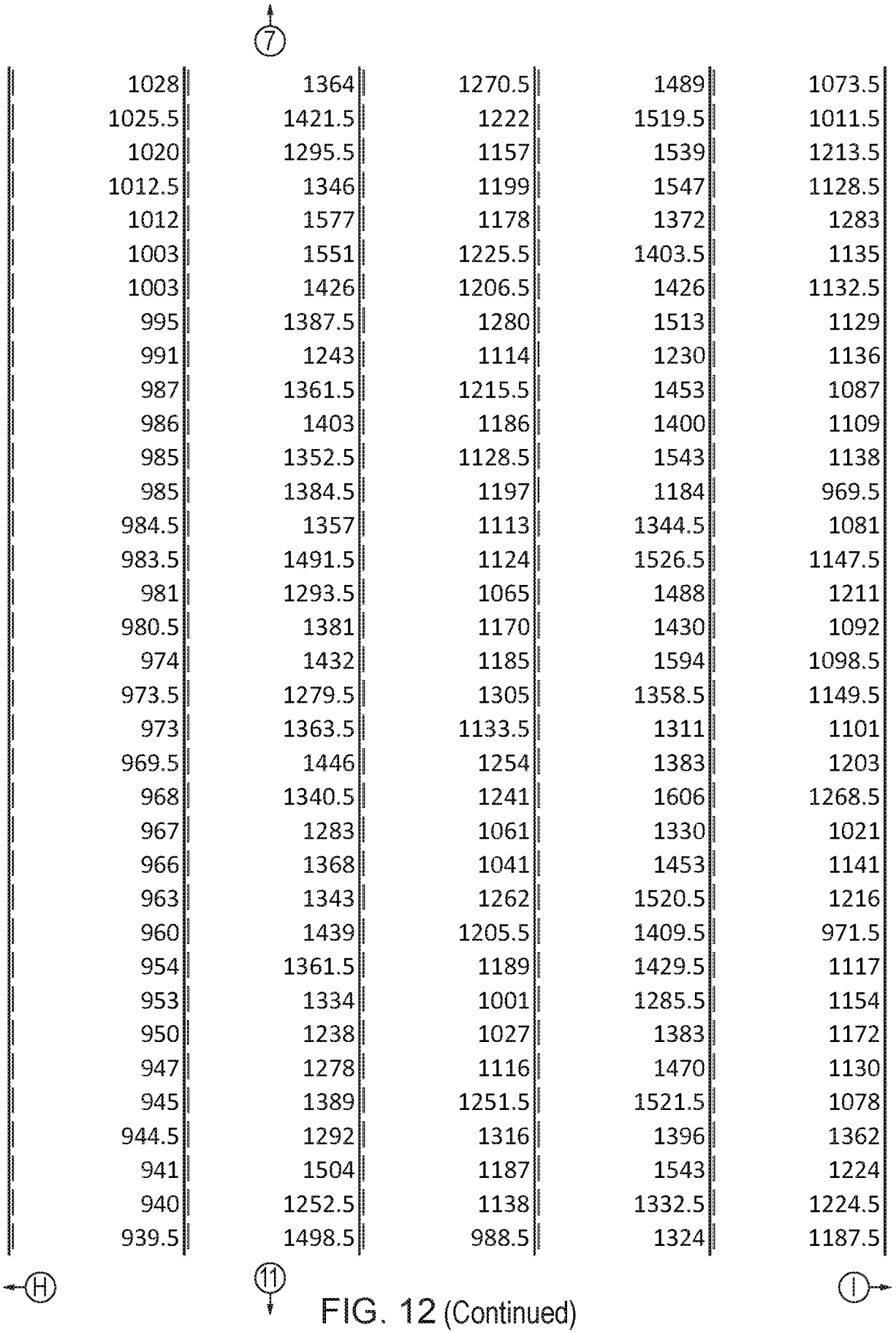


FIG. 12 (Continued)

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1016	2116	1103	1456
871	2112	1136	1531.5
1089	1925	1144	1411
1011	2010.5	1117	1415
1019.5	1991.5	1084	1561
1039	1995	1025	1528
957	2162	1258	1339
1018.5	1935	1027	1379.5
990	1945	1068	1307.5
971	2079.5	1133	1448
1007.5	2025	1068	1480.5
1017.5	1997.5	1093	1429
832	1854	969	1383
952.5	2040	1076	1210
1122	2102.5	1292	1489
971	2110.5	1095	1483
995.5	2115	1197.5	1290.5
995.5	2086.5	1150.5	1448
1069	2173	1186	1397
952	2000	990	1531
904	1949	1045	1434
1065	2013.5	1052	1415
963	1889	1108	1413
937	1923	1167	1424.5
1028	1987	1224.5	1367
1086	2082.5	1273	1437
890	2061.5	1051.5	1385
1007	2021.5	1177	1427
980.5	2054	1043	1434
930.5	1972.5	1122	1454
949	2068	1076	1348
1077	2060	1125.5	1394
897.5	2173	1130	1553
920	2078	1195	1357
934	2066.5	1260	1519





FIG. 12 (Continued)

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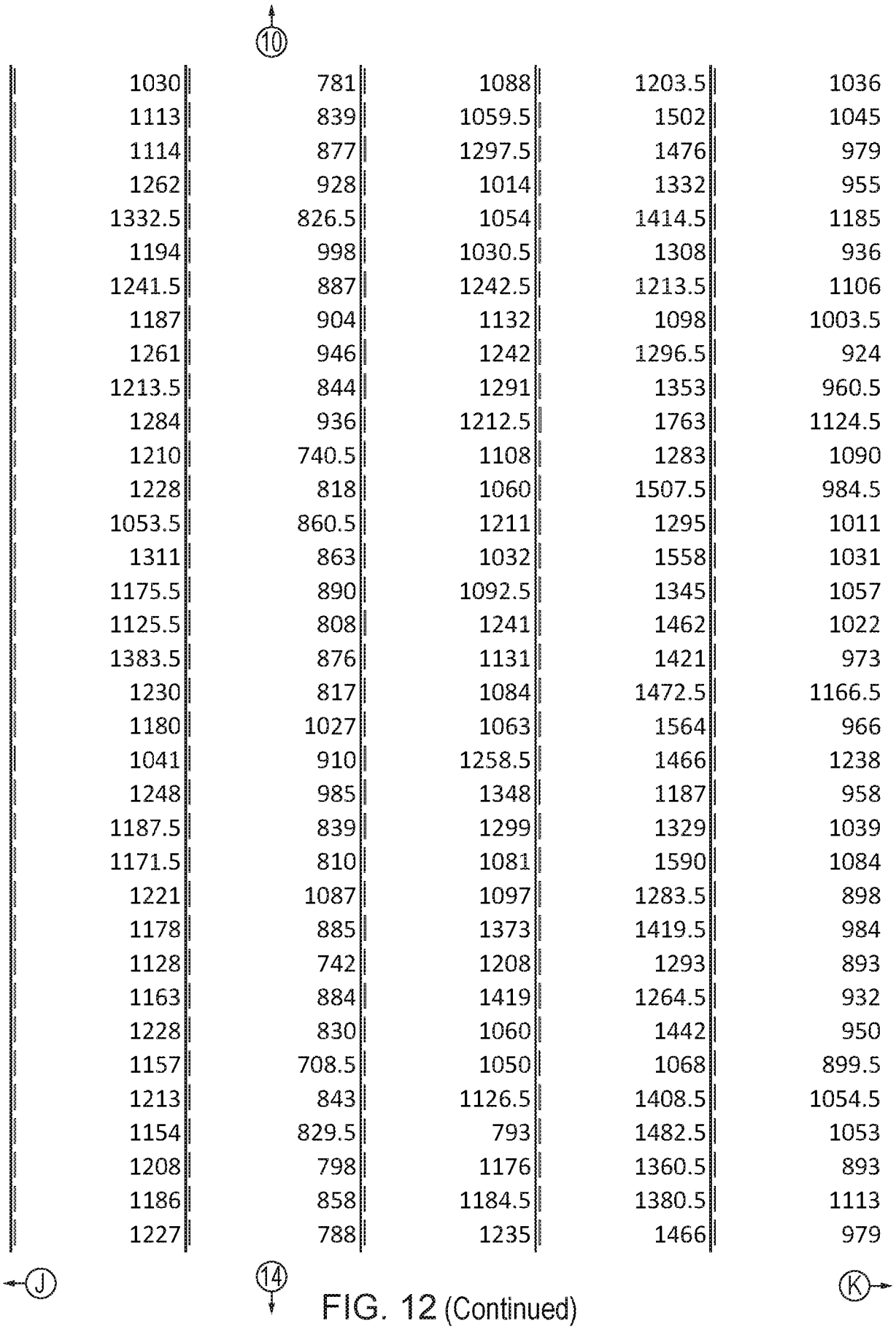
00451_644648	1739	1155	1114
00451_644669	1823	1081	1054
00451_644705	1765	1058	1082
00451_644604	1701	1270	1184
00451_644679	1848	1458	1144
00451_644675	1707	1152	1251
00451_644623	1809	1136	1037.5
00451_644595	1677	1186.5	1139.5
00451_644625	1596	1159.5	1144
00451_644681	1767	1183	1193
00451_644683	1833	1070	1139.5
00451_644601	1783.5	1046.5	1117
00451_644694	1908	1108	1275.5
00451_644678	1817	1108.5	1174
00451_644704	1896	1158.5	1100
00451_644649	1656	1131.5	1206
00451_644608	1809	1136	1139.5
00451_644718	1811	1098	971
00451_644637	1722	1294	1099.5
00451_644615	1747	1227	1044
00451_644699	1671.5	1161	1035.5
00451_644626	1501	1101.5	1184
00451_644639	1531.5	1170	1046
00451_644701	1734	1159	1132
00451_644723	1691	1273	1175.5
00451_644610	1812	1216	1215
00451_644647	1737.5	1041	1077
00451_644618	1817.5	1056	1079
00451_644727	1761	1270	1031
00451_644662	1583	1150.5	1052
00451_644638	1763	1100	1070.5
00451_644698	1704	1173	1213
00451_644709	1792	1103	1023.5
00451_644602	1702.5	1121	1159





FIG. 12 (Continued)

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1030	781	1088	1203.5	1036
1113	839	1059.5	1502	1045
1114	877	1297.5	1476	979
1262	928	1014	1332	955
1332.5	826.5	1054	1414.5	1185
1194	998	1030.5	1308	936
1241.5	887	1242.5	1213.5	1106
1187	904	1132	1098	1003.5
1261	946	1242	1296.5	924
1213.5	844	1291	1353	960.5
1284	936	1212.5	1763	1124.5
1210	740.5	1108	1283	1090
1228	818	1060	1507.5	984.5
1053.5	860.5	1211	1295	1011
1311	863	1032	1558	1031
1175.5	890	1092.5	1345	1057
1125.5	808	1241	1462	1022
1383.5	876	1131	1421	973
1230	817	1084	1472.5	1166.5
1180	1027	1063	1564	966
1041	910	1258.5	1466	1238
1248	985	1348	1187	958
1187.5	839	1299	1329	1039
1171.5	810	1081	1590	1084
1221	1087	1097	1283.5	898
1178	885	1373	1419.5	984
1128	742	1208	1293	893
1163	884	1419	1264.5	932
1228	830	1060	1442	950
1157	708.5	1050	1068	899.5
1213	843	1126.5	1408.5	1054.5
1154	829.5	793	1482.5	1053
1208	798	1176	1360.5	893
1186	858	1184.5	1380.5	1113
1227	788	1235	1466	979

FIG. 12 (Continued)

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(11)

938.5	1373	1065	1328	955
938	1348.5	1132	1479	1027
935.5	1286	1106	1265	1132
930.5	1423	1099	1398.5	1197
925	1470.5	1395	1487.5	1078
922	1338	1300	1162	1193
921.5	1454.5	960	1445	1144.5
920	1313	1171	1421	1144
919	1337	1159.5	1300	1048
915	1302.5	1182.5	1515.5	1342
912	1329	1248.5	1534	1241
911.5	1318	1036.5	1443	1246
904.5	1477.5	1016	1329	1237.5
904	1353.5	937	1456	974
904	1446	1235.5	1451	1185
903.5	1382.5	1045	1259	1006
901.5	1462	1093	1488.5	963
901	1262	1144	1388	1096.5
888.5	1255	1114.5	1227.5	1097.5
885	1275.5	1176	1317	1090
878	1421	1194	1415	1336
871.5	1378.5	1235	1515	1246
868	1329	1140	1320	1207
867.5	1389.5	1216	1329	1020
860	1434	1043.5	1305	1132
857	1356	1368.5	1360	1149
853	1279.5	1092	1354	1196
849	1265	1117	1463.5	1007.5
848	1308	1292	1461	1042.5
847	1310.5	1005.5	1381	1076
845.5	1329	1262	1411	1124.5
839	1405	1056	1448	1249
837	1366.5	1187	1318	1156
837	1272	1198.5	1363	1157
833.5	1479	1122	1504	1106

(K) (15) (L)

FIG. 12 (Continued)

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892	1954	950	1305
1074	1976	1113.5	1262
880	1880.5	1185.5	1367
893	2094	1145.5	1424
1037	2254	1181	1446
956	1927	1164	1374.5
1149	1996	1176	1384
1019	1933	1090	1308
928	2078.5	1220	1351
915	1973	1035	1434
1006	2076	1357	1427.5
915	2059	996	1366
1006	2055	1163	1330
974	2005	1010	1399
955	2079	1176	1555
895.5	1967	1168.5	1456
952	2059	1184	1507.5
1024	2132	1165	1435
964.5	2022	1227	1446
907	2097	1200	1402
979	2008.5	1026.5	1310
926.5	2102	1105.5	1424
950	1908	1028	1234
1003	2009	1105	1342
1012	2087	1110.5	1238
902	1962	1213.5	1447
963	1900.5	1057	1217
959	1924.5	1073	1457.5
891.5	1989	1152	1356.5
1008	1777	963.5	1349
901	1979	1039	1388
930	1998	1219	1391
974	1821.5	1060	1204
963	2016	1209	1334
934	2148	1217.5	1446.5

L

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FIG. 12 (Continued)

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00451_644700	1600	1249	1239
00451_644606	1617	1162	1107
00451_644728	1777	1198	1065
00451_644635	1702	1109	1022
00451_644688	1752.5	1263	1167
00451_644616	1774	1182.5	1043.5
00451_644725	1837.5	1122	1200
00451_644663	1677	1108	1081.5
00451_644650	1795	1148	1052
00451_644640	1612	1206	1144.5
00451_644720	1785	1167	1073
00451_644651	1634	1188.5	1182
00451_644672	1617	1210.5	963
00451_644721	1648	1130.5	950
00451_644715	1625	1081	1101



FIG. 12 (Continued)

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1227	788	1235	1466	979
1252	913	1235	1214	1025
1154	923	1381	1391	1011
1070.5	863	927	1402.5	1017
1164.5	769.5	1184	1235	1020
1222	855	1117	1223.5	1047.5
1283.5	912	1086	1520	1122.5
1364	933	1233	1370	1097
1128	1021	990.5	1505.5	892.5
1232.5	818	1243	1356.5	952
1280	789	1238	1380	1157
1256	938.5	1165	1499	807
1136	833.5	1030.5	1340	988
1098	782	1183	1308.5	1033
1258.5	778.5	1203.5	1434	937

M N

FIG. 12 (Continued)

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833.5	1479	1122	1504	1106
830	1407.5	1198	1376.5	1218
830	1386	1284	1510	1216
824	1375	1080	1486.5	1183.5
823	1434	1022	1351	1216
816	1397.5	1095	1432	1127.5
811	1361	1171	1477	1170
810	1460	1124	1410	1047.5
792	1318	1215	1397	1083
781	1347.5	1200	1466.5	1030
778.5	1349	1039	1422	1197.5
757	1204	981	1377	1087
753	1417	1124	1402	1135
740.5	1429	1106	1429	1033.5
669	1390	995	1457.5	1205

N O

FIG. 12 (Continued)

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934	2148	1217.5	1446.5
785	1955	1103	1373
910	1936.5	1056	1380
962	1995	962	1310
963	1928	1210	1475
875.5	2065.5	1168	1393.5
1000	2071	1144.5	1442
920	2009.5	1138	1175.5
833	1939	998	1372.5
829.5	1966	1169	1536
893	1955	1106	1421
930	1965	1031.5	1288
837.5	1925	1008	1221
1043	2017	1089.5	1278.5
855	1906	1116	1371.5

O

FIG. 12 (Continued)

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Affimer Working ID	EGFR (FL1-A of QSH01)	mIgG2b (FL1-A of QSH03)	hIgG1 Fc (FL1-A of QSH05)
00453_644900	1857.5	1053.5	1830.5
00453_644808	1639.5	1022	1091.5
00453_645051	1630	1108	1086.5
00453_644819	1652	1198.5	1213
00453_644802	1671	1029	1179.5
00453_644873	1556	1036.5	1052
00453_644769	1736	1245	1065.5
00453_644869	1620	1086	1011.5
00453_644810	1796	1164	1482
00453_644809	1541.5	1119	1032.5
00453_644888	1704	1099.5	1448
00453_645069	1623	1230	1319.5
00453_644803	1743.5	1135	1416
00453_644823	1715.5	1044	1130
00453_644864	1675.5	1078	1295
00453_644924	1734	1272	1689
00453_645089	1809	1071	1466
00453_644861	1691	1106	1146
00453_644824	1548	1099	1064.5
00453_644919	1683	982.5	1205.5
00453_645036	1712	1136.5	1136
00453_644833	1672	1159.5	1151
00453_644851	1737.5	993	1454.5
00453_644926	1936	1216	1698.5
00453_645085	1679.5	1122	1213
00453_645010	1842	1123.5	1710
00453_644825	1796	1206.5	1248
00453_644912	1648.5	1793	1091
00453_644844	1878	1147	1240
00453_645033	1739	1033	1335
00453_644772	1690	1098	1239
00453_644751	1842.5	1093	1284
00453_644832	1752	1071	1221





FIG. 13

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hPD-L2 (FL1-A of QSH06)	Her2 (R&D) (FL1-A of QSH08)	hPD-L1 (FL1-A of QSH10)	Trastuzumab (FL1-A of QSH11)	hIgG (FL1-A of QSH13)
1736.5	882	1163	180145	1255.5
1211.5	769	1144	175577	1054
1119	783	1178	167951	1021
1210.5	852.5	1230	163608.5	1100
1208	884.5	1035	161481	1078
1186	863	1035	150133.5	985.5
1151.5	883	1357	133402.5	971
1134	863	1148	123517.5	868
1423	828.5	1217.5	113116	1004
1109	802	1206.5	108140	925
1383	723	1138.5	106234	1073
1433	872	1214	98995	1089
1411	862.5	1362	98358	1140
1202	905.5	928	97128	1146
1219	812	1111	84553.5	967
1508	801.5	1250.5	83304	1035
1470	760	1105	78338	1022
1178	721.5	1177	77833	1068
1178	823	1078.5	77752	1020
1160	802	1143	75438	960
1163	768.5	1217.5	74310.5	995
1259	687	1153	70321	1004
1337	778.5	1163.5	68725	1011
1536	1022	1424	68205	1206
1227	919	1328.5	64471	968
1337	763	1264	63598	1071
1161	935.5	1335.5	63035.5	1063
1171.5	804	1003	61262	1000
1227	695	1027	59343	988
1456	750	1152	50967	1227
1278	787.5	1372	48521.5	982.5
1216	811	1232	44657	1140
1299	1001	1062	43627	992

(A) (2) (B)

FIG. 13 (Continued)

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Her2 (Sino) (FL1-A of QSH15)	Her3 (FL1-A of QSH16)	mPD-L1 (FL1-A of QSH18)	No target ctr (FL1-A of QSH22)	Avastin (FL1-A of QSH23)
728	1502.5	1364	1387	1574.5
861	1324	1079	1425	1132
748	1485.5	1092	1444	1162
861	1427	1136.5	1580	1212.5
893	1418	1006	1437	1202
785	1253.5	1145	1531	1101
846.5	2106	1170	1512	1121
849	1334	1080.5	1349	1012
927	1423.5	1268	1362	1415
885	1354	1025.5	1400	1025
842	1351.5	1046.5	1216	1421
887	1346.5	1151.5	1405	1442
834	1452	1161.5	1373	1418
868	1271.5	1033.5	1307	1140
718	1265	1060	1326	1345
884.5	1547	1308	1483	1515
922	1365	1214.5	1435.5	1402
793	1293	1144	1402.5	1090.5
842	1265	1077.5	1415	1141
969	1330	1138	1329.5	1211
920	1459	1148	1392	1136
802	1387	1135	1461	1171.5
839.5	1329	1315.5	1287	1340
876	1591	1524	1469	1795.5
752	1349	1256	1391.5	1346
857	1521	1443	1507	1646
927	1433	1157	1546	1175
723	1380	1095	1237	1067
861.5	1453	1077.5	1421	1118.5
817	1363.5	1233	1351	1360
729	1431	1205	1503	1265.5
903	1378	1221	1492	1267
820	1321	1140	1395	1241

Ⓑ ③ Ⓒ

FIG. 13 (Continued)

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Humira (FL1-A of QSH25)	Rituximab (FL1-A of QSH26)	mPD-L2 (FL1-A of QSH27)	Her4 (FL1-A of QSH28)
1639	2222	1586	1332
1009	1949	1189.5	1394
1017	1970	1227	1357
1041	2014	1233.5	1304.5
1016	1839	1192	1408
1019	1912.5	1075.5	1255.5
1132	1970.5	1642	1492.5
853	2048	1197	1330
1278	2057	1310	1357
1052.5	1925	1052	1303
1353.5	1987	1152.5	1245
1168	1978.5	1138	1343
1291	2129	1289	1396
901	1912	1236	1295
1227	1954	1141	1461
1454	2035	1251.5	1493
1488	2075.5	1194	1256.5
901	1857	1070	1384
884.5	2027	1006	1305
1113	1943.5	1060	1303
995	1995	1171	1334.5
1052	1925	1290.5	1370
1130	2143.5	1304	1443.5
1722.5	2115.5	1526	1379.5
1208	2033	1290.5	1418
1574	2050	1411	1292
1071	1987	1237.5	1375
952	1808	963	1184
1121	1998.5	1229.5	1432
1315	2110	1127	1355
1181	2024	1216	1304
1238	1993	1225	1264.5
1095	2136.5	1272	1351

← C

4 ↓

FIG. 13 (Continued)

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00453_644874	1760	1141	1243.5
00453_644887	1642	996	1122
00453_644921	1809	1241	1553.5
00453_645038	1717	1078	1561
00453_645035	1802.5	1148	1596
00453_645041	1746.5	1204	1480.5
00453_645030	1713	1057	1112
00453_644904	1737	995	1506.5
00453_644798	1793	1120	1259
00453_644753	1720	1221	986
00453_644891	1866	1159.5	1877.5
00453_644886	1770	1117	1539
00453_644853	1803	1066	1030
00453_644977	1599	1058	1393.5
00453_644777	1708	1091	1081
00453_644797	1629.5	1205	1020.5
00453_645111	1629	999	1108
00453_644754	1737.5	1224	1417
00453_644760	1580	1127	1563
00453_645094	1860	1117	1736
00453_644838	1856	1084	1508
00453_644781	1618	1059	1078
00453_644759	1652.5	1114	1084
00453_644868	1469	1012	1257
00453_644792	1645.5	1073.5	1518
00453_644991	1692	1047	1419.5
00453_644880	1801	1074	1752.5
00453_644764	1772	1191	1590
00453_644848	1844	1103	1529
00453_645043	1614	1030	1088.5
00453_644987	1788.5	1016	1689.5
00453_644997	1677.5	1165	1261
00453_644950	1739	1111	1367

⑤

FIG. 13 (Continued)

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1453	877	1302	41091.5	1019.5
1206.5	810	1102.5	35642	1012
1369	929	1148	34638	1167.5
1507	855	1386	32862.5	1124
1365.5	788.5	1119	28013	1119.5
1327	887	1091	24984.5	1009
1205	837.5	1093	23350	1012
1489	806	1431	22227	1128
1290	710	1070.5	19871	1089
1323.5	862.5	1073	19301	1001.5
1747	826.5	1273.5	18955.5	1152.5
1519.5	786.5	1218	18790	1074.5
1291	910.5	1210	18587.5	1036
1202.5	657	1213.5	17713	964.5
1227	906.5	1318	17648	1006
1221.5	922	1189	17306	953
1096.5	763	1090	16978	1011
1335.5	780	1248.5	16454	1147
1429	890	1213	16277	1071
1690	798.5	1207	16163	1236
1552.5	886	1231	15900	1148
1210	806	904	15468	1020.5
1152.5	849.5	1213.5	15110	1028
1214.5	777	1181.5	14888	1051.5
1399	863	1229	14886	1044
1359	777	1082.5	14746	931
1600	841	1222	14666.5	1011
1593	868	1440	14637	1177
1364	739	1288	14554	1132
1152	829.5	1073	14458.5	1003
1696	775	1249.5	14342	1093
1086	732.5	1287.5	14283	1132
1434	867	1244	14058.5	1168







FIG. 13 (Continued)

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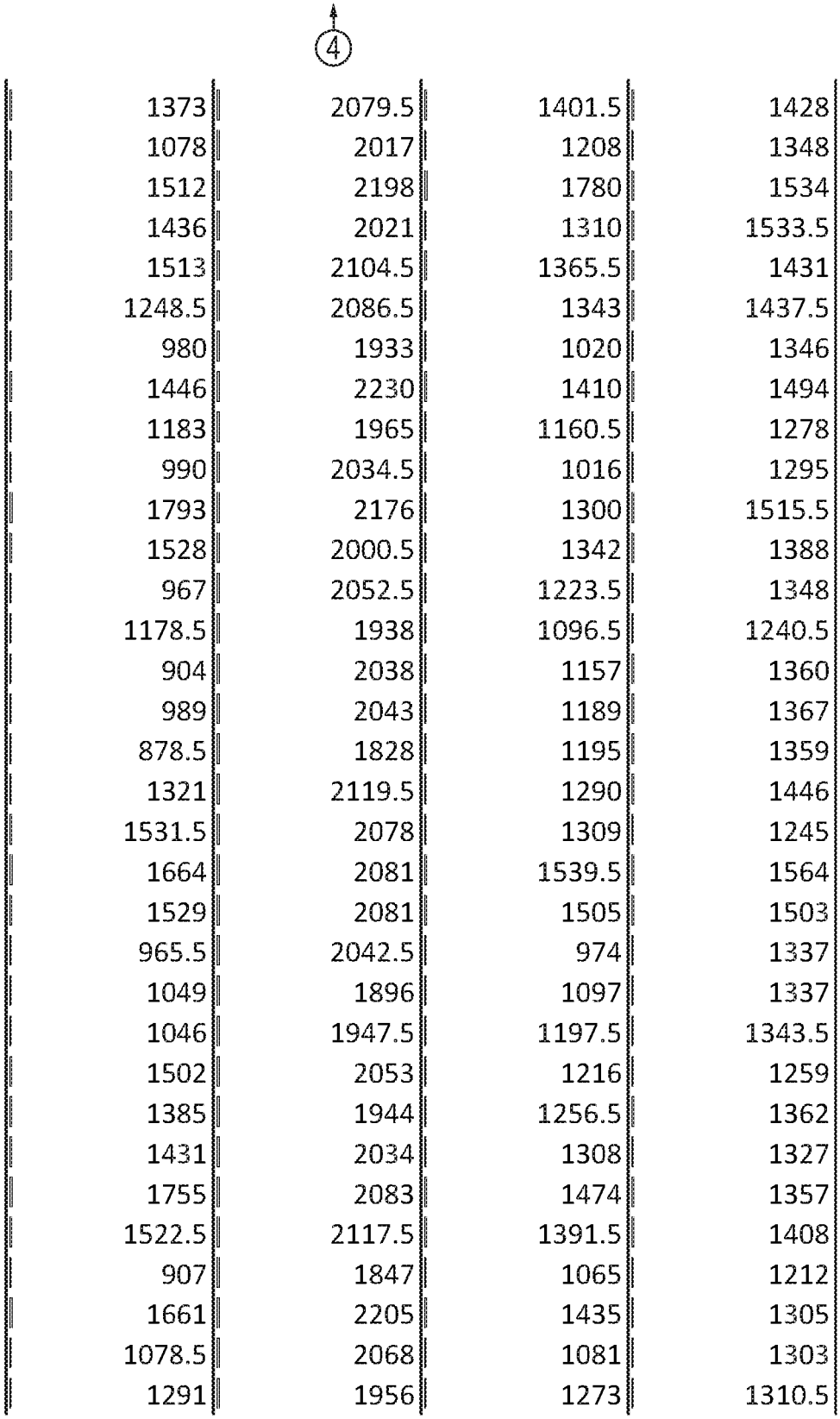
③

761	1333	1251	1440	1310.5
853	1445.5	1210	1524	1246
984.5	1609	1288	1574.5	1499
847	1399	1316.5	1472	1571
702	1420	1266	1570.5	1588
785	1413	1303.5	1407	1316
780	1363	1113.5	1356.5	1284
824	1419.5	1233.5	1415	1536
755	1302.5	1063	1399.5	1264
720	1286	1096.5	1450	1097
880	1429	1253.5	1388	1806
979	1410	1324	1440	1605.5
857	1354	1069.5	1467	1057.5
960.5	1363	1040	1360.5	1294
973	1402	1001	1432	1189.5
841.5	1362	1167	1397	1045.5
850	1342.5	1138	1438	1011.5
888	1405	1318	1372.5	1311
888	1421	1212	1516	1374
874	1523	1542	1491	1782
778.5	1408	1302.5	1395.5	1497
783.5	1356	1189	1420.5	1235
909	1210	1019.5	1472	1139
904.5	1218	1045.5	1295	1169
831	1295.5	1295	1410	1448
774.5	1248.5	1131.5	1353.5	1343
661	1452	1322	1244	1484
833	1370	1383	1430.5	1666
833	1424	1327	1440	1475
742	1290	1122	1408	1086
883.5	1311	1435	1450	1564
726	1371	1025	1349	1180
851	1308.5	1335	1316	1451.5

Ⓔ ⑦ Ⓕ

FIG. 13 (Continued)

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1373	2079.5	1401.5	1428
1078	2017	1208	1348
1512	2198	1780	1534
1436	2021	1310	1533.5
1513	2104.5	1365.5	1431
1248.5	2086.5	1343	1437.5
980	1933	1020	1346
1446	2230	1410	1494
1183	1965	1160.5	1278
990	2034.5	1016	1295
1793	2176	1300	1515.5
1528	2000.5	1342	1388
967	2052.5	1223.5	1348
1178.5	1938	1096.5	1240.5
904	2038	1157	1360
989	2043	1189	1367
878.5	1828	1195	1359
1321	2119.5	1290	1446
1531.5	2078	1309	1245
1664	2081	1539.5	1564
1529	2081	1505	1503
965.5	2042.5	974	1337
1049	1896	1097	1337
1046	1947.5	1197.5	1343.5
1502	2053	1216	1259
1385	1944	1256.5	1362
1431	2034	1308	1327
1755	2083	1474	1357
1522.5	2117.5	1391.5	1408
907	1847	1065	1212
1661	2205	1435	1305
1078.5	2068	1081	1303
1291	1956	1273	1310.5

FIG. 13 (Continued)

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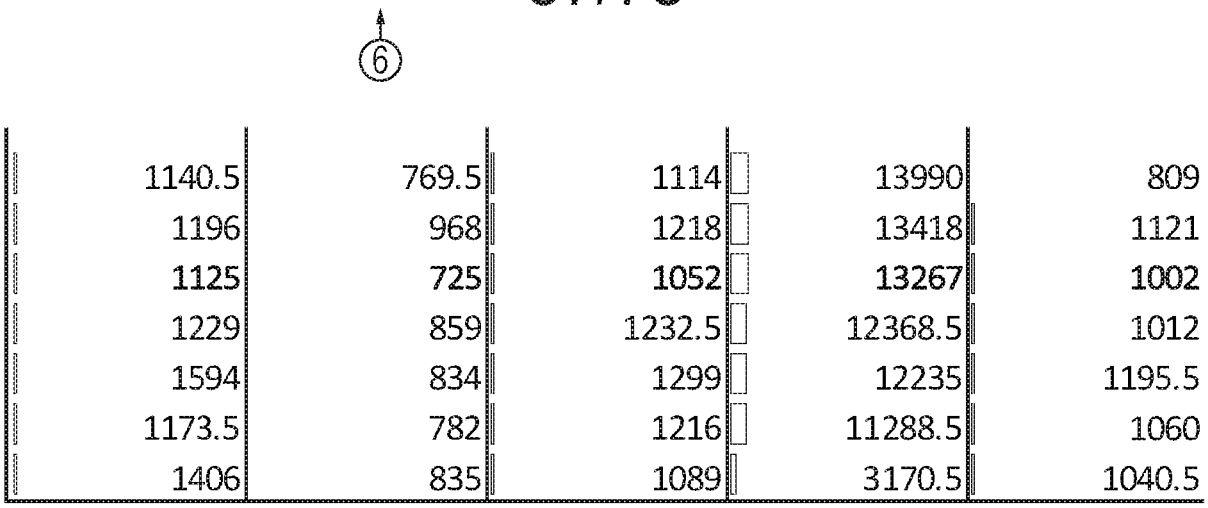
5

00453_645057	1723	1147.5	1010.5
00453_645048	1645	1221	938
00453_644892	1647.5	1113	1057
00453_645037	1661	1113.5	962
00453_644938	1916	1125	1745
00453_644925	1708	1157	1145
00453_644915	1672	1107	1195

G

FIG. 13 (Continued)

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G H

FIG. 13 (Continued)

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920	1337.5	1114	1301	1124
664	1308.5	1154.5	1390	1235
892	1219.5	1133	1406	1074
892	1377	1079.5	1411	1101.5
847	1439.5	1291	1432	1543
807	1305	1139.5	1494	1219
807	1413	1292	1297	1268





FIG. 13 (Continued)

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946	2060	1023.5	1337
1070	2108	1095	1297
873.5	2087	1353	1335
975	1963	1014	1359
1694.5	2101	1287	1432
1004	2140	1241	1402
1208	2031.5	1252	1283.5

FIG. 13 (Continued)

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Affimer Working ID	EGFR (FL1-A of QSH01)	mIgG2b (FL1-A of QSH03)	hIgG1 Fc (FL1-A of QSH05)
PBS (BLANK)	1630.5	1071	1111
PBS (BLANK)	1710.5	1084	903
PBS (BLANK)	1554.5	1078	1044
PBS (BLANK)	1543	992	1043
PBS (BLANK)	1621	1051	1093
PBS (BLANK)	1697	1055	1000.5
NEGATIVE - - HA (NEGATIVE)	1656	1101	1089
NEGATIVE - - HA (NEGATIVE)	1628	996	971
NEGATIVE - - HA (NEGATIVE)	1643.5	1116	1118
NEGATIVE - - HA (NEGATIVE)	1640	1130	1144
NEGATIVE - - HA (NEGATIVE)	1598.5	1055	1007
NEGATIVE - - HA (NEGATIVE)	1664	1163	1049
ZIKA NS1 C12 () - HA (POSITIVE)	1663	54687	1014
ZIKA NS1 C12 () - HA (POSITIVE)	1588	55225	1158.5
ZIKA NS1 C12 () - HA (POSITIVE)	1766	43772.5	1031
ZIKA NS1 C12 () - HA (POSITIVE)	1736	45644	1063
ZIKA NS1 C12 () - HA (POSITIVE)	1658	52196	1033.5
ZIKA NS1 C12 () - HA (POSITIVE)	1734	41868	1143
00454_645193	1785	1090	1137
00454_645325	1648	1120.5	1001
00454_645324	1701	1190	1101
00454_645296	18689	1211	108570
00454_645277	1616	1097	1030
00454_645140	17415.5	1071	79251
00454_645250	14422	1168.5	73151
00454_645222	1621	1020	1109
00454_645156	1726	1059	1176
00454_645174	1528	1100	1163.5
00454_645178	1607	1219	1124.5
00454_645271	1737	1265	1160
00454_645234	1593	1092	1177
00454_645290	1725	1019	1202
00454_645304	1724.5	1089	1167

1

A

FIG. 14

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hPD-L2 (FL1-A of QSH06)	Her2 (R&D) (FL1-A of QSH08)	hPD-L1 (FL1-A of QSH10)	Trastuzumab (FL1-A of QSH11)	hIgG (FL1-A of QSH13)
1089	820	1343	1206	996
1094.5	830	1047	1359	928
1132	783	1062	1391	884.5
1122	811	1087	1367	1006
1079	810	1117	1361	920
1088.5	863	1148	1298.5	951
1085	957.5	990	1402	1015
1062	861	1014	1379.5	973
1181	745	1048	1246	963.5
1147	909	1135	1254	976
1215	833.5	1152	1310.5	1026
1132.5	850	1207.5	1431	1041
1062	825	1034.5	1381	1009
1192.5	814	1028	1500.5	960.5
1152	766	1052	1471	1038
1187	781.5	1063	1356.5	1041
1133	887	1082	1262	987
1127	969	1235	1426	1082
1349	1004.5	29696	1539	1128
1264	974	23210	1411	1079
1287	1046.5	22917.5	1504	1237
54516.5	1536.5	16156	139013	20297
1166	855	15552	1272	1004
43622	1201.5	14844	114642	16430
36800	1464	14150	105700	13898
1222	815	13524	1254	1045.5
1119	1056	13461	1284.5	1241
1240	937.5	13186	1493	1187.5
1211.5	834	13119	1450	1047.5
1299	887	12822	1370.5	1127
1280.5	1011	12603	1424	1068
1202	990.5	12387	1347	1088.5
1219	899	12054	1327	1025

A 2 B

FIG. 14 (Continued)

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Her2 (Sino) (FL1-A of QSH15)	Her3 (FL1-A of QSH16)	mPD-L1 (FL1-A of QSH18)	No target ctr (FL1-A of QSH22)	Avastin (FL1-A of QSH23)
882	1276	1035	1397	1075.5
957	1354	1093	1315	1151
831	1267	1069	1407	1012.5
863	1317	996.5	1349	1130
845	1308	1114	1330	1101
721	1349	1044	1357.5	1063
807	1216	974	1297	1121.5
746	1353	1022	1316	1001
785	1289	1001	1378	1041
833.5	1257	1047	1449.5	1027
715	1310	1060.5	1371.5	1093.5
869.5	1276.5	1192	1316	1058.5
904	1295	929	1513	1025
791	1244	1196	1414.5	1140
969	1310	1167	1465.5	1065
920.5	1368.5	1124	1345	1073
758	1313	1175	1301	1118
930	1376	1079	1451	1153.5
791.5	1416	1139.5	1570.5	1213
755	1373	1171.5	1491	1050.5
1000	1446.5	1203.5	1619	1087
1558	9050	34447	1722	80937.5
977	1355	998	1542	1043
1086	7678	31591	1498	67202.5
1450.5	8058	26664.5	1723	59332.5
966	1370	1046	1411	1019.5
853	1402	1176	1577.5	1049.5
827	1402	1076	1559	1103
912	1359.5	1179	1401	1225.5
835	1397	1117	1483	1116
752	1492	1087	1489.5	1187
758.5	1403.5	954	1521	1125.5
775	1356	1140	1307	1133

B 3 C

FIG. 14 (Continued)

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Humira (FL1-A of QSH25)	Rituximab (FL1-A of QSH26)	mPD-L2 (FL1-A of QSH27)	Her4 (FL1-A of QSH28)
848.5	1987	1148	1390.5
915	1962	987	1355.5
898	1952	1111	1354
953.5	1862.5	1133	1316
981	2065	1132.5	1375
861	1943	1125	1302
905	1807	1073.5	1346
937	1874	1026	1381
949.5	1986	1082	1291.5
933.5	1936	1138	1356.5
827	1970	1044	1334.5
931.5	2066.5	1190	1407
874	1903	1113	1278
961.5	1944	1095	1261
953.5	2009	1093	1247
901	2010	1085	1211
936	1989.5	1098	1413
996.5	1990	1104	1445
954	2070	1114	1488
1045.5	2066.5	954.5	1495.5
1111	2078.5	1242	1518
131315	22615	40253	4399
946	2029.5	1087	1437
86596	19134.5	37128	3722
84929	15932	30973.5	3826
986	1978	1042	1423
949	2012.5	1084	1502
1079	2023	1171	1610
890	1882	1085	1353
1040	2003	1138.5	1367
973	1957	1054	1476
1069	1952	1101	1571
977.5	2025.5	1163	1357.5

← C

4 ↓

FIG. 14 (Continued)

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1

00454_645287	14587.5	1176	67151
00454_645143	1798	1170	1084
00454_645169	13680	1101.5	82856
00454_645167	1600	1175	1022
00454_645175	1589	1144	960
00454_645160	1590	1088	1088
00454_645297	1664	1081	1015.5
00454_645152	1722	1119	1013.5
00454_645155	1690	1191.5	1076
00454_645202	13487	1059.5	83252
00454_645291	1673	1165	1018
00454_645144	1631	1143	978
00454_645326	1605.5	1170	1171
00454_645136	1634	1109	1094
00454_645281	1595.5	1121.5	1047
00454_645244	9506	1111.5	45050
00454_645289	1520	1117	1114
00454_645205	1609.5	1173	1110.5
00454_645120	1661	1173	1082
00454_645298	8834	946	35873
00454_645195	1539	1028	1019.5
00454_645268	1708.5	1093	1146
00454_645131	1781	1118.5	1047.5
00454_645116	8608	1222	32338
00454_645262	1666.5	1133	1200
00454_645121	1661	1240	1256.5
00454_645173	5170.5	1081	45807
00454_645284	5839	1165	23132.5
00454_645280	1623	1098	1060
00454_645132	1591	1200	1179
00454_645212	1566	1050.5	917
00454_645260	4442	1084	32130.5
00454_645166	2528.5	1087	13169
00454_645164	1691.5	1155	1128
00454_645118	1696	1138.5	1143.5

5

D

FIG. 14 (Continued)

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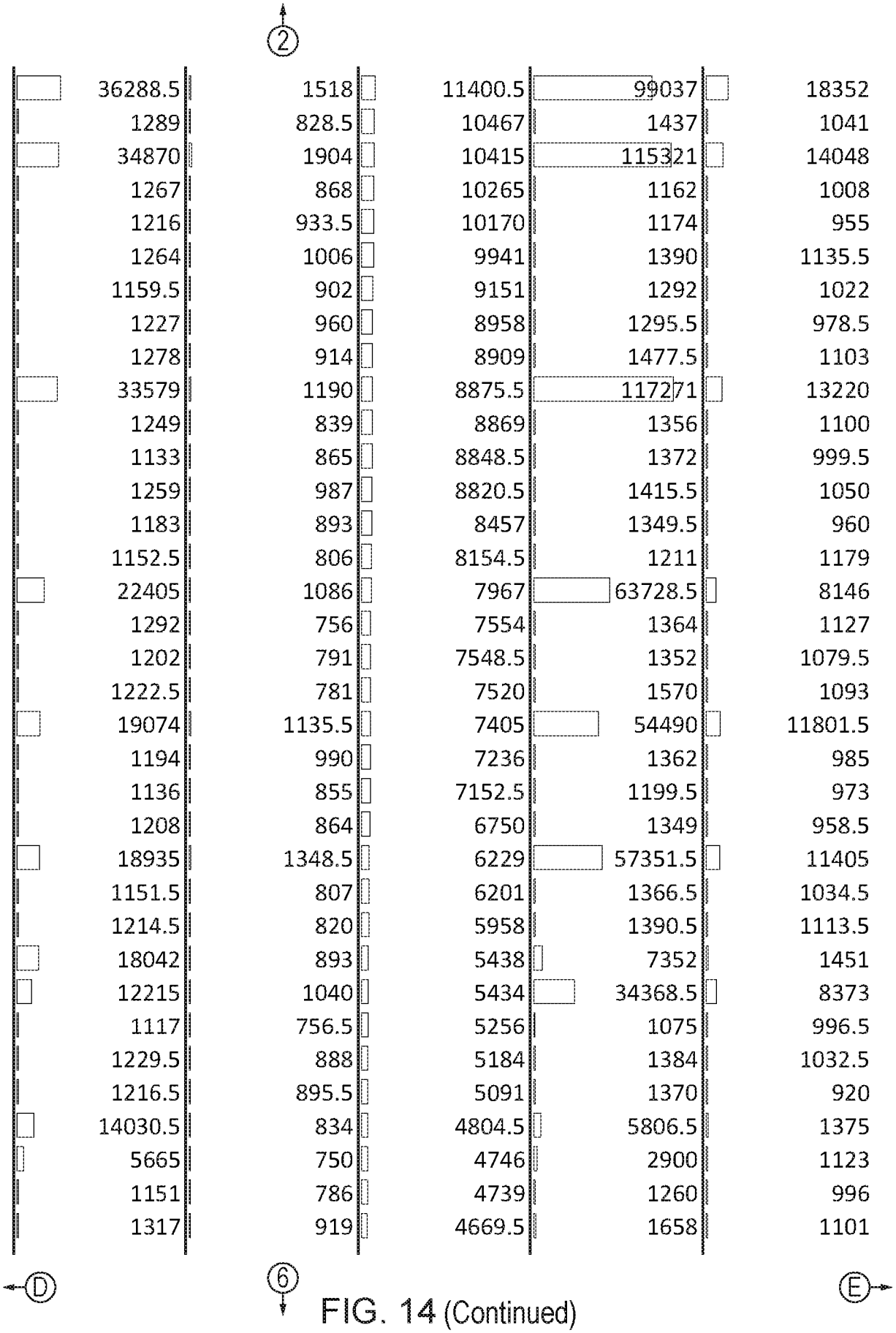


FIG. 14 (Continued)

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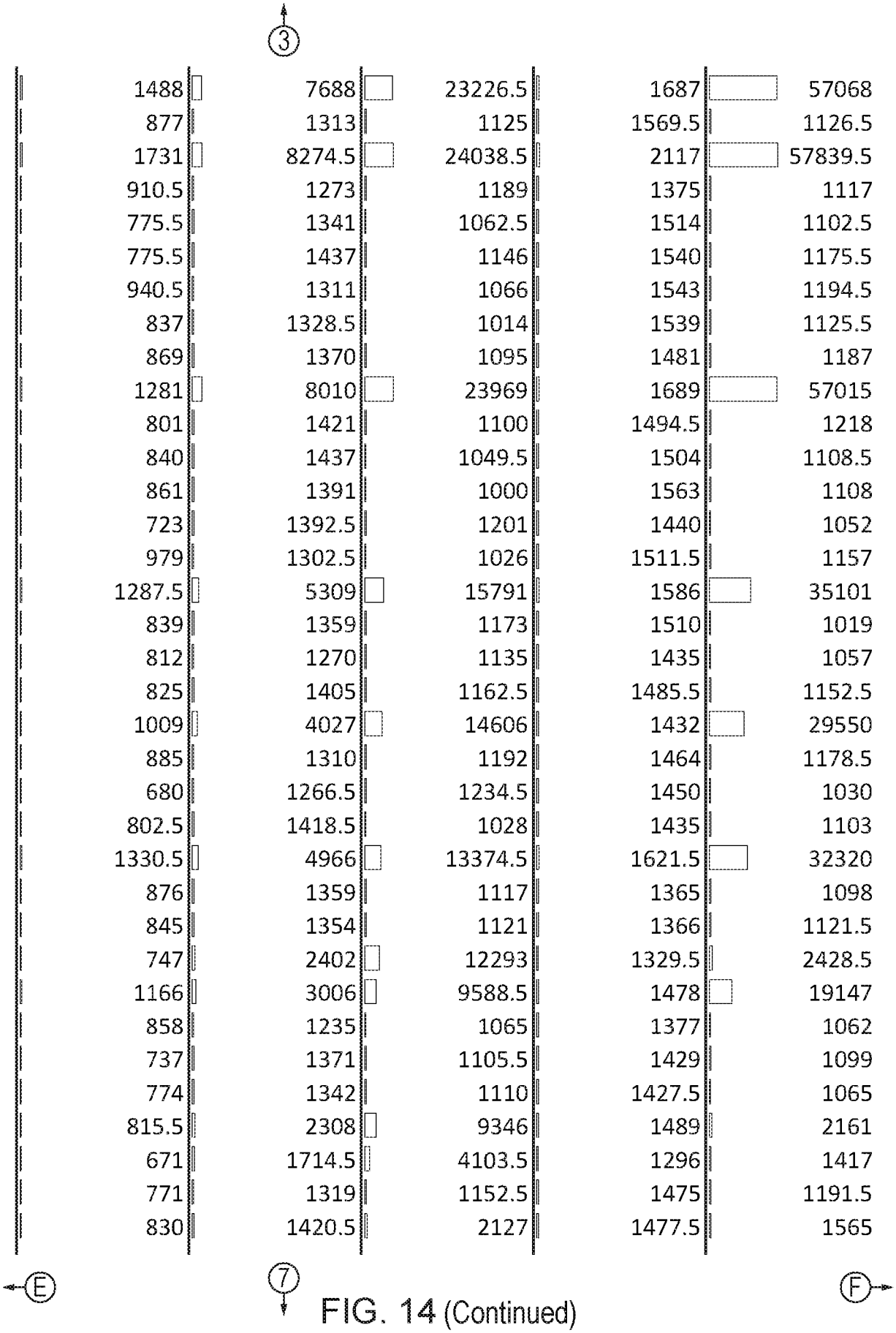


FIG. 14 (Continued)

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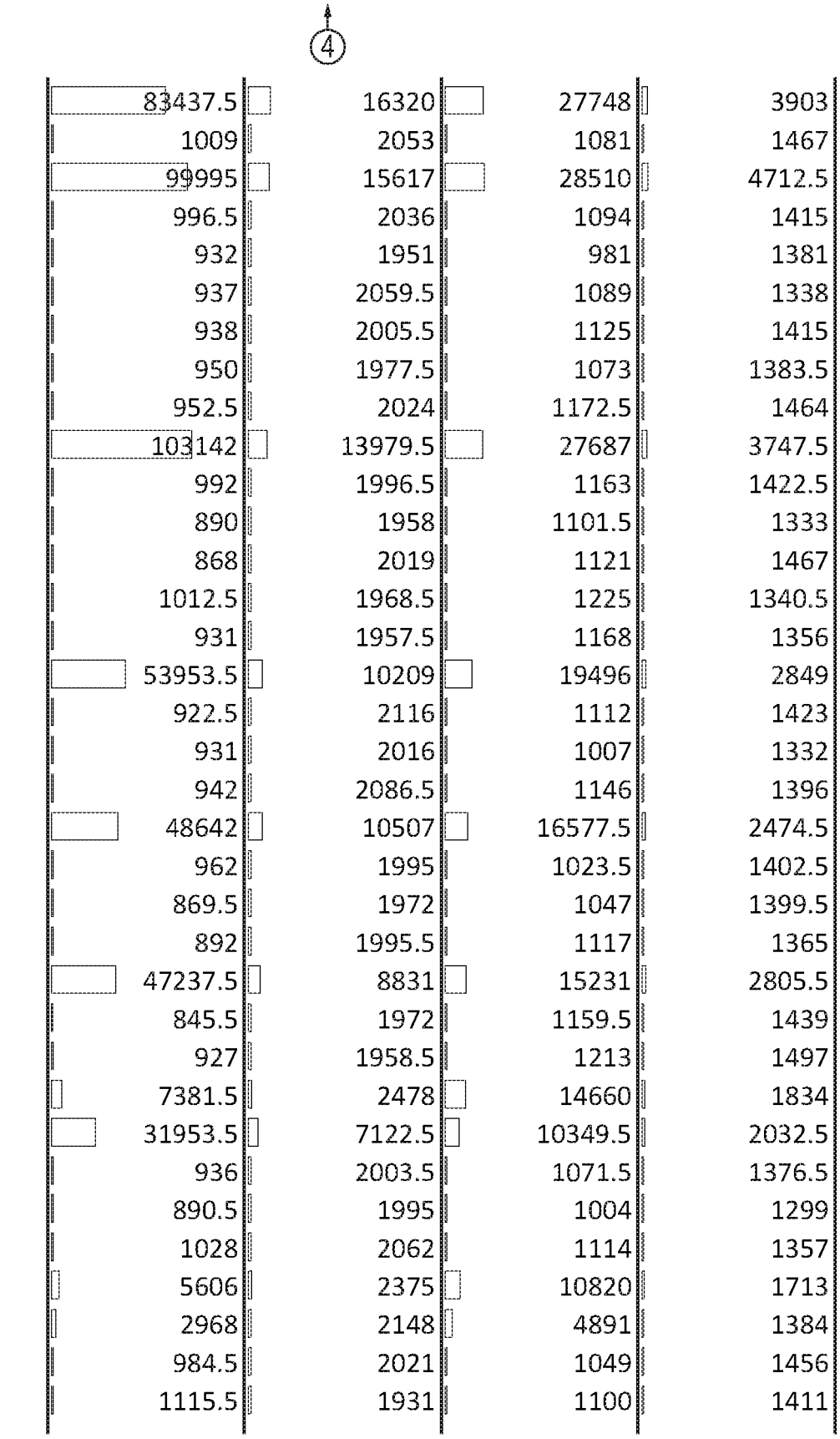


FIG. 14 (Continued)

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5

00454_645171	1620.5	1111	995
00454_645142	5730	1082	39717.5
00454_645276	4595	1058	38717
00454_645130	1683.5	1020.5	1006
00454_645162	1567	998.5	1103.5
00454_645299	1655.5	1094	1141
00454_645138	1674	1119	1072
00454_645285	1759	1173	1061
00454_645282	1558	1060	1123.5
00454_645165	1685	1046	1173
00454_645185	1720	1102.5	1092
00454_645134	1699	1114	1224
00454_645141	1628.5	1128	1210
00454_645209	1525.5	1154	1219
00454_645267	1661	1198	1055.5
00454_645214	1817	1151.5	1174.5
00454_645179	1545	1070	879
00454_645117	4995.5	1075.5	36788
00454_645157	1710	1054.5	1162
00454_645213	1687.5	1097	1106
00454_645180	1585.5	1012.5	1048
00454_645286	1647	1059	1070
00454_645259	4787	1100	23727
00454_645129	3095.5	1135.5	14084
00454_645242	1660	1124	968
00454_645145	1683	1101.5	1107
00454_645170	4318	1144	25645
00454_645181	4500	1049	25746
00454_645216	1701	1221	1210
00454_645201	1610	1185.5	1063
00454_645261	4576	1103	24225
00454_645308	1759	1218	1155
00454_645275	1671	1049	1086
00454_645264	1782	1130.5	1052
00454_645249	1760	1054	996

9

G

FIG. 14 (Continued)

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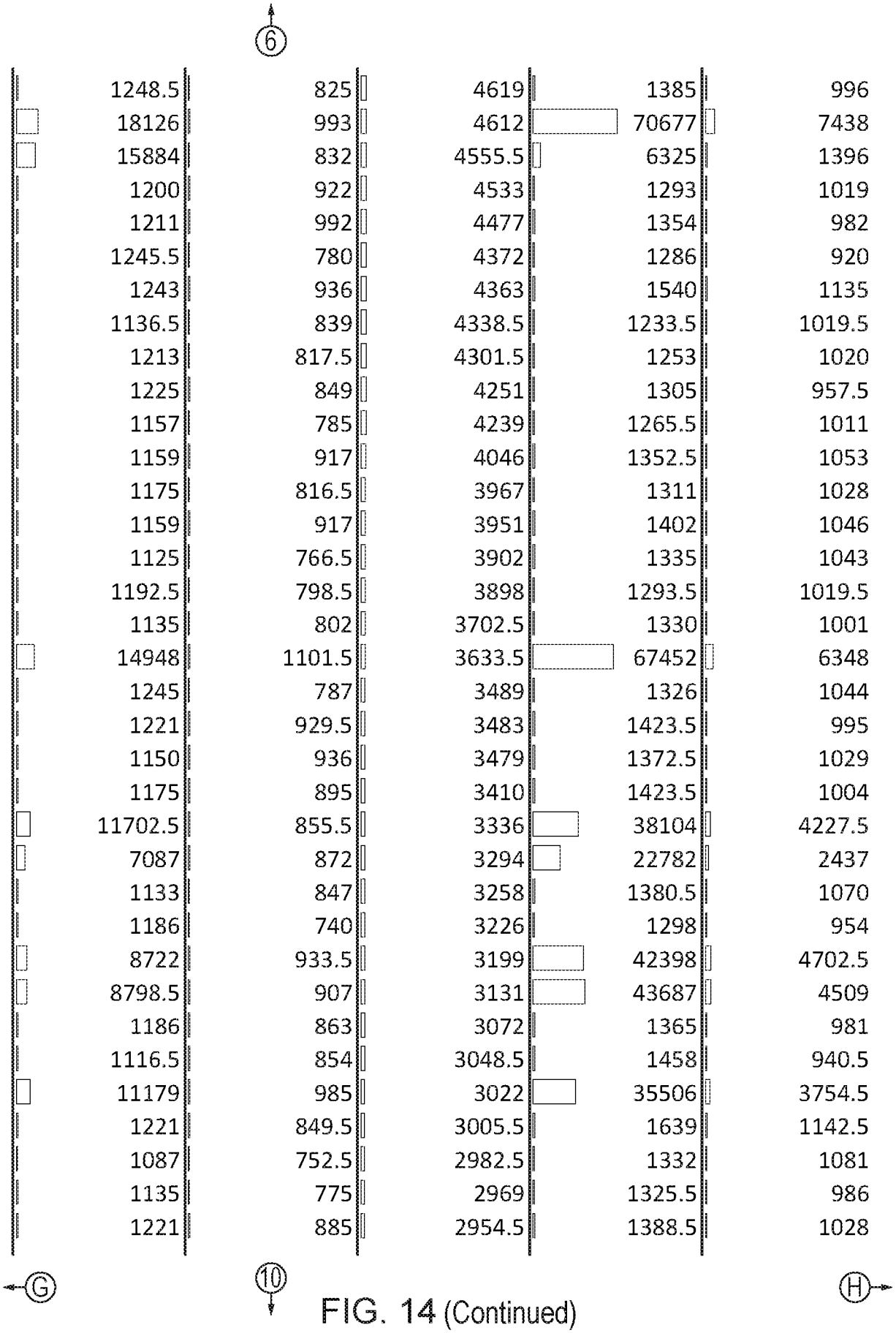


FIG. 14 (Continued)

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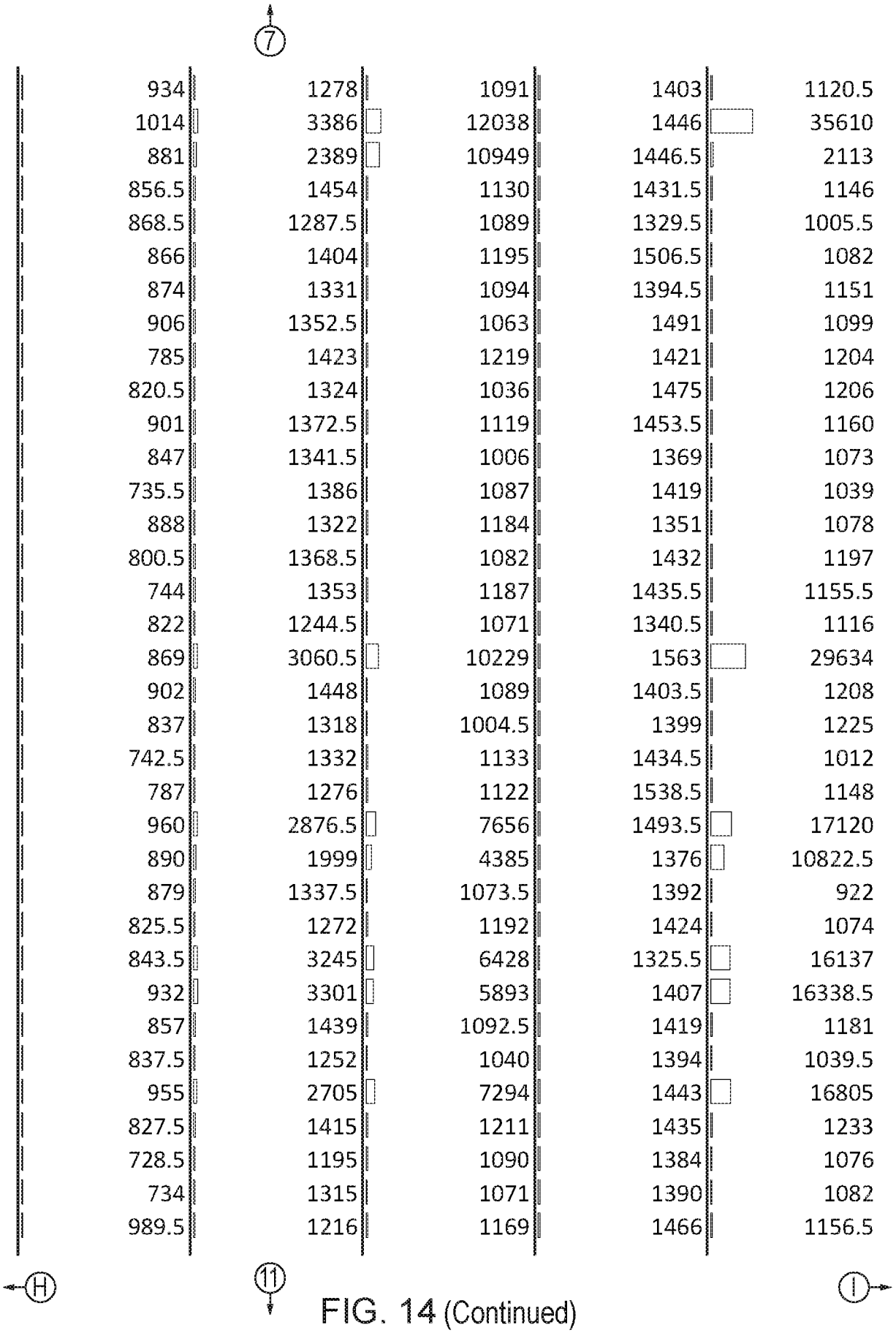


FIG. 14 (Continued)

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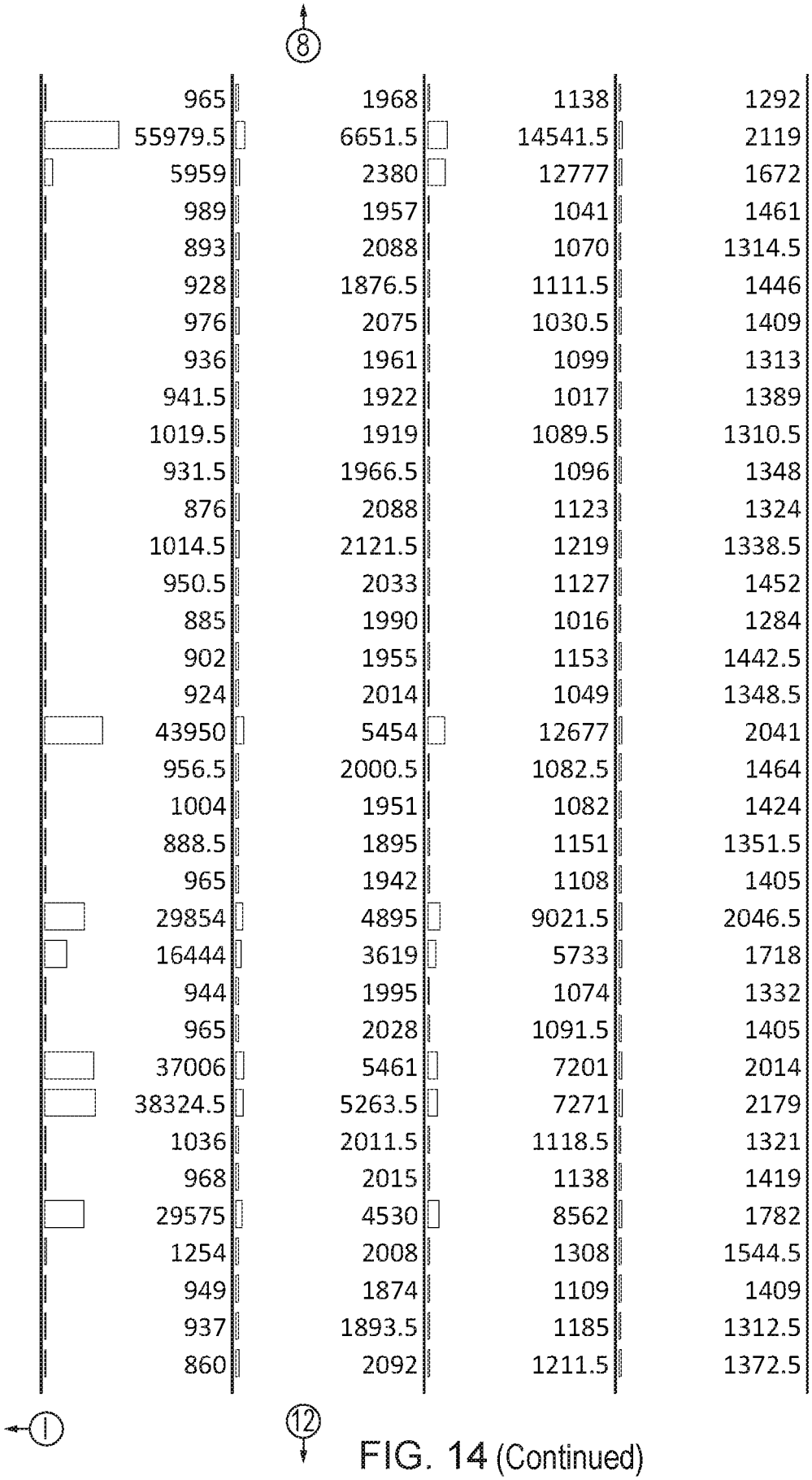


FIG. 14 (Continued)

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00454_645306	1632.5	1148	1089
00454_645196	4067	1122	30337
00454_645263	1671	1114	1049
00454_645188	4239	1045.5	32917
00454_645194	4131	1108	35041
00454_645190	1742	1142	1010
00454_645204	1540	1036.5	1023.5
00454_645191	1540.5	1114	1066.5
00454_645310	1696	1133.5	1198.5
00454_645227	1677	1054	1047
00454_645208	3960	1100	30807
00454_645311	1701	1238	1163.5
00454_645309	3898.5	1217.5	14158.5
00454_645224	1674	1149	1165
00454_645251	1768.5	1116	1020
00454_645319	1634	1151	1033
00454_645318	1659.5	1177	994.5
00454_645302	1696	1019	1025
00454_645307	1727	1126.5	1093
00454_645115	2696	1147.5	8206
00454_645328	1733	1104	1233
00454_645210	1663	1139.5	1024
00454_645113	1728	1157	937.5
00454_645288	1666	1004.5	1041
00454_645303	1590.5	1113.5	1076
00454_645317	1769	1084	1112
00454_645158	1640.5	971	1435
00454_645161	1644	1135.5	1113.5
00454_645147	1647	1159.5	1023.5
00454_645322	1644	1205	928
00454_645177	1738.5	1098	1114
00454_645203	1601	1154	988.5
00454_645320	1725.5	1144	1081.5
00454_645270	1650	1100	1214.5
00454_645327	1672	1057	1115.5





FIG. 14 (Continued)

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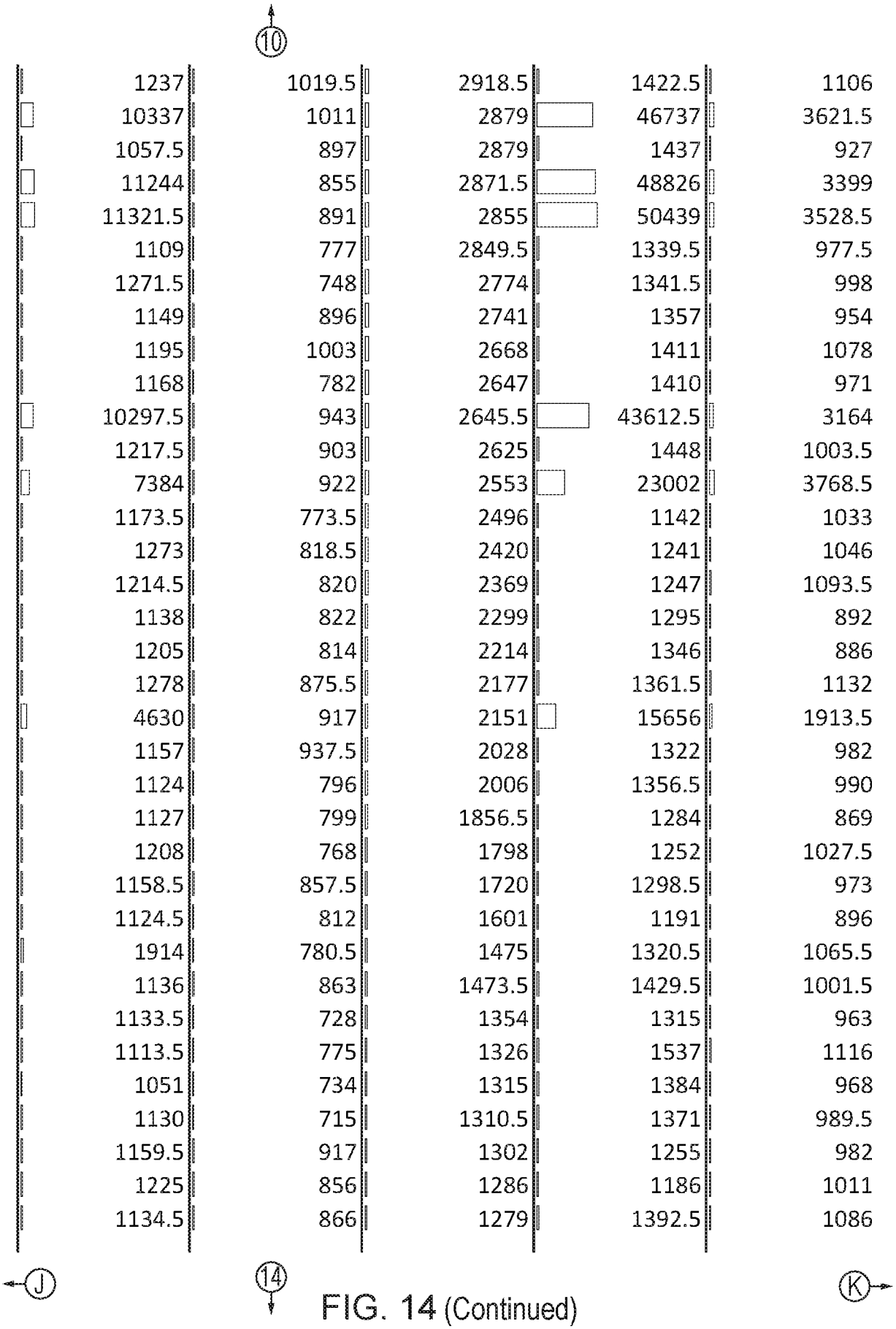


FIG. 14 (Continued)

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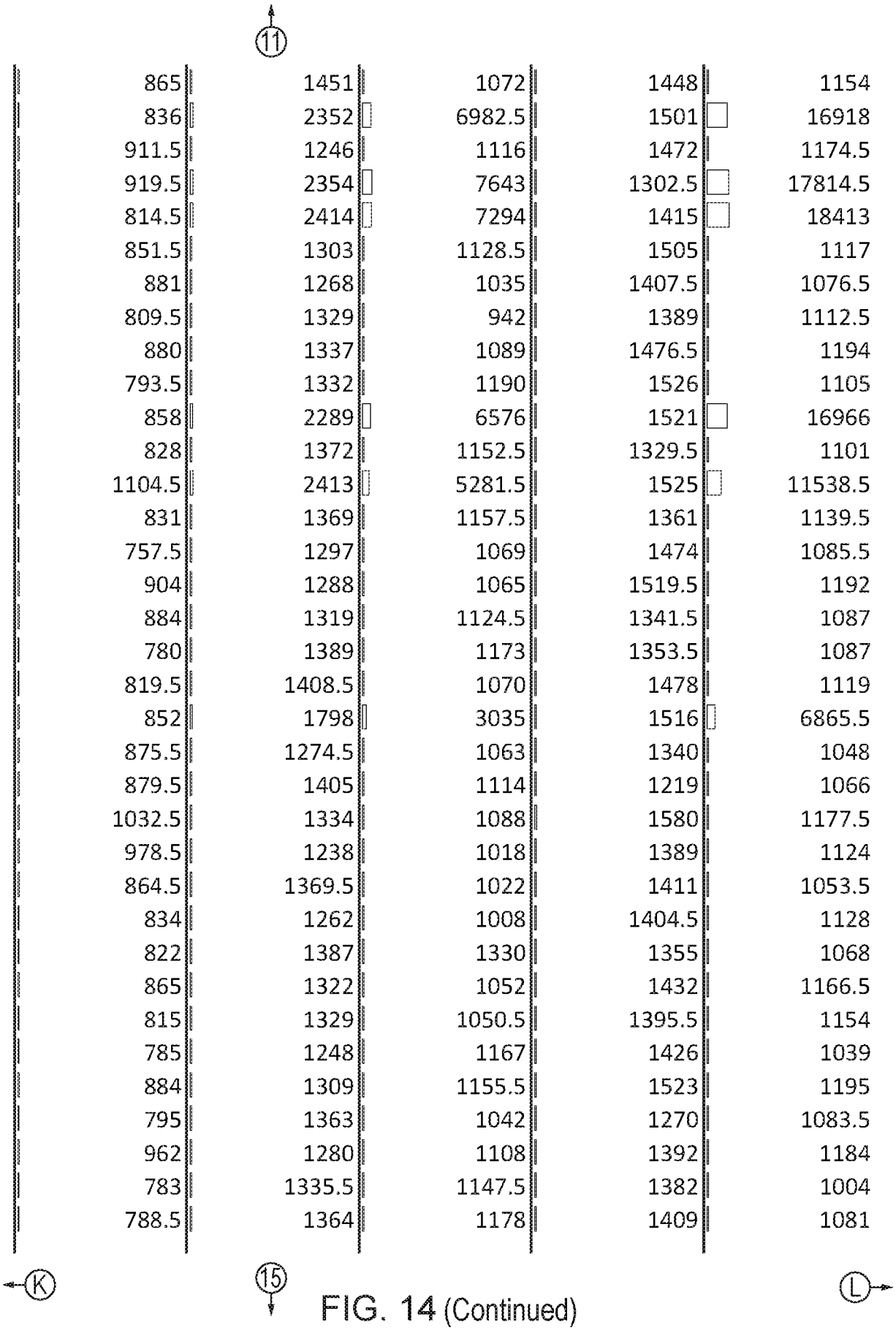


FIG. 14 (Continued)

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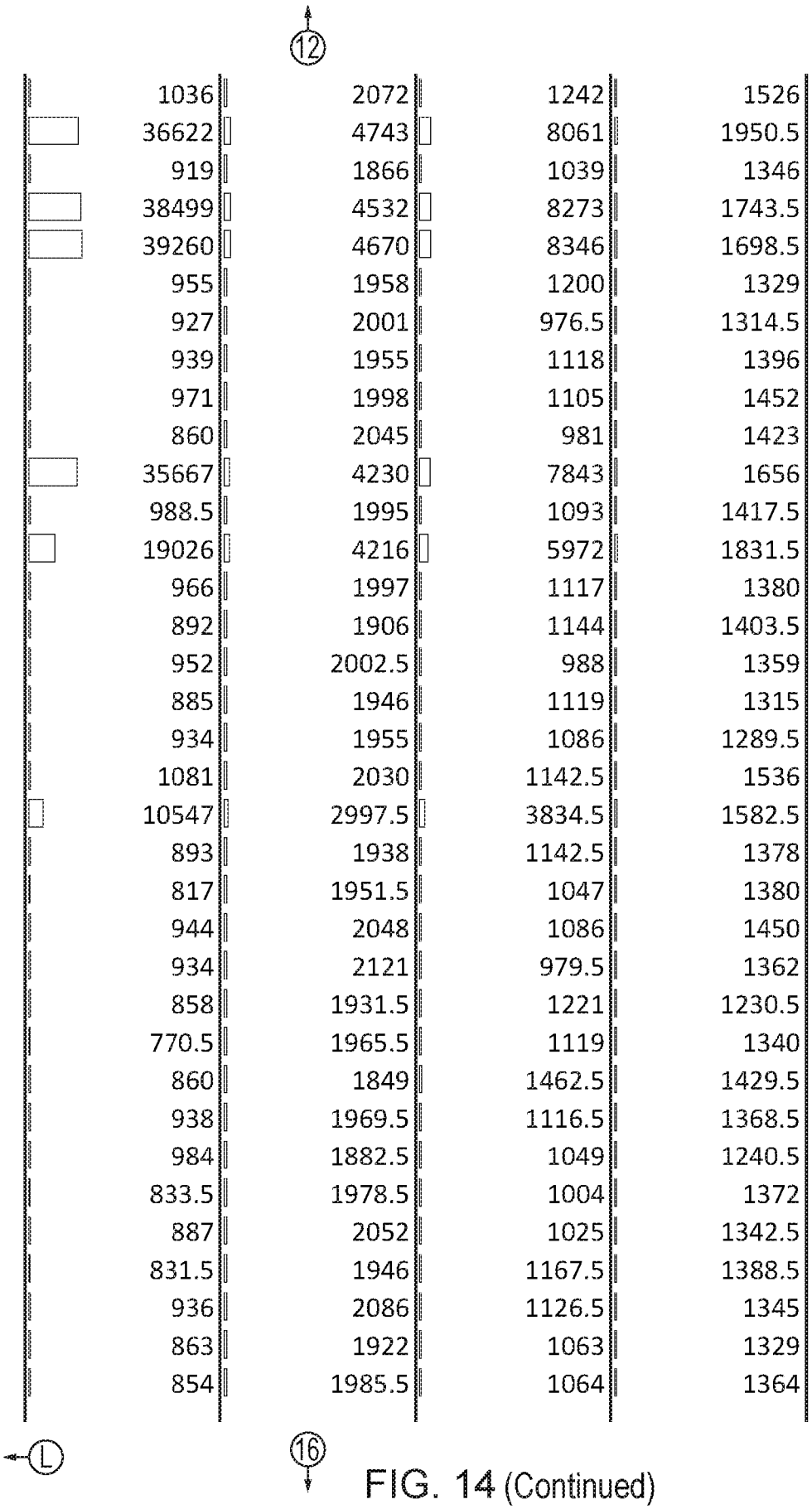


FIG. 14 (Continued)

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00454_645128	1670.5	1112	1082
00454_645151	1674	1108.5	930.5
00454_645226	1696	1208	1083.5
00454_645215	1759	1165	1170.5
00454_645329	1820.5	1134	983.5
00454_645235	1650	1008.5	1061.5
00454_645269	1668	1056	1103
00454_645217	1665	1106	1054
00454_645237	1663.5	1229	984.5
00454_645278	1706	1046	1069
00454_645127	1736	1157	1160
00454_645253	1678.5	1127	999
00454_645272	1561	1081	989
00454_645189	1619	1188.5	1117.5
00454_645200	1550	1426	1186
00454_645313	1640	1066	1035
00454_645148	1623	1135.5	1049
00454_645258	1648	1094.5	1054
00454_645206	1623	1086	1082
00454_645239	1682	1085.5	1056.5
00454_645187	1714	1109	1051.5
00454_645150	1691	1079	1027.5
00454_645323	1674	1097.5	1082
00454_645135	1634.5	1055.5	1011
00454_645241	1685	1152	1135
00454_645186	1711.5	1106	1011
00454_645153	1673.5	1208	971.5
00454_645125	1724.5	1176	1149
00454_645248	1593	1070	1044
00454_645154	1658	1099	1114.5
00454_645172	1650	1108	992
00454_645139	1832.5	1107.5	1022
00454_645252	1737.5	1226	960
00454_645321	1705.5	1118.5	1082
00454_645133	1601	1151.5	1044





FIG. 14 (Continued)

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1136	779	1277.5	1295	909
1158	822.5	1270	1350	1003.5
1170	943.5	1265	1388	1042
1181	886.5	1265	1189	982.5
1206	815	1262	1330	998
1221.5	753	1262	1360.5	853
1176	796	1256	1381.5	979
1136	782.5	1255.5	1243	1014
1251	836	1254	1367	1060
1123	825.5	1254	1292	992
1288.5	868	1252	1411	1074
1229	857.5	1250.5	1237	989
1230	863	1250	1222	998.5
1074	694.5	1246	1344	910
1153.5	701	1246	1419	968.5
1159	813	1241	1164	1063
1224	812	1232.5	1187.5	979
1044	827.5	1226.5	1318	1014
1152.5	799	1222	1307	947
1311	761	1220.5	1319	1068.5
1110	766	1216.5	1346	1031
1173.5	865.5	1208	1278.5	1027.5
1136	735	1197	1430.5	934
1297	685	1197	1319	1036
1197.5	783	1194	1540	1012
1070.5	852	1189	1321	922
1160	787.5	1182.5	1384	979
1127.5	868	1174.5	1365.5	986
1024	780	1168	1284	1024.5
1100.5	869	1167.5	1341.5	936
1114	828.5	1166.5	1175.5	966
1138	814	1165	1451	957
1089	869.5	1161.5	1425.5	1054.5
1203	829	1161.5	1400	987
1173	748.5	1148	1356	974.5

FIG. 14 (Continued)

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643.5	1292	1162.5	1399	1200
790	1349	1148	1445	1154
863	1348	1136	1521	1119
903.5	1301	1197	1435	1098
892.5	1233	979	1521	1145
954	1247	1185	1307	1098.5
890	1334	1222	1439	1213
797.5	1297	1060	1414.5	1068
934	1315	1083.5	1423	1115.5
814	1274	1076	1407	1096.5
874	1313	1127	1533	1050.5
783	1374	1044	1478.5	1093
726	1321.5	1051	1419	998
962	1381	1041	1413	1067
860	1272.5	1074	1322	1026.5
899	1364	1101	1364	1045
812	1307	987.5	1340	1024
790	1289	1049	1367	1154
906	1269.5	1070	1287	1115
896	1350.5	1022	1415	1117.5
854.5	1287.5	1007.5	1294	1106
879.5	1332	995	1412.5	1119.5
809	1327.5	1091.5	1317	1109.5
984.5	1318	1060	1386.5	1086
853	1390	1108	1388	1097
755	1268	1077.5	1415	1004
925	1189	1025.5	1431.5	1068
814	1310	1143	1391	1080
901.5	1228	1079	1494	1127.5
771.5	1291	1143	1478.5	1009
843.5	1286.5	1256	1364.5	982
819.5	1380.5	1031	1453	1212
703	1368.5	1127	1298.5	1160.5
817.5	1351	1081.5	1459	1181
837	1302.5	1097	1454	1101

FIG. 14 (Continued)

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904	2126	1051.5	1411
891.5	1896	1117	1370
931	1979	1047	1429.5
896	1906	1176	1391
853	1939.5	1127	1335
972.5	1986	1033	1404
989.5	2052.5	1175.5	1408.5
893	1984	1185	1287
888	1966	1080	1264.5
951	1998	1184	1270.5
883.5	2017	1110.5	1397
920	2059.5	1179	1362
886.5	1966	1056.5	1310
913	1964	1118	1404
938.5	1864	980.5	1295
876	1999	990	1241
965	1876	1114	1361
833	1769.5	1007	1370
1002	1920	1109	1326.5
965	2052.5	1111	1444.5
883	1931	1080	1356
984.5	1988.5	1155	1355.5
810	1990.5	997	1393
1036	1931	1089	1369
928	2020	1175	1353.5
934.5	1979	1095	1257
981.5	1909	1163	1335
966	1941	1073.5	1424
801	1813	1051.5	1263
948	2009.5	1163.5	1385.5
890	1931.5	1072	1257
914	1998	1133	1332
841	2044.5	1186	1313
922	2047.5	1018	1263.5
922	1864	1135	1357.5

FIG. 14 (Continued)

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00454_645182	1580	1157.5	1103
00454_645223	1600	967.5	1127
00454_645149	1651.5	1160	1143
00454_645168	1622	1175	1174.5
00454_645274	1646.5	1010.5	1150.5
00454_645231	1645	1082.5	1111
00454_645137	1672	1165.5	1047
00454_645192	1632.5	1199	1097
00454_645159	1599.5	1089.5	1100
00454_645183	1799	1106	1047
00454_645163	1631	1124	1073
00454_645305	1775	1076	1166
00454_645243	1663.5	1118	1055
00454_645301	1630	1133	1061.5
00454_645236	1768.5	1172.5	950
00454_645238	1745	1234	1109.5
00454_645312	1682	1086	983.5
00454_645119	1677	1243	1109
00454_645114	1792	1143.5	1047.5
00454_645273	1571.5	1042.5	1165
00454_645256	1655	1235	1064
00454_645300	1612.5	1044.5	1071
00454_645232	1602	1167	1008
00454_645221	1674.5	1197	1138
00454_645184	1620	1144	1097
00454_645254	1537	1133	1007.5
00454_645265	1648	1148	1070.5
00454_645246	1600	1128.5	1055
00454_645225	1624.5	1099.5	1071
00454_645211	1617	1065.5	1093
00454_645257	1648	1118	1106
00454_645229	1620	1155	1020
00454_645228	1596	1115	976.5
00454_645283	1699	1138.5	1118
00454_645292	1588.5	1097	1148

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P

FIG. 14 (Continued)

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1195	825	1145	1396	1037.5
1155	723	1143	1154	1043.5
1111	831.5	1142	1292.5	965
1248	868	1138	1317	985
1155.5	804	1134	1273	1068
1162.5	862.5	1133.5	1356	899.5
1157	885	1132	1399	968
1115	795.5	1128.5	1371	1047
1162.5	812	1125	1294	878
1149.5	831.5	1124	1318	1028
1108	804.5	1122	1307	985.5
1168.5	874.5	1122	1335	1074
1159.5	807	1121.5	1361.5	987
1107.5	778	1120.5	1280	1044
1127	895.5	1120	1302	942
1269	808	1119	1321	847
1200	920	1119	1486	886.5
1124	836	1118.5	1426	989
1240	857	1109	1311	1041
1141.5	756	1108.5	1260	956
1200.5	887	1103	1235	952
1184	851.5	1102.5	1353	1019
1108	863	1101	1340.5	931
1159	871	1100	1254	995
1127	799	1091	1332	998.5
1073.5	853	1087	1254.5	998.5
1174	852	1086	1369	1029
1179.5	908	1086	1287	1028.5
1252	910.5	1085.5	1586	1065
1135.5	761	1084	1385	1006
1202	717	1076	1252	933
1197	794.5	1065	1218.5	1006
1130	857	1060	1108	1016
1190	815	1059	1225.5	942
1079	793	1055	1325	974.5

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FIG. 14 (Continued)

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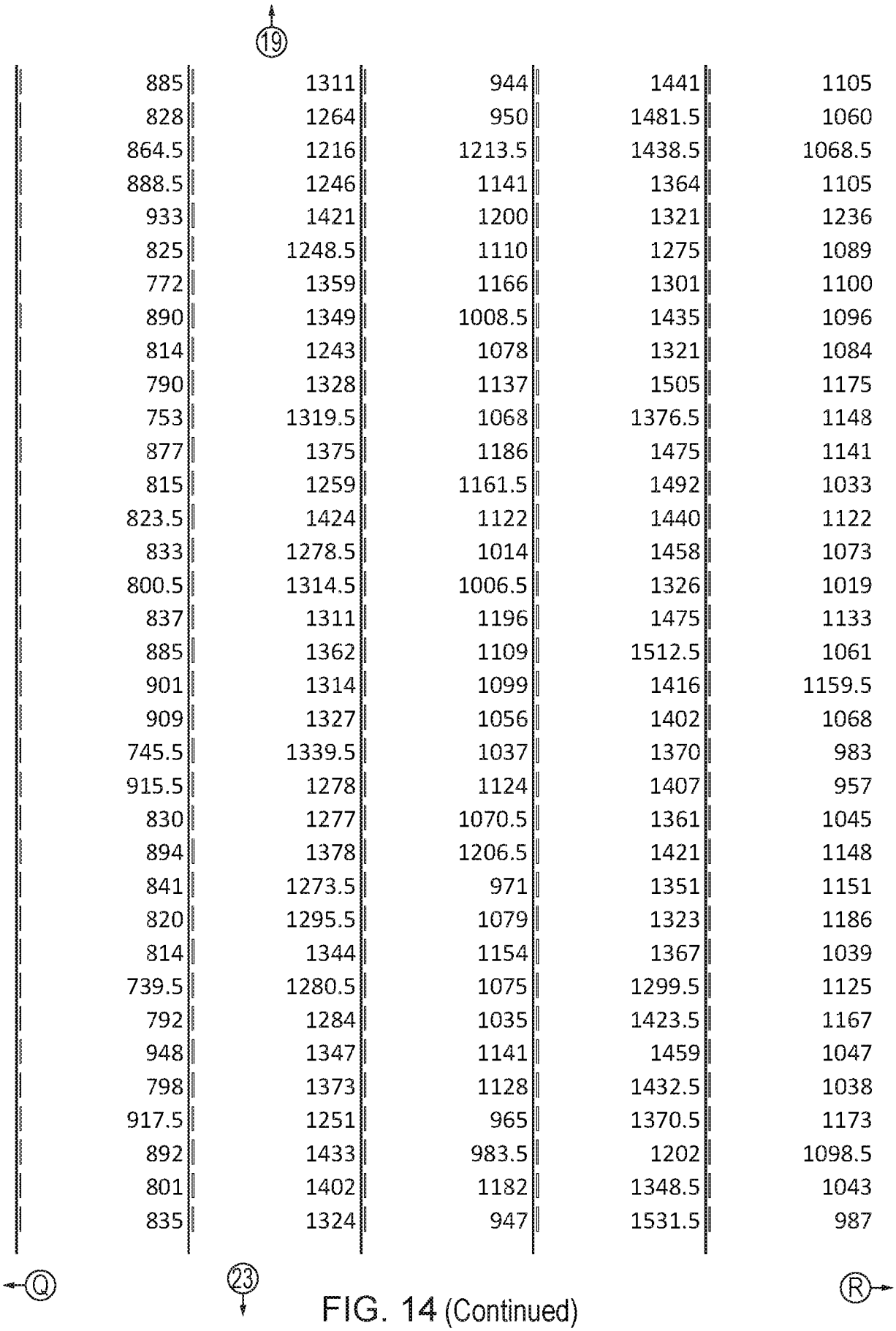


FIG. 14 (Continued)

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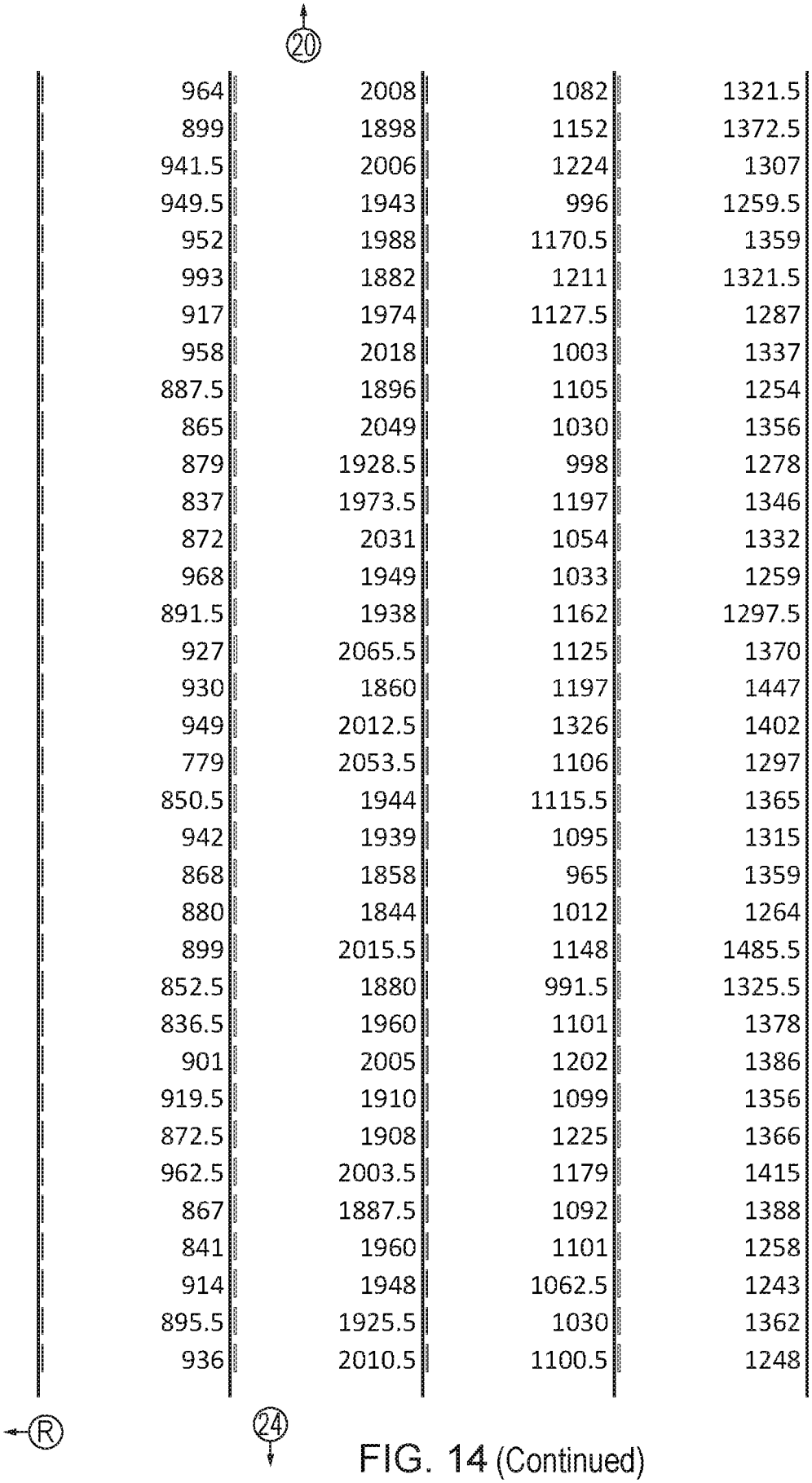


FIG. 14 (Continued)

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00454_645255	1710	1024	1092
00454_645245	1666.5	1119	1088.5
00454_645266	1679	1022	913
00454_645126	1683	1135	1139
00454_645146	1704.5	971	936.5
00454_645233	1704	1063	1117
00454_645230	1744	971	1208
00454_645279	1726.5	1108	1109
00454_645207	1762	1115	995
00454_645240	1756	1088	1098
00454_645247	1620.5	1094.5	936
00454_645176	1553	1138	941



FIG. 14 (Continued)

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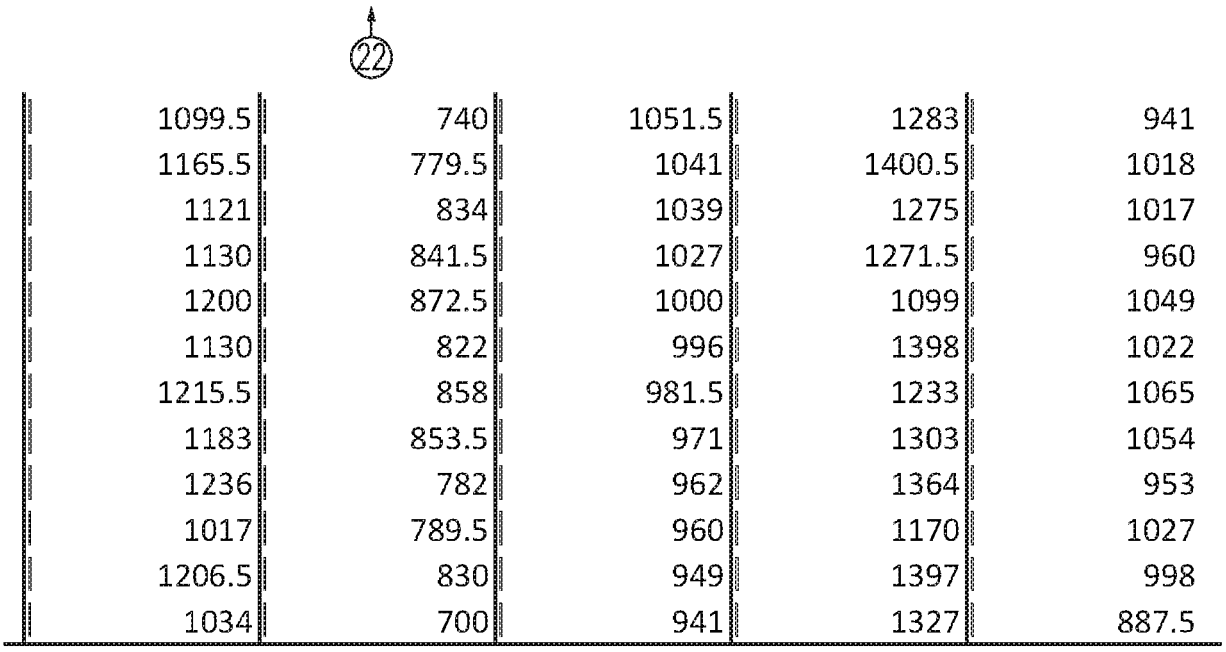


FIG. 14 (Continued)

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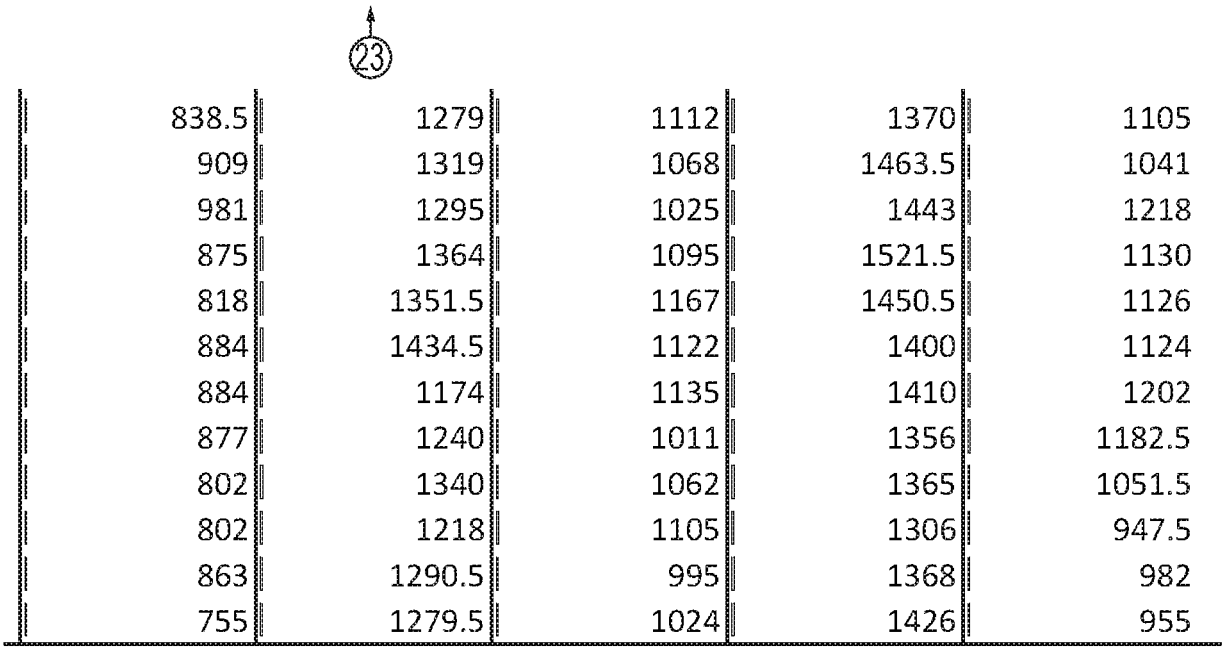


FIG. 14 (Continued)

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876.5	1962	1198	1421
1019	1933	1141	1311
866	1847	1114	1318.5
874	2027	960	1412.5
896	2000	1071	1280
971	2075.5	1180.5	1404
902.5	1981	950	1360.5
851	1965	969	1304
946	1927	1159	1287
912	1868	1024	1273
896.5	1979	1166	1443
787.5	1904.5	1062.5	1261.5



FIG. 14 (Continued)

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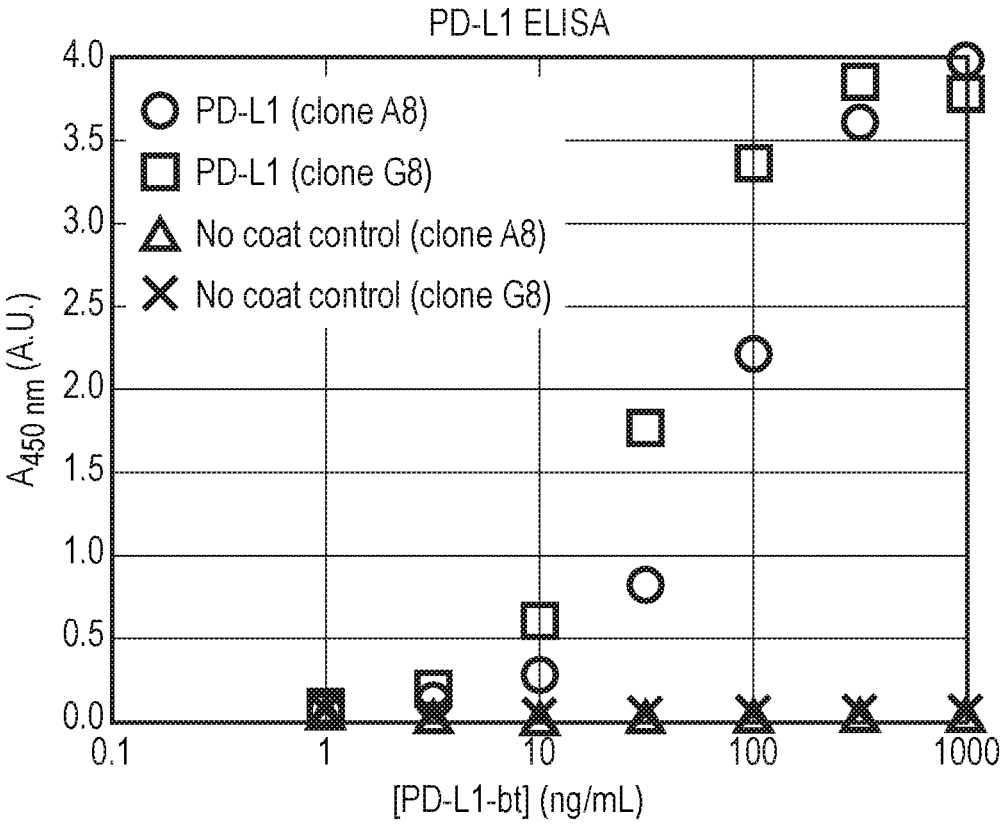


FIG. 15

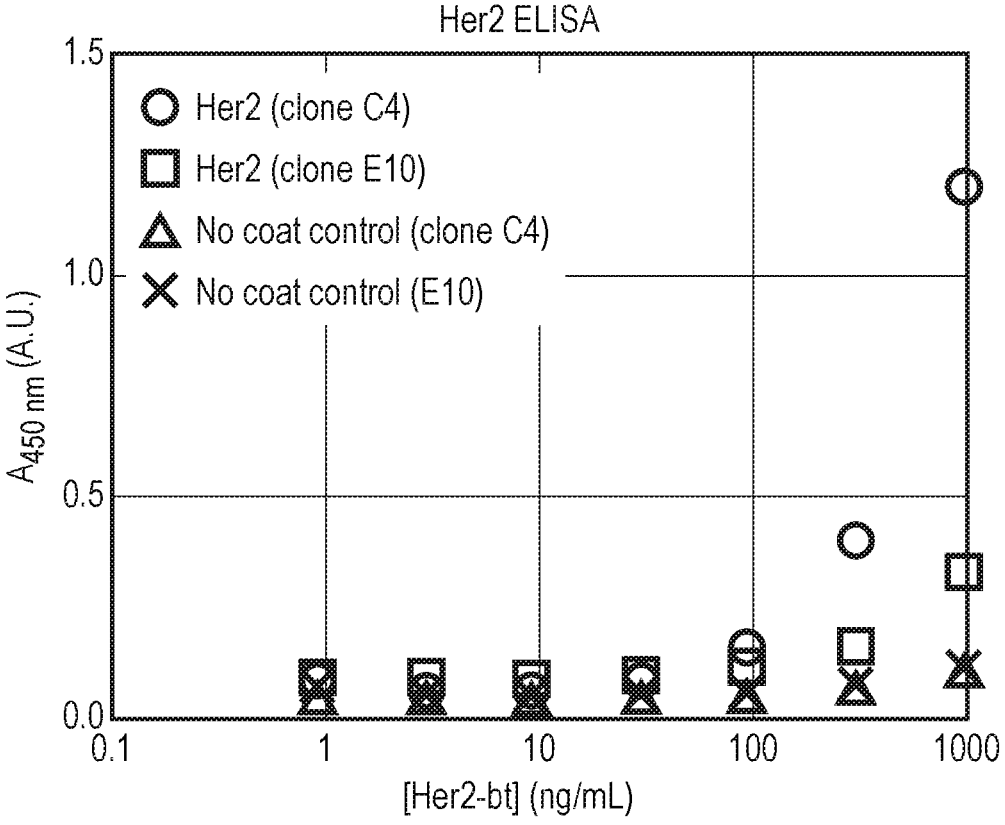


FIG. 16

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Affimer Working ID	Canine PDL_1 (FL1-A of QSH02)	Human VEGFR2 (FL1-A of QSH04)
00492_655534	487686	1046.5
00492_655606	300170	968
00492_655478	265975	973
00492_655610	198066	1053
00492_655494	192523.5	1148
00492_655614	185154.5	1134.5
00492_655480	174787	1126.5
00492_655573	157161	962.5
00492_655572	156490.5	1171
00492_655611	150997.5	1025
00492_655638	139829	1028.5
00492_655648	133745	963
00492_655481	116414.5	1016.5
00492_655608	114909	949
00492_655505	109482.5	1066.5
00492_655533	107343	1116
00492_655652	106006	1052
00492_655495	105968	882
00492_655472	100676	979
00492_655585	100161	1178
00492_655521	99513.5	896.5
00492_655468	99499	1070
00492_655552	99086.5	984
00492_655471	98638	961.5
00492_655519	92743	843

1

FIG. 17

A

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SMA(4A6)+BA-Peptide (FL1-A of QSH05)	Human FC (FL1-A of QSH10)	Canine PD1 (FL1-A of QSH18)	Amine Peg11 (FL1-A of QSH15)
1515	1362	1976	1057.5
1441	1235	1489	1087.5
1268	1569	1512	1031
1270	1458.5	1414	1163
1341.5	1284	1467.5	966.5
1489	1330	1602	1180
1248	1364	1371	1173
1132	1338	1404	1112
1558	1510	1332	1105.5
1343	1202.5	1261	914
1502.5	1299	1623	970.5
1310	1254	1448	976
1190	1149	1241	934
1334	1346.5	1406	1055
1278	1245	1357	1086
1275	1252	1278	920
1196	1205	1178	858
1254	1178.5	1427	1031.5
1297	1301.5	1319	928.5
1585.5	1233	1291.5	1140
1262	1197	1186	935
1308	1324	1259.5	999
1224	1232	1168	967.5
1389.5	1462.5	1245	1066.5
1245	1179	1346	1148

A 2 B

FIG. 17 (Continued)

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mIgG2b (Control) (FL1-A of QSH21)	No Target (Control) (FL1-A of QSH26)	Fumonisin B1 (FL1-A of QSH29)	SMA (2A9) + BA-Peptide (FL1-A of QSH22)
2661	2272	1297	2172
2557.5	2138	1283	2493
2674	2396	1466	2397.5
2872.5	2102	1257	1679
2447	2272	1182.5	2143
2378	2214	1250.5	1848
2598	2221.5	1300.5	1982
2609	2267	2566	2648
2811	2219.5	1763	2302
2345	1946.5	1558.5	2286
2722.5	2202.5	1674	2299.5
2672	2157	1741.5	2216.5
2397.5	2214	1271	1734
2642	2091	1561	1972
2429	2129	1295	2071
2432	2019	1349	1992
2302	2075	1173.5	2039.5
2234	2034	1047	1874
2647	2243	1255.5	2030
2459.5	2375	2440	2723.5
2497	2138	1315.5	1929
2589.5	2040.5	1406	1850
2214.5	2029	1233	1693
2359	1979	1089	2259
2217	2110	1311.5	1739

←B ③

FIG. 17 (Continued)

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①

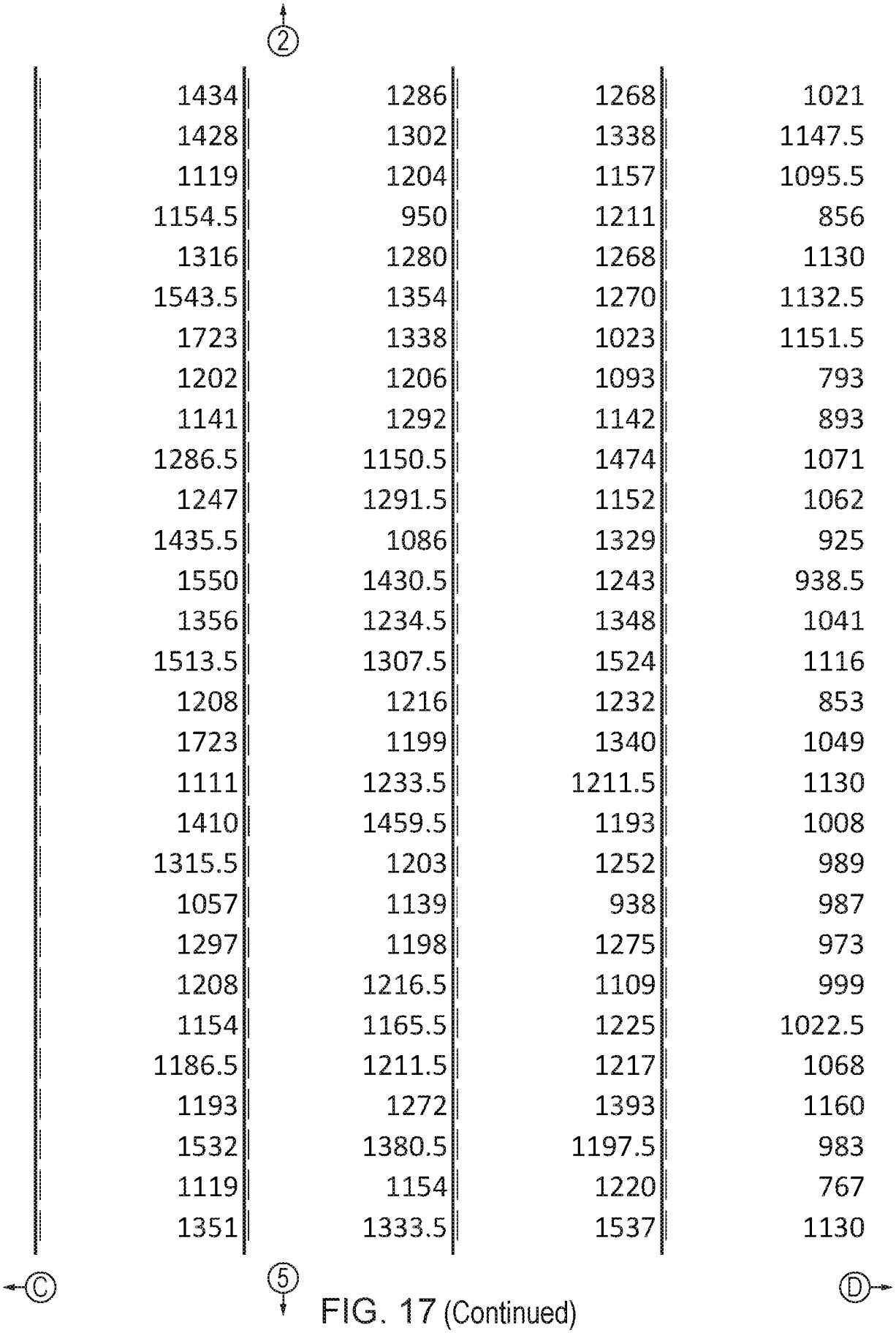
00492_655541	89444	1081.5
00492_655601	84042	1041.5
00492_655551	81090.5	1011
00492_655639	81068.5	941
00492_655465	80613.5	1054
00492_655477	77681.5	1065
00492_655520	75087	1121
00492_655591	75010	958
00492_655627	71011.5	1057
00492_655605	67170	1096
00492_655654	65882	971.5
00492_655563	60901	1081
00492_655574	56010.5	1122
00492_655562	54184.5	1021.5
00492_655625	53675	1036
00492_655612	52102	1092
00492_655488	51106.5	947
00492_655583	50260.5	965.5
00492_655530	50008.5	1019
00492_655514	49577	1040
00492_655650	48880	1038
00492_655482	48521	994
00492_655643	46995.5	1035
00492_655511	44026	951.5
00492_655518	43791	1001
00492_655576	42227	1021
00492_655641	38459	1173
00492_655628	37765	918.5
00492_655607	36757.5	985

④

Ⓒ

FIG. 17 (Continued)

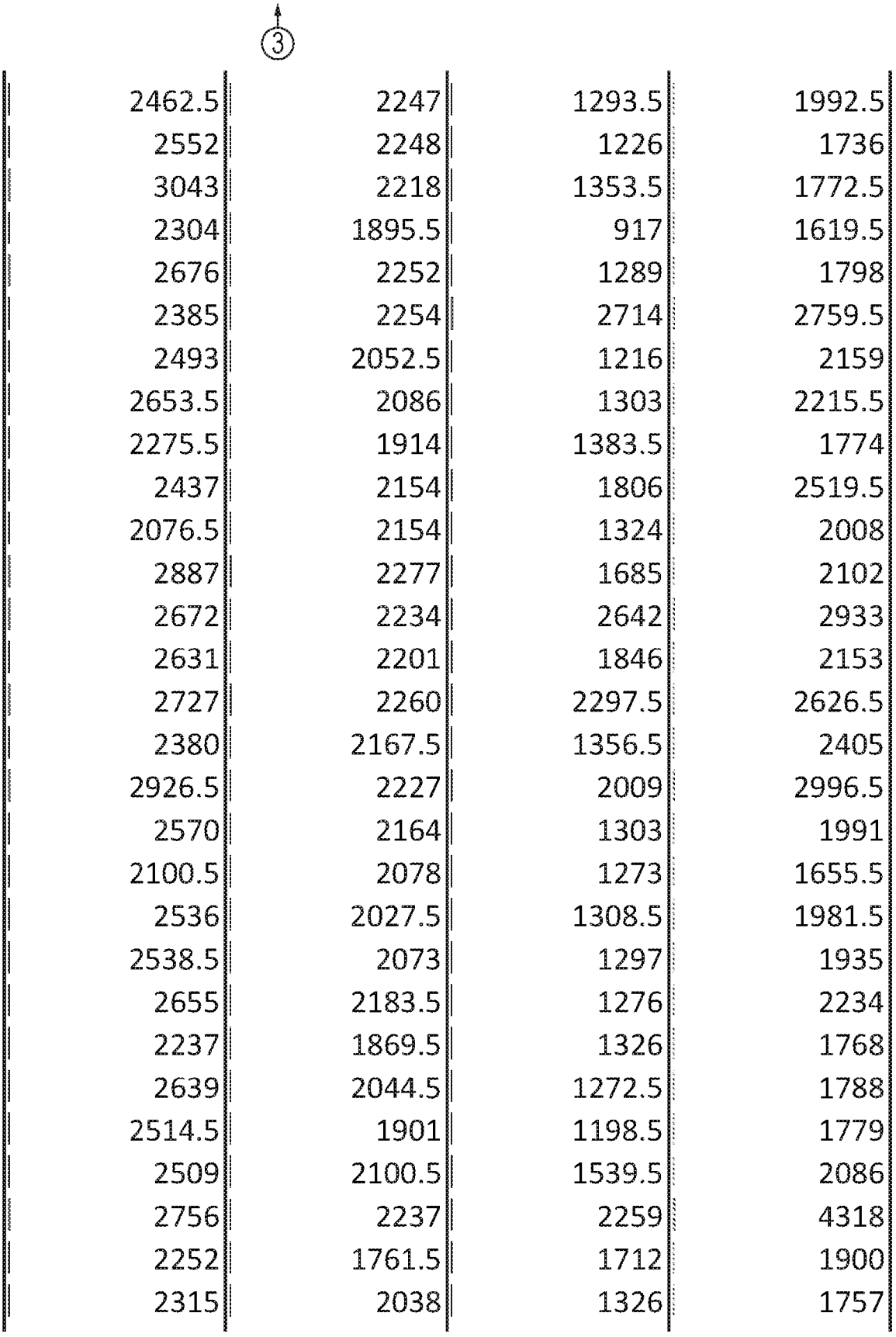
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1434	1286	1268	1021
1428	1302	1338	1147.5
1119	1204	1157	1095.5
1154.5	950	1211	856
1316	1280	1268	1130
1543.5	1354	1270	1132.5
1723	1338	1023	1151.5
1202	1206	1093	793
1141	1292	1142	893
1286.5	1150.5	1474	1071
1247	1291.5	1152	1062
1435.5	1086	1329	925
1550	1430.5	1243	938.5
1356	1234.5	1348	1041
1513.5	1307.5	1524	1116
1208	1216	1232	853
1723	1199	1340	1049
1111	1233.5	1211.5	1130
1410	1459.5	1193	1008
1315.5	1203	1252	989
1057	1139	938	987
1297	1198	1275	973
1208	1216.5	1109	999
1154	1165.5	1225	1022.5
1186.5	1211.5	1217	1068
1193	1272	1393	1160
1532	1380.5	1197.5	983
1119	1154	1220	767
1351	1333.5	1537	1130

FIG. 17 (Continued)

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2462.5	2247	1293.5	1992.5
2552	2248	1226	1736
3043	2218	1353.5	1772.5
2304	1895.5	917	1619.5
2676	2252	1289	1798
2385	2254	2714	2759.5
2493	2052.5	1216	2159
2653.5	2086	1303	2215.5
2275.5	1914	1383.5	1774
2437	2154	1806	2519.5
2076.5	2154	1324	2008
2887	2277	1685	2102
2672	2234	2642	2933
2631	2201	1846	2153
2727	2260	2297.5	2626.5
2380	2167.5	1356.5	2405
2926.5	2227	2009	2996.5
2570	2164	1303	1991
2100.5	2078	1273	1655.5
2536	2027.5	1308.5	1981.5
2538.5	2073	1297	1935
2655	2183.5	1276	2234
2237	1869.5	1326	1768
2639	2044.5	1272.5	1788
2514.5	1901	1198.5	1779
2509	2100.5	1539.5	2086
2756	2237	2259	4318
2252	1761.5	1712	1900
2315	2038	1326	1757

FIG. 17 (Continued)

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④

00492_655529	32520.5	1035
00492_655567	31602	1066
00492_655528	29826	1039
00492_655557	27151	981.5
00492_655564	25662	877
00492_655582	25377	992
00492_655613	24028.5	1218
00492_655485	21284.5	928
00492_655501	20252	1077
00492_655634	20036	1079
00492_655543	19493	1022
00492_655587	19437	975
00492_655577	18999.5	1113
00492_655633	16266	1014
00492_655594	15210.5	1010
00492_655542	12009	1021
00492_655655	10450	1018
00492_655609	8522	1073.5
00492_655490	7667	1136
00492_655584	6709	1021.5
00492_655487	4646	960

Ⓔ

FIG. 17 (Continued)

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1151.5	1206	1454	1136.5
1015.5	1183	1250.5	1185.5
1285	1178	1510	1095
1321	1189	1170	923
1114	1130	1400	1000.5
1174.5	1153	1199	957
1566	1448	1323	1116
1380	1170	1175	1052
1206	1145.5	1204.5	1046
2175.5	1472	1252.5	1168
1702.5	1305.5	1319	1008
1089	1137	1065	834
1335	1273	1446	1065
1380.5	1260	1161	1141
1133	1304.5	1119	1029
1646.5	1493	1329	892.5
1211	1190	1192.5	1018
1319	1338.5	1289	1057
1218	1204	1253	938.5
1482	1484.5	1316	989
1571	1163	1158.5	912





FIG. 17 (Continued)

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⑥

2536	2199.5	1440.5	2305
2299	2203	1276	1742.5
2622.5	2121.5	1349	1942
2292	1892	1251	1724
2399	1935.5	1214	1623
2510	1922	1194	2507.5
2504	2278	1285	2286
2386	2078.5	1213	2206.5
2456	2211	1309.5	1639
2509	2216	3030.5	4665
2343	2054	2326	2610
2431	2052	1077.5	1520
2680.5	2226	2042	2366
2951	1986	2174	2346
2597	2017	1805.5	2535.5
2466	2195.5	1311.5	2585
2647	2038	1515	2277
2525	2159	1426.5	2049
2763	2213	1385.5	2079
2540	2195	2603.5	2525
2677.5	1989.5	1224	2006

Ⓕ

FIG. 17 (Continued)

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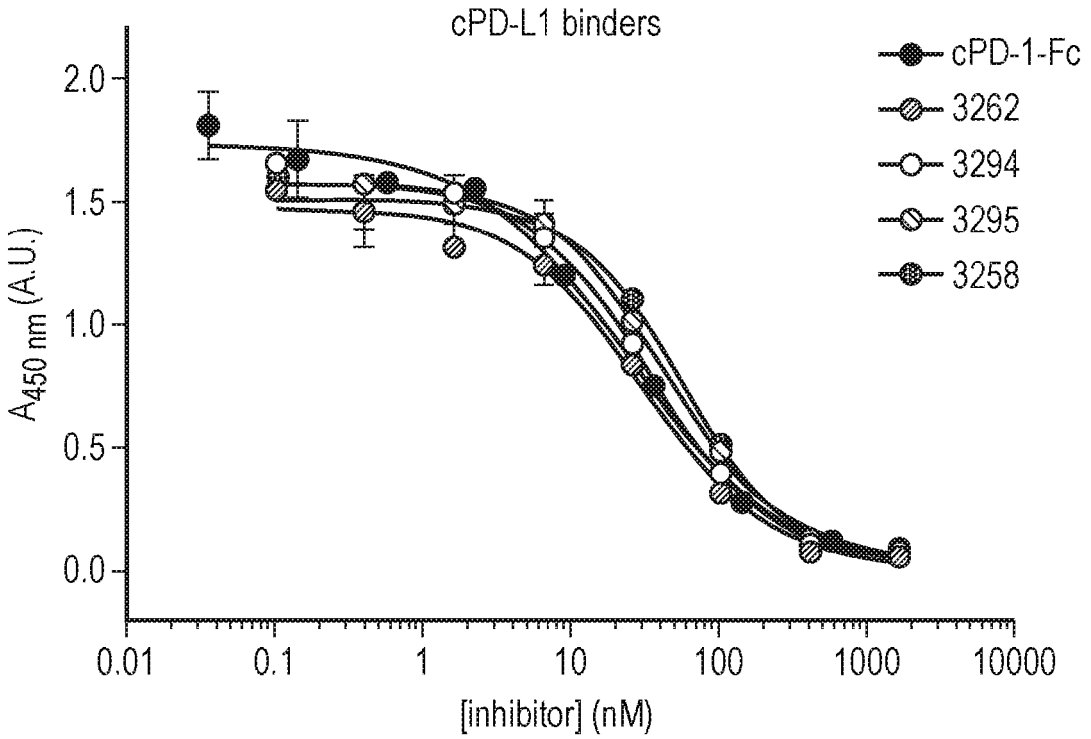


FIG. 18

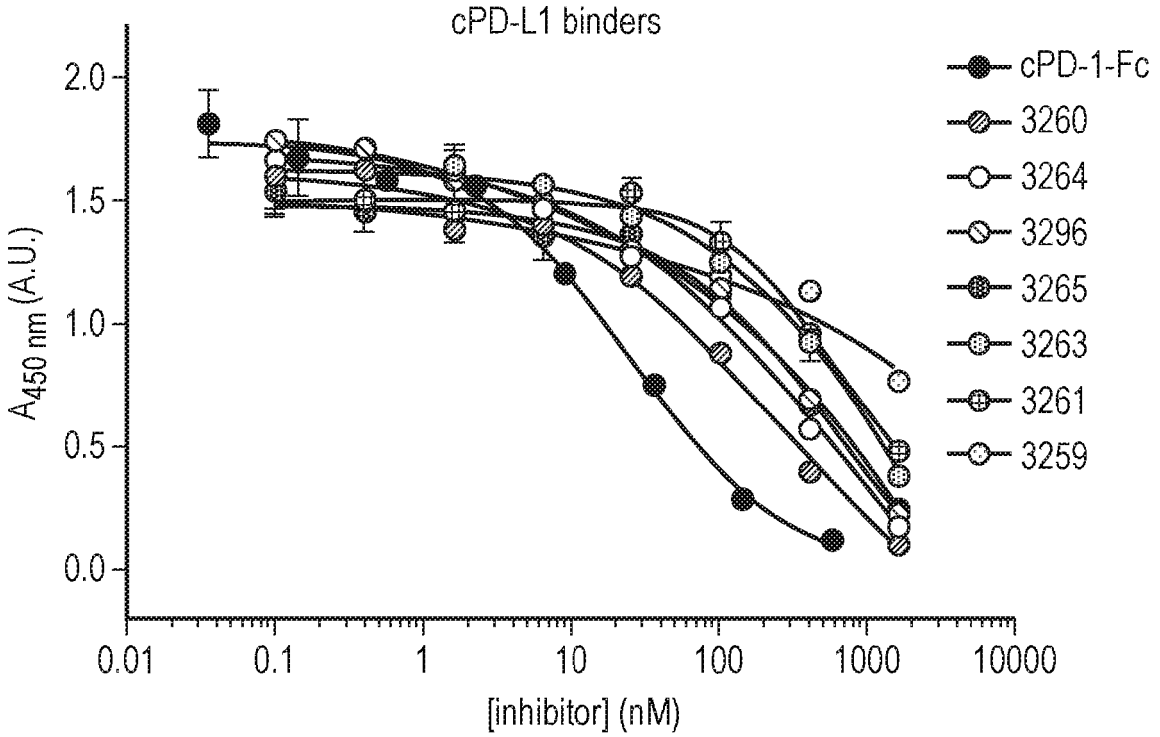


FIG. 19

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Sequence Listing

1	**Sequence Listing Information**	
1-1	File Name	P1924AU01-SEQ.xml
1-2	DTD Version	V1_3
1-3	Software Name	WIPO Sequence
1-4	Software Version	2.3.0
1-5	Production Date	2023-09-22
1-6	Original free text language code	
1-7	Non English free text language code	
2	**General Information**	
2-1	Current application: IP Office	AU
2-2	Current application: Application number	2023208125
2-3	Current application: Filing date	2023-07-26
2-4	Current application: Applicant file reference	P1924AU01
2-5	Earliest priority application: IP Office	GB
2-6	Earliest priority application: Application number	1710973.7
2-7	Earliest priority application: Filing date	2017-07-07
2-8en	Applicant name	Avacta Life Sciences Limited
2-8	Applicant name: Name Latin	
2-9en	Inventor name	
2-9	Inventor name: Name Latin	
2-10en	Invention title	SCAFFOLD PROTEINS
2-11	Sequence Total Quantity	107

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3-1	**Sequences**	
3-1-1	Sequence Number [ID]	1
3-1-2	Molecule Type	AA
3-1-3	Length	98
3-1-4	Features	**source 1..98**
	Location/Qualifiers	mol_type=protein
		organism=Homo sapiens
	NonEnglishQualifier Value	
3-1-5	Residues	MIPGGLSEAK PATPEIQEIV DKVKPQLEEK TNETYGKLEA VQYKTQVVAG TNYYIKVRAG 60 DNKYMHLKVF KSLPGQNEDL VLTGYQVDKN KDDELTGF 98
3-2	**Sequences**	
3-2-1	Sequence Number [ID]	2
3-2-2	Molecule Type	DNA
3-2-3	Length	294
3-2-4	Features	**misc_feature 1..294**
	Location/Qualifiers	note=codon optimised nucleic acid encoding SEQ ID NO:1
		source 1..294
		mol_type=other DNA
		organism=synthetic construct
	NonEnglishQualifier Value	
3-2-5	Residues	atgattcctg gtggtttgtc ggaagccaaa ccggctactc cggaaatcca ggagattgtg 60 gacaaagtca aaccgcaact ggaggaaaag accaatgaaa cctatggcaa actcgaagcg 120 gtacagtaca aaacccaagt cgttgcgggt acgaactact acatcaaagt acgcgcagga 180 gataacaagt atatgcatct gaaagtgttc aaaagcttac cagggcagaa tgaggatctg 240 gttcttacgg gctatcaggt ggataagaac aaagacgatg aactgacagg cttt 294
3-3	**Sequences**	
3-3-1	Sequence Number [ID]	3
3-3-2	Molecule Type	AA
3-3-3	Length	98
3-3-4	Features	**source 1..98**
	Location/Qualifiers	mol_type=protein
		organism=Canis familiaris
	NonEnglishQualifier Value	
3-3-5	Residues	MMPGGLTEAK PATPEVQEIA NEVKPQLEEK TNETYQEFEA VEYKTQVVAG INYYIKVRVG 60 DNSYIHLKIF KGLPGQNPTL TLTGYQTDKS KDDELTGF 98
3-4	**Sequences**	
3-4-1	Sequence Number [ID]	4
3-4-2	Molecule Type	AA
3-4-3	Length	227
3-4-4	Features	**REGION 1..227**
	Location/Qualifiers	note=hIgG1a_191
		source 1..227
		mol_type=protein
		organism=synthetic construct
	NonEnglishQualifier Value	
3-4-5	Residues	DKTHTCPPCP APELLGGPSV FLFPPKPKDT LMISRTPEVT CVVVDVSHED PEVKFNWYVD 60 GVEVHNAKTK PREEQYNSTY RVVSVLTVLH QDWLNGKEYK CKVSNKALPA PIEKTISKAK 120 GQPREPQVYT LPPSRDELTK NQVSLTCLVK GFYPSDIAVE WESNGQPENN YKTTPPVLDS 180 DGSFFLYSKL TVDKSRWQQG NVFSCSVMHE ALHNHYTQKS LSLSPGK 227
3-5	**Sequences**	
3-5-1	Sequence Number [ID]	5
3-5-2	Molecule Type	AA
3-5-3	Length	225
3-5-4	Features	**REGION 1..225**
	Location/Qualifiers	note=hIgG1a_189 [hIgG1a_191 sans "GK" on C term
		source 1..225
		mol_type=protein
		organism=synthetic construct
	NonEnglishQualifier Value	
3-5-5	Residues	DKTHTCPPCP APELLGGPSV FLFPPKPKDT LMISRTPEVT CVVVDVSHED PEVKFNWYVD 60 GVEVHNAKTK PREEQYNSTY RVVSVLTVLH QDWLNGKEYK CKVSNKALPA PIEKTISKAK 120 GQPREPQVYT LPPSRDELTK NQVSLTCLVK GFYPSDIAVE WESNGQPENN YKTTPPVLDS 180 DGSFFLYSKL TVDKSRWQQG NVFSCSVMHE ALHNHYTQKS LSLSP 225
3-6	**Sequences**	
3-6-1	Sequence Number [ID]	6
3-6-2	Molecule Type	AA
3-6-3	Length	227
3-6-4	Features	**REGION 1..227**
	Location/Qualifiers	note=hIgG1a_191b [A/F subtype]
		source 1..227

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		mol_type=protein
		organism=synthetic construct
	NonEnglishQualifier Value	
3-6-5	Residues	DKTHTCPPCP APELLGGPSV FLFPPKPKDT LMISRTPEVT CVVVDVSHED PEVKFNWYVD 60 GVEVHNAKTK PREEQYNSTY RVVSVLTVLH QDWLNGKEYK CKVSNKALPA PIEKTISKAK 120 GQPREPQVYT LPPSRDEMTK NQVSLTCLVK GFYPSDIAVE WESNGQPENN YKTTPPVLDS 180 DGSFFLYSKL TVDKSRWQQG NVFSCSVMHE ALHNHYTQKS LSLSPGK 227
3-7	**Sequences**	
3-7-1	Sequence Number [ID]	7
3-7-2	Molecule Type	AA
3-7-3	Length	227
3-7-4	Features	**REGION 1..227**
	Location/Qualifiers	note=hIgG1f_1.1_191 [Contains 5 point mutations to alter ADCC functio n, F subtype]
		source 1..227
		mol_type=protein
		organism=synthetic construct
	NonEnglishQualifier Value	
3-7-5	Residues	DKTHTCPPCP APEAEGAPSV FLFPPKPKDT LMISRTPEVT CVVVDVSHED PEVKFNWYVD 60 GVEVHNAKTK PREEQYNSTY RVVSVLTVLH QDWLNGKEYK CKVSNKALPS SIEKTISKAK 120 GQPREPQVYT LPPSREEMTK NQVSLTCLVK GFYPSDIAVE WESNGQPENN YKTTPPVLDS 180 DGSFFLYSKL TVDKSRWQQG NVFSCSVMHE ALHNHYTQKS LSLSPGK 227
3-8	**Sequences**	
3-8-1	Sequence Number [ID]	8
3-8-2	Molecule Type	AA
3-8-3	Length	232
3-8-4	Features	**REGION 1..232**
	Location/Qualifiers	note=hIgG1f_1.1_186 [Contains 5 point mutations to alter ADCC functio n and C225S
		source 1..232
		mol_type=protein
		organism=synthetic construct
	NonEnglishQualifier Value	
3-8-5	Residues	EPKSSDKTHT CPPCPAPEAE GAPSVFLFPP KPKDTLMISR TPEVTCVVVD VSHEDPEVKF 60 NWYVDGVEVH NAKTKPREEQ YNSTYRVVSV LTVLHQDWLN GKEYKCKVSN KALPSSIEKT 120 ISKAKGQPRE PQVYTLPPSR EEMTKNQVSL TCLVKGFYPS DIAVEWESNG QPENNYKTTP 180 PVLDSDGSFF LYSKLTVDKS RWQQGNVFSC SVMHEALHNH YTQKSLSLSP GK 232
3-9	**Sequences**	
3-9-1	Sequence Number [ID]	9
3-9-2	Molecule Type	AA
3-9-3	Length	227
3-9-4	Features	**REGION 1..227**
	Location/Qualifiers	note=hIgG1a_(N297G)_191
		source 1..227
		mol_type=protein
		organism=synthetic construct
	NonEnglishQualifier Value	
3-9-5	Residues	DKTHTCPPCP APELLGGPSV FLFPPKPKDT LMISRTPEVT CVVVDVSHED PEVKFNWYVD 60 GVEVHNAKTK PREEQYGSTY RVVSVLTVLH QDWLNGKEYK CKVSNKALPA PIEKTISKAK 120 GQPREPQVYT LPPSRDELTK NQVSLTCLVK GFYPSDIAVE WESNGQPENN YKTTPPVLDS 180 DGSFFLYSKL TVDKSRWQQG NVFSCSVMHE ALHNHYTQKS LSLSPGK 227
3-10	**Sequences**	
3-10-1	Sequence Number [ID]	10
3-10-2	Molecule Type	AA
3-10-3	Length	226
3-10-4	Features	**REGION 1..226**
	Location/Qualifiers	note=hIgG1a_190 [hIgG1a_190 sans "K" on C term; A subtype]
		source 1..226
		mol_type=protein
		organism=synthetic construct
	NonEnglishQualifier Value	
3-10-5	Residues	DKTHTCPPCP APELLGGPSV FLFPPKPKDT LMISRTPEVT CVVVDVSHED PEVKFNWYVD 60 GVEVHNAKTK PREEQYNSTY RVVSVLTVLH QDWLNGKEYK CKVSNKALPA PIEKTISKAK 120 GQPREPQVYT LPPSRDELTK NQVSLTCLVK GFYPSDIAVE WESNGQPENN YKTTPPVLDS 180 DGSFFLYSKL TVDKSRWQQG NVFSCSVMHE ALHNHYTQKS LSLSPG 226
3-11	**Sequences**	
3-11-1	Sequence Number [ID]	11
3-11-2	Molecule Type	AA
3-11-3	Length	227
3-11-4	Features	**REGION 1..227**
	Location/Qualifiers	note=hIgG1a_(N297Q)_191
		source 1..227

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		mol_type=protein
		organism=synthetic construct
	NonEnglishQualifier Value	
3-11-5	Residues	DKTHTCPPCP APELLGGPSV FLFPPKPKDT LMISRTPEVT CVVVDVSHED PEVKFNWYVD 60 GVEVHNAKTK PREEQYQSTY RVVSVLTVLH QDWLNGKEYK CKVSNKALPA PIEKTISKAK 120 GQPREPQVYT LPPSRDELTK NQVSLTCLVK GFYPSDIAVE WESNGQPENN YKTTPPVLDS 180 DGSFFLYSKL TVDKSRWQQG NVFSCSVMHE ALHNHYTQKS LSLSPGK 227
3-12	**Sequences**	
3-12-1	Sequence Number [ID]	12
3-12-2	Molecule Type	AA
3-12-3	Length	227
3-12-4	Features	**REGION 1..227**
	Location/Qualifiers	note=hIgG1a_(N297S)_191
		source 1..227
		mol_type=protein
		organism=synthetic construct
	NonEnglishQualifier Value	
3-12-5	Residues	DKTHTCPPCP APELLGGPSV FLFPPKPKDT LMISRTPEVT CVVVDVSHED PEVKFNWYVD 60 GVEVHNAKTK PREEQYSSTY RVVSVLTVLH QDWLNGKEYK CKVSNKALPA PIEKTISKAK 120 GQPREPQVYT LPPSRDELTK NQVSLTCLVK GFYPSDIAVE WESNGQPENN YKTTPPVLDS 180 DGSFFLYSKL TVDKSRWQQG NVFSCSVMHE ALHNHYTQKS LSLSPGK 227
3-13	**Sequences**	
3-13-1	Sequence Number [ID]	13
3-13-2	Molecule Type	AA
3-13-3	Length	227
3-13-4	Features	**REGION 1..227**
	Location/Qualifiers	note=hIgG1a_(N297A)_191
		source 1..227
		mol_type=protein
		organism=synthetic construct
	NonEnglishQualifier Value	
3-13-5	Residues	DKTHTCPPCP APELLGGPSV FLFPPKPKDT LMISRTPEVT CVVVDVSHED PEVKFNWYVD 60 GVEVHNAKTK PREEQYASTY RVVSVLTVLH QDWLNGKEYK CKVSNKALPA PIEKTISKAK 120 GQPREPQVYT LPPSRDELTK NQVSLTCLVK GFYPSDIAVE WESNGQPENN YKTTPPVLDS 180 DGSFFLYSKL TVDKSRWQQG NVFSCSVMHE ALHNHYTQKS LSLSPGK 227
3-14	**Sequences**	
3-14-1	Sequence Number [ID]	14
3-14-2	Molecule Type	AA
3-14-3	Length	227
3-14-4	Features	**REGION 1..227**
	Location/Qualifiers	note=hIgG1a_(N297H)_191
		source 1..227
		mol_type=protein
		organism=synthetic construct
	NonEnglishQualifier Value	
3-14-5	Residues	DKTHTCPPCP APELLGGPSV FLFPPKPKDT LMISRTPEVT CVVVDVSHED PEVKFNWYVD 60 GVEVHNAKTK PREEQYHSTY RVVSVLTVLH QDWLNGKEYK CKVSNKALPA PIEKTISKAK 120 GQPREPQVYT LPPSRDELTK NQVSLTCLVK GFYPSDIAVE WESNGQPENN YKTTPPVLDS 180 DGSFFLYSKL TVDKSRWQQG NVFSCSVMHE ALHNHYTQKS LSLSPGK 227
3-15	**Sequences**	
3-15-1	Sequence Number [ID]	15
3-15-2	Molecule Type	AA
3-15-3	Length	233
3-15-4	Features	**REGION 1..233**
	Location/Qualifiers	note=hIgG4
		source 1..233
		mol_type=protein
		organism=synthetic construct
	NonEnglishQualifier Value	
3-15-5	Residues	DKRVESKYGP PCPSCPAPEF LGGPSVFLFP PKPKDTLMIS RTPEVTCVVV DVSQEDPEVQ 60 FNWYVDGVEV HNAKTKPREE QFNSTYRVVS VLTVLHQDWL NGKEYKCKVS NKGLPSSIEK 120 TISKAKGQPR EPQVYTLPPS QEEMTKNQVS LTCLVKGFYP SDIAVEWESN GQPENNYKTT 180 PPVLDSDGSF FLYSRLTVDK SRWQEGNVFS CSVMHEALHN HYTQKSLSLS LGK 233
3-16	**Sequences**	
3-16-1	Sequence Number [ID]	16
3-16-2	Molecule Type	AA
3-16-3	Length	233
3-16-4	Features	**REGION 1..233**
	Location/Qualifiers	note=hIgG4_(S241P)
		source 1..233

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		mol_type=protein
		organism=synthetic construct
	NonEnglishQualifier Value	
3-16-5	Residues	DKRVESKYGP PCPPCPAPEF LGGPSVFLFP PKPKDTLMIS RTPEVTCVVV DVSQEDPEVQ 60 FNWYVDGVEV HNAKTKPREE QFNSTYRVVS VLTVLHQDWL NGKEYKCKVS NKGLPSSIEK 120 TISKAKGQPR EPQVYTLPPS QEEMTKNQVS LTCLVKGFYP SDIAVEWESN GQPENNYKTT 180 PPVLDSDGSF FLYSRLTVDK SRWQEGNVFS CSVMHEALHN HYTQKSLSLS LGK 233
3-17	**Sequences**	
3-17-1	Sequence Number [ID]	17
3-17-2	Molecule Type	AA
3-17-3	Length	585
3-17-4	Features	**source 1..585**
	Location/Qualifiers	mol_type=protein
		organism=Homo sapiens
	NonEnglishQualifier Value	
3-17-5	Residues	DAHKSEVAHR FKDLGEENFK ALVLIAFAQY LQQCPFEDHV KLVNEVTEFA KTCVADESAE 60 NCDKSLHTLF GDKLCTVATL RETYGEMADC CAKQEPERNE CFLQHKDDNP NLPRLVRPEV 120 DVMCTAFHDN EETFLKKYLY EIARRHPYFY APELLFFAKR YKAAFTECCQ AADKAACLLP 180 KLDELRDEGK ASSAKQRLKC ASLQKFGERA FKAWAVARLS QRFPKAEFAE VSKLVTDLTK 240 VHTECCHGDL LECADDRADL AKYICENQDS ISSKLKECCE KPLLEKSHCI AEVENDEMPA 300 DLPSLAADFV ESKDVCKNYA EAKDVFLGMF LYEYARRHPD YSVVLLLRLA KTYETTLEKC 360 CAAADPHECY AKVFDEFKPL VEEPQNLIKQ NCELFEQLGE YKFQNALLVR YTKKVPQVST 420 PTLVEVSRNL GKVGSKCCKH PEAKRMPCAE DYLSVVLNQL CVLHEKTPVS DRVTKCCTES 480 LVNRRPCFSA LEVDETYVPK EFNAETFTFH ADICTLSEKE RQIKKQTALV ELVKHKPKAT 540 KEQLKAVMDD FAAFVEKCCK ADDKETCFAE EGKKLVAASQ AALGL 585
3-18	**Sequences**	
3-18-1	Sequence Number [ID]	18
3-18-2	Molecule Type	AA
3-18-3	Length	96
3-18-4	Features	**REGION 1..96**
	Location/Qualifiers	note=3r1 with insertions after TQVD and FKSL
		source 1..96
		mol_type=protein
		organism=synthetic construct
	NonEnglishQualifier Value	
3-18-5	Residues	MIPGGLSEAK PATPEIQEIV DKVKPQLEKE TGKTWGKLEA VEYKTQVDXN GLNYYIKVRV 60 GNKYIHLKVF KSLXNEDLVL TGYQVDKNKD DELTGF 96
3-19	**Sequences**	
3-19-1	Sequence Number [ID]	19
3-19-2	Molecule Type	AA
3-19-3	Length	96
3-19-4	Features	**REGION 1..96**
	Location/Qualifiers	note=3r2 with insertions after TQVD and FKSL
		source 1..96
		mol_type=protein
		organism=synthetic construct
	NonEnglishQualifier Value	
3-19-5	Residues	MIPGGLSEAK PATPEIQEIV DKVKPQLEKE TGKTWGKLEA VEYKTQVDXN GLNYYIKVRV 60 NGKYIHLKVF KSLXNEDLVL TGYQVDKNKD DELTGF 96
3-20	**Sequences**	
3-20-1	Sequence Number [ID]	20
3-20-2	Molecule Type	AA
3-20-3	Length	97
3-20-4	Features	**REGION 1..97**
	Location/Qualifiers	note=3t1 with insertions after TQVD and FKSL
		source 1..97
		mol_type=protein
		organism=synthetic construct
	NonEnglishQualifier Value	
3-20-5	Residues	MIPGGLSEAK PATPEIQEIV DKVKPQLEEK TGETYGKLEA VQYKTQVDXN GTNYYIKVRA 60 GDNKYMHLKV FKSLXNEDLV LTGYQVDKNK DDELTGF 97
3-21	**Sequences**	
3-21-1	Sequence Number [ID]	21
3-21-2	Molecule Type	AA
3-21-3	Length	97
3-21-4	Features	**REGION 1..97**
	Location/Qualifiers	note=3t2 with insertions after TQVD and FKSL
		source 1..97
		mol_type=protein
		organism=synthetic construct

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	NonEnglishQualifier Value	
3-21-5	Residues	MIPGGLSEAK PATPEIQEIV DKVKPQLEEK TGETYGKLEA VQYKTQVDXN GTNYYIKVRA 60 GDNKYIHLKV FKSLXNEDLV LTGYQVDKNK DDELTGF 97
3-22	**Sequences**	
3-22-1	Sequence Number [ID]	22
3-22-2	Molecule Type	AA
3-22-3	Length	97
3-22-4	Features	**REGION 1..97**
	Location/Qualifiers	note=3t3 with insertions after TQVD and FKSL **source 1..97** mol_type=protein organism=synthetic construct
	NonEnglishQualifier Value	
3-22-5	Residues	MIPGGLSEAK PATPEIQEIV DKVKPQLEEK TGETYGKLEA VQYKTQVDXN GLNYYIKVRA 60 GDNKYIHLKV FKSLXNEDLV LTGYQVDKNK DDELTGF 97
3-23	**Sequences**	
3-23-1	Sequence Number [ID]	23
3-23-2	Molecule Type	AA
3-23-3	Length	97
3-23-4	Features	**REGION 1..97**
	Location/Qualifiers	note=3t4 with insertions after TQVD and FKSL **source 1..97** mol_type=protein organism=synthetic construct
	NonEnglishQualifier Value	
3-23-5	Residues	MIPGGLSEAK PATPEIQEIV DKVKPQLEEK TGETYGKLEA VEYKTQVDXN GTNYYIKVRA 60 GDNKYIHLKV FKSLXNEDLV LTGYQVDKNK DDELTGF 97
3-24	**Sequences**	
3-24-1	Sequence Number [ID]	24
3-24-2	Molecule Type	AA
3-24-3	Length	97
3-24-4	Features	**REGION 1..97**
	Location/Qualifiers	note=3t5 with insertions after TQVD and FKSL **source 1..97** mol_type=protein organism=synthetic construct
	NonEnglishQualifier Value	
3-24-5	Residues	MIPGGLSEAK PATPEIQEIV DKVKPQLEEK TGETYGKLEA VEYKTQVDXN GLNYYIKVRA 60 GDNKYIHLKV FKSLXNEDLV LTGYQVDKNK DDELTGF 97
3-25	**Sequences**	
3-25-1	Sequence Number [ID]	25
3-25-2	Molecule Type	AA
3-25-3	Length	18
3-25-4	Features	**REGION 1..18**
	Location/Qualifiers	note=signal peptide **source 1..18** mol_type=protein organism=synthetic construct
	NonEnglishQualifier Value	
3-25-5	Residues	MKWVTFISLL FLFSSAYS 18
3-26	**Sequences**	
3-26-1	Sequence Number [ID]	26
3-26-2	Molecule Type	AA
3-26-3	Length	22
3-26-4	Features	**REGION 1..22**
	Location/Qualifiers	note=signal peptide **source 1..22** mol_type=protein organism=synthetic construct
	NonEnglishQualifier Value	
3-26-5	Residues	MDMRAPAGIF GFLLVLFPGY RS 22
3-27	**Sequences**	
3-27-1	Sequence Number [ID]	27
3-27-2	Molecule Type	AA
3-27-3	Length	19
3-27-4	Features	**REGION 1..19**
	Location/Qualifiers	note=signal peptide **source 1..19** mol_type=protein

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		organism=synthetic construct	
	NonEnglishQualifier Value		
3-27-5	Residues	MTRLTVLALL AGLLASSRA	19
3-28	**Sequences**		
3-28-1	Sequence Number [ID]	28	
3-28-2	Molecule Type	AA	
3-28-3	Length	19	
3-28-4	Features	**REGION 1..19**	
	Location/Qualifiers	note=signal peptide	
		source 1..19	
		mol_type=protein	
		organism=synthetic construct	
	NonEnglishQualifier Value		
3-28-5	Residues	MELGLSWIFL LAILKGVQC	19
3-29	**Sequences**		
3-29-1	Sequence Number [ID]	29	
3-29-2	Molecule Type	AA	
3-29-3	Length	19	
3-29-4	Features	**REGION 1..19**	
	Location/Qualifiers	note=signal peptide	
		source 1..19	
		mol_type=protein	
		organism=synthetic construct	
	NonEnglishQualifier Value		
3-29-5	Residues	MELGLRWVFL VAILEGVQC	19
3-30	**Sequences**		
3-30-1	Sequence Number [ID]	30	
3-30-2	Molecule Type	AA	
3-30-3	Length	19	
3-30-4	Features	**REGION 1..19**	
	Location/Qualifiers	note=signal peptide	
		source 1..19	
		mol_type=protein	
		organism=synthetic construct	
	NonEnglishQualifier Value		
3-30-5	Residues	MKHLWFFLLL VAAPRWVLS	19
3-31	**Sequences**		
3-31-1	Sequence Number [ID]	31	
3-31-2	Molecule Type	AA	
3-31-3	Length	19	
3-31-4	Features	**REGION 1..19**	
	Location/Qualifiers	note=signal peptide	
		source 1..19	
		mol_type=protein	
		organism=synthetic construct	
	NonEnglishQualifier Value		
3-31-5	Residues	MDWTWRILFL VAAATGAHS	19
3-32	**Sequences**		
3-32-1	Sequence Number [ID]	32	
3-32-2	Molecule Type	AA	
3-32-3	Length	19	
3-32-4	Features	**REGION 1..19**	
	Location/Qualifiers	note=signal peptide	
		source 1..19	
		mol_type=protein	
		organism=synthetic construct	
	NonEnglishQualifier Value		
3-32-5	Residues	MDWTWRFLFV VAAATGVQS	19
3-33	**Sequences**		
3-33-1	Sequence Number [ID]	33	
3-33-2	Molecule Type	AA	
3-33-3	Length	19	
3-33-4	Features	**REGION 1..19**	
	Location/Qualifiers	note=signal peptide	
		source 1..19	
		mol_type=protein	
		organism=synthetic construct	
	NonEnglishQualifier Value		

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3-33-5	Residues	MEFGLSWLFL VAILKGVQC	19
3-34	**Sequences**		
3-34-1	Sequence Number [ID]	34	
3-34-2	Molecule Type	AA	
3-34-3	Length	19	
3-34-4	Features	**REGION 1..19**	
	Location/Qualifiers	note=signal peptide	
		source 1..19	
		mol_type=protein	
		organism=synthetic construct	
	NonEnglishQualifier Value		
3-34-5	Residues	MEFGLSWVFL VALFRGVQC	19
3-35	**Sequences**		
3-35-1	Sequence Number [ID]	35	
3-35-2	Molecule Type	AA	
3-35-3	Length	26	
3-35-4	Features	**REGION 1..26**	
	Location/Qualifiers	note=signal peptide	
		source 1..26	
		mol_type=protein	
		organism=synthetic construct	
	NonEnglishQualifier Value		
3-35-5	Residues	MDLLHKNMKH LWFFLLLVAA PRWVLS	26
3-36	**Sequences**		
3-36-1	Sequence Number [ID]	36	
3-36-2	Molecule Type	AA	
3-36-3	Length	22	
3-36-4	Features	**REGION 1..22**	
	Location/Qualifiers	note=signal peptide	
		source 1..22	
		mol_type=protein	
		organism=synthetic construct	
	NonEnglishQualifier Value		
3-36-5	Residues	MDMRVPAQLL GLLLLWLSGA RC	22
3-37	**Sequences**		
3-37-1	Sequence Number [ID]	37	
3-37-2	Molecule Type	AA	
3-37-3	Length	22	
3-37-4	Features	**REGION 1..22**	
	Location/Qualifiers	note=signal peptide	
		source 1..22	
		mol_type=protein	
		organism=synthetic construct	
	NonEnglishQualifier Value		
3-37-5	Residues	MKYLLPTAAA GLLLLAAQPA MA	22
3-38	**Sequences**		
3-38-1	Sequence Number [ID]	38	
3-38-2	Molecule Type	AA	
3-38-3	Length	17	
3-38-4	Features	**REGION 1..17**	
	Location/Qualifiers	note=signal peptide	
		source 1..17	
		mol_type=protein	
		organism=synthetic construct	
	NonEnglishQualifier Value		
3-38-5	Residues	MGVKVLFALI CIAVAEA	17
3-39	**Sequences**		
3-39-1	Sequence Number [ID]	39	
3-39-2	Molecule Type	AA	
3-39-3	Length	18	
3-39-4	Features	**REGION 1..18**	
	Location/Qualifiers	note=signal peptide	
		source 1..18	
		mol_type=protein	
		organism=synthetic construct	
	NonEnglishQualifier Value		
3-39-5	Residues	MKWVTFISLL FLFSSAYS	18
3-40	**Sequences**		

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3-40-1	Sequence Number [ID]	40	
3-40-2	Molecule Type	AA	
3-40-3	Length	18	
3-40-4	Features	**REGION 1..18**	
	Location/Qualifiers	note=signal peptide	
		source 1..18	
		mol_type=protein	
		organism=synthetic construct	
	NonEnglishQualifier Value		
3-40-5	Residues	MAFLWLLSCW ALLGTTFG	18
3-41	**Sequences**		
3-41-1	Sequence Number [ID]	41	
3-41-2	Molecule Type	AA	
3-41-3	Length	14	
3-41-4	Features	**REGION 1..14**	
	Location/Qualifiers	note=signal peptide	
		source 1..14	
		mol_type=protein	
		organism=synthetic construct	
	NonEnglishQualifier Value		
3-41-5	Residues	MQLLSCIALI LALV	14
3-42	**Sequences**		
3-42-1	Sequence Number [ID]	42	
3-42-2	Molecule Type	AA	
3-42-3	Length	15	
3-42-4	Features	**REGION 1..15**	
	Location/Qualifiers	note=signal peptide	
		source 1..15	
		mol_type=protein	
		organism=synthetic construct	
	NonEnglishQualifier Value		
3-42-5	Residues	MNLLLILTFV AAAVA	15
3-43	**Sequences**		
3-43-1	Sequence Number [ID]	43	
3-43-2	Molecule Type	AA	
3-43-3	Length	16	
3-43-4	Features	**REGION 1..16**	
	Location/Qualifiers	note=signal peptide	
		source 1..16	
		mol_type=protein	
		organism=synthetic construct	
	NonEnglishQualifier Value		
3-43-5	Residues	MPLLLLLPLL WAGALA	16
3-44	**Sequences**		
3-44-1	Sequence Number [ID]	44	
3-44-2	Molecule Type	AA	
3-44-3	Length	30	
3-44-4	Features	**REGION 1..30**	
	Location/Qualifiers	note=signal peptide	
		source 1..30	
		mol_type=protein	
		organism=synthetic construct	
	NonEnglishQualifier Value		
3-44-5	Residues	MDSKGSSQKG SRLLLLLVVS NLLLCQGVVS	30
3-45	**Sequences**		
3-45-1	Sequence Number [ID]	45	
3-45-2	Molecule Type	AA	
3-45-3	Length	23	
3-45-4	Features	**REGION 1..23**	
	Location/Qualifiers	note=signal peptide	
		source 1..23	
		mol_type=protein	
		organism=synthetic construct	
	NonEnglishQualifier Value		
3-45-5	Residues	MDAMKRGLCC VLLLCGAVFV SPS	23
3-46	**Sequences**		
3-46-1	Sequence Number [ID]	46	
3-46-2	Molecule Type	AA	

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3-46-3	Length	16	
3-46-4	Features	**REGION 1..16**	
	Location/Qualifiers	note=signal peptide	
		source 1..16	
		mol_type=protein	
		organism=synthetic construct	
	NonEnglishQualifier Value		
3-46-5	Residues	MLLLLLLLLL LALALA	16
3-47	**Sequences**		
3-47-1	Sequence Number [ID]	47	
3-47-2	Molecule Type	AA	
3-47-3	Length	21	
3-47-4	Features	**REGION 1..21**	
	Location/Qualifiers	note=signal peptide	
		source 1..21	
		mol_type=protein	
		organism=synthetic construct	
	NonEnglishQualifier Value		
3-47-5	Residues	MWWRLWWLLL LLLLLWPMVW A	21
3-48	**Sequences**		
3-48-1	Sequence Number [ID]	48	
3-48-2	Molecule Type	AA	
3-48-3	Length	5	
3-48-4	Features	**REGION 1..5**	
	Location/Qualifiers	note=linker	
		source 1..5	
		mol_type=protein	
		organism=synthetic construct	
	NonEnglishQualifier Value		
3-48-5	Residues	GGGGS	5
3-49	**Sequences**		
3-49-1	Sequence Number [ID]	49	
3-49-2	Molecule Type	AA	
3-49-3	Length	8	
3-49-4	Features	**REGION 1..8**	
	Location/Qualifiers	note=linker	
		source 1..8	
		mol_type=protein	
		organism=synthetic construct	
	NonEnglishQualifier Value		
3-49-5	Residues	GGGGGGGG	8
3-50	**Sequences**		
3-50-1	Sequence Number [ID]	50	
3-50-2	Molecule Type	AA	
3-50-3	Length	6	
3-50-4	Features	**REGION 1..6**	
	Location/Qualifiers	note=linker	
		source 1..6	
		mol_type=protein	
		organism=synthetic construct	
	NonEnglishQualifier Value		
3-50-5	Residues	GGGGGG	6
3-51	**Sequences**		
3-51-1	Sequence Number [ID]	51	
3-51-2	Molecule Type	AA	
3-51-3	Length	18	
3-51-4	Features	**REGION 1..18**	
	Location/Qualifiers	note=linker	
		source 1..18	
		mol_type=protein	
		organism=synthetic construct	
	NonEnglishQualifier Value		
3-51-5	Residues	KESGSVSSEQ LAQFRSLD	18
3-52	**Sequences**		
3-52-1	Sequence Number [ID]	52	
3-52-2	Molecule Type	AA	
3-52-3	Length	14	
3-52-4	Features	**REGION 1..14**	

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	Location/Qualifiers	note=linker	
		source 1..14	
		mol_type=protein	
		organism=synthetic construct	
	NonEnglishQualifier Value		
3-52-5	Residues	EGKSSGSGSE SKST	14
3-53	**Sequences**		
3-53-1	Sequence Number [ID]	53	
3-53-2	Molecule Type	AA	
3-53-3	Length	12	
3-53-4	Features	**REGION 1..12**	
	Location/Qualifiers	note=linker	
		source 1..12	
		mol_type=protein	
		organism=synthetic construct	
	NonEnglishQualifier Value		
3-53-5	Residues	GSAGSAAGSG EF	12
3-54	**Sequences**		
3-54-1	Sequence Number [ID]	54	
3-54-2	Molecule Type	AA	
3-54-3	Length	5	
3-54-4	Features	**REGION 1..5**	
	Location/Qualifiers	note=linker	
		source 1..5	
		mol_type=protein	
		organism=synthetic construct	
	NonEnglishQualifier Value		
3-54-5	Residues	EAAAK	5
3-55	**Sequences**		
3-55-1	Sequence Number [ID]	55	
3-55-2	Molecule Type	AA	
3-55-3	Length	46	
3-55-4	Features	**REGION 1..46**	
	Location/Qualifiers	note=linker	
		source 1..46	
		mol_type=protein	
		organism=synthetic construct	
	NonEnglishQualifier Value		
3-55-5	Residues	AEAAAKEAAA KEAAAKEAAA KALEAEAAAK EAAAKEAAAK EAAAKA	46
3-56	**Sequences**		
3-56-1	Sequence Number [ID]	56	
3-56-2	Molecule Type	AA	
3-56-3	Length	5	
3-56-4	Features	**REGION 1..5**	
	Location/Qualifiers	note=linker	
		source 1..5	
		mol_type=protein	
		organism=synthetic construct	
	NonEnglishQualifier Value		
3-56-5	Residues	PAPAP	5
3-57	**Sequences**		
3-57-1	Sequence Number [ID]	57	
3-57-2	Molecule Type	AA	
3-57-3	Length	12	
3-57-4	Features	**REGION 1..12**	
	Location/Qualifiers	note=linker	
		source 1..12	
		mol_type=protein	
		organism=synthetic construct	
	NonEnglishQualifier Value		
3-57-5	Residues	AEAAAKEAAA KA	12
3-58	**Sequences**		
3-58-1	Sequence Number [ID]	58	
3-58-2	Molecule Type	AA	
3-58-3	Length	10	
3-58-4	Features	**REGION 1..10**	
	Location/Qualifiers	note=linker	
		source 1..10	

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		mol_type=protein	
		organism=synthetic construct	
	NonEnglishQualifier Value		
3-58-5	Residues	APAPAPAPAP	10
3-59	**Sequences**		
3-59-1	Sequence Number [ID]	59	
3-59-2	Molecule Type	AA	
3-59-3	Length	4	
3-59-4	Features	**REGION 1..4**	
	Location/Qualifiers	note=linker	
		source 1..4	
		mol_type=protein	
		organism=synthetic construct	
	NonEnglishQualifier Value		
3-59-5	Residues	GGSG	4
3-60	**Sequences**		
3-60-1	Sequence Number [ID]	60	
3-60-2	Molecule Type	AA	
3-60-3	Length	4	
3-60-4	Features	**REGION 1..4**	
	Location/Qualifiers	note=linker	
		source 1..4	
		mol_type=protein	
		organism=synthetic construct	
	NonEnglishQualifier Value		
3-60-5	Residues	GSGS	4
3-61	**Sequences**		
3-61-1	Sequence Number [ID]	61	
3-61-2	Molecule Type	AA	
3-61-3	Length	4	
3-61-4	Features	**REGION 1..4**	
	Location/Qualifiers	note=linker	
		source 1..4	
		mol_type=protein	
		organism=synthetic construct	
	NonEnglishQualifier Value		
3-61-5	Residues	GGGS	4
3-62	**Sequences**		
3-62-1	Sequence Number [ID]	62	
3-62-2	Molecule Type	AA	
3-62-3	Length	4	
3-62-4	Features	**REGION 1..4**	
	Location/Qualifiers	note=linker	
		source 1..4	
		mol_type=protein	
		organism=synthetic construct	
	NonEnglishQualifier Value		
3-62-5	Residues	SGGS	4
3-63	**Sequences**		
3-63-1	Sequence Number [ID]	63	
3-63-2	Molecule Type	AA	
3-63-3	Length	7	
3-63-4	Features	**REGION 1..7**	
	Location/Qualifiers	note=linker	
		source 1..7	
		mol_type=protein	
		organism=synthetic construct	
	NonEnglishQualifier Value		
3-63-5	Residues	GGGSGGG	7
3-64	**Sequences**		
3-64-1	Sequence Number [ID]	64	
3-64-2	Molecule Type	AA	
3-64-3	Length	8	
3-64-4	Features	**REGION 1..8**	
	Location/Qualifiers	note=linker	
		source 1..8	
		mol_type=protein	
		organism=synthetic construct	

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	NonEnglishQualifier Value		
3-64-5	Residues	ESGGGGVT	8
3-65	**Sequences**		
3-65-1	Sequence Number [ID]	65	
3-65-2	Molecule Type	AA	
3-65-3	Length	9	
3-65-4	Features	**REGION 1..9**	
	Location/Qualifiers	note=linker	
		source 1..9	
		mol_type=protein	
		organism=synthetic construct	
	NonEnglishQualifier Value		
3-65-5	Residues	LESGGGGVT	9
3-66	**Sequences**		
3-66-1	Sequence Number [ID]	66	
3-66-2	Molecule Type	AA	
3-66-3	Length	6	
3-66-4	Features	**REGION 1..6**	
	Location/Qualifiers	note=linker	
		source 1..6	
		mol_type=protein	
		organism=synthetic construct	
	NonEnglishQualifier Value		
3-66-5	Residues	GRAQVT	6
3-67	**Sequences**		
3-67-1	Sequence Number [ID]	67	
3-67-2	Molecule Type	AA	
3-67-3	Length	6	
3-67-4	Features	**REGION 1..6**	
	Location/Qualifiers	note=linker	
		source 1..6	
		mol_type=protein	
		organism=synthetic construct	
	NonEnglishQualifier Value		
3-67-5	Residues	WRAQVT	6
3-68	**Sequences**		
3-68-1	Sequence Number [ID]	68	
3-68-2	Molecule Type	AA	
3-68-3	Length	8	
3-68-4	Features	**REGION 1..8**	
	Location/Qualifiers	note=linker	
		source 1..8	
		mol_type=protein	
		organism=synthetic construct	
	NonEnglishQualifier Value		
3-68-5	Residues	ARGRAQVT	8
3-69	**Sequences**		
3-69-1	Sequence Number [ID]	69	
3-69-2	Molecule Type	AA	
3-69-3	Length	33	
3-69-4	Features	**REGION 1..33**	
	Location/Qualifiers	note=GPI anchor domain	
		source 1..33	
		mol_type=protein	
		organism=synthetic construct	
	NonEnglishQualifier Value		
3-69-5	Residues	SGTTSGTTRL LSGHTCFTLT GLLGTLVTMG LLT	33
3-70	**Sequences**		
3-70-1	Sequence Number [ID]	70	
3-70-2	Molecule Type	AA	
3-70-3	Length	26	
3-70-4	Features	**REGION 1..26**	
	Location/Qualifiers	note=GPI anchor domain	
		source 1..26	
		mol_type=protein	
		organism=synthetic construct	
	NonEnglishQualifier Value		
3-70-5	Residues	SGTSPGLSAG ATVGIMIGVL VGVALI	26

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3-71	**Sequences**		
3-71-1	Sequence Number [ID]	71	
3-71-2	Molecule Type	AA	
3-71-3	Length	24	
3-71-4	Features	**REGION 1..24**	
	Location/Qualifiers	note=GPI anchor domain	
		source 1..24	
		mol_type=protein	
		organism=synthetic construct	
	NonEnglishQualifier Value		
3-71-5	Residues	SAPVLSAVAT VGITIGVLAR VALI	24
3-72	**Sequences**		
3-72-1	Sequence Number [ID]	72	
3-72-2	Molecule Type	AA	
3-72-3	Length	24	
3-72-4	Features	**REGION 1..24**	
	Location/Qualifiers	note=GPI anchor domain	
		source 1..24	
		mol_type=protein	
		organism=synthetic construct	
	NonEnglishQualifier Value		
3-72-5	Residues	SSPDLSAGTA VSIMIGVLAG MALI	24
3-73	**Sequences**		
3-73-1	Sequence Number [ID]	73	
3-73-2	Molecule Type	AA	
3-73-3	Length	25	
3-73-4	Features	**REGION 1..25**	
	Location/Qualifiers	note=GPI anchor domain	
		source 1..25	
		mol_type=protein	
		organism=synthetic construct	
	NonEnglishQualifier Value		
3-73-5	Residues	TLGGNSASYT FVSLLFSAVT LLLLC	25
3-74	**Sequences**		
3-74-1	Sequence Number [ID]	74	
3-74-2	Molecule Type	AA	
3-74-3	Length	26	
3-74-4	Features	**REGION 1..26**	
	Location/Qualifiers	note=GPI anchor domain	
		source 1..26	
		mol_type=protein	
		organism=synthetic construct	
	NonEnglishQualifier Value		
3-74-5	Residues	SGTSPGLSAG ATVGIMIGVL VGVALI	26
3-75	**Sequences**		
3-75-1	Sequence Number [ID]	75	
3-75-2	Molecule Type	AA	
3-75-3	Length	227	
3-75-4	Features	**REGION 1..227**	
	Location/Qualifiers	note=human IgG1 immunoglobulin Fc domain	
		source 1..227	
		mol_type=protein	
		organism=synthetic construct	
	NonEnglishQualifier Value		
3-75-5	Residues	DKTHTCPPCP APELLGGPSV FLFPPKPKDT LMISRTPEVT CVVVDVSHED PEVKFNWYVD	60
		GVEVHNAKTK PREEQYNSTY RVVSVLTVLH QDWLNGKEYK CKVSNKALPA PIEKTISKAK	120
		GQPREPQVYT LPPSRDELTK NQVSLTCLVK GFYPSDIAVE WESNGQPENN YKTTPPVLDS	180
		DGSFFLYSKL TVDKSRWQQG NVFSCSVMHE ALHNHYTQKS LSLSPGK	227
3-76	**Sequences**		
3-76-1	Sequence Number [ID]	76	
3-76-2	Molecule Type	AA	
3-76-3	Length	16	
3-76-4	Features	**REGION 1..16**	
	Location/Qualifiers	note=hinge	
		source 1..16	
		mol_type=protein	
		organism=synthetic construct	
	NonEnglishQualifier Value		

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3-76-5	Residues	DKTHTCPPCP APELLG	16
3-77	**Sequences**		
3-77-1	Sequence Number [ID]	77	
3-77-2	Molecule Type	AA	
3-77-3	Length	16	
3-77-4	Features	**REGION 1..16**	
	Location/Qualifiers	note=hinge	
		source 1..16	
		mol_type=protein	
		organism=synthetic construct	
	NonEnglishQualifier Value		
3-77-5	Residues	GSTHTCPPCP APELLG	16
3-78	**Sequences**		
3-78-1	Sequence Number [ID]	78	
3-78-2	Molecule Type	AA	
3-78-3	Length	21	
3-78-4	Features	**REGION 1..21**	
	Location/Qualifiers	note=hinge	
		source 1..21	
		mol_type=protein	
		organism=synthetic construct	
	NonEnglishQualifier Value		
3-78-5	Residues	EPKSCDKTHT CPPCPAPELL G	21
3-79	**Sequences**		
3-79-1	Sequence Number [ID]	79	
3-79-2	Molecule Type	AA	
3-79-3	Length	24	
3-79-4	Features	**REGION 1..24**	
	Location/Qualifiers	note=hinge	
		source 1..24	
		mol_type=protein	
		organism=synthetic construct	
	NonEnglishQualifier Value		
3-79-5	Residues	EPKSCDKTHT CPPCPAPELL GGPS	24
3-80	**Sequences**		
3-80-1	Sequence Number [ID]	80	
3-80-2	Molecule Type	AA	
3-80-3	Length	24	
3-80-4	Features	**REGION 1..24**	
	Location/Qualifiers	note=hinge	
		source 1..24	
		mol_type=protein	
		organism=synthetic construct	
	NonEnglishQualifier Value		
3-80-5	Residues	EPKSSDKTHT CPPCPAPELL GGPS	24
3-81	**Sequences**		
3-81-1	Sequence Number [ID]	81	
3-81-2	Molecule Type	AA	
3-81-3	Length	24	
3-81-4	Features	**REGION 1..24**	
	Location/Qualifiers	note=hinge	
		source 1..24	
		mol_type=protein	
		organism=synthetic construct	
	NonEnglishQualifier Value		
3-81-5	Residues	EPKSSDKTHT CPPCPAPELL GGSS	24
3-82	**Sequences**		
3-82-1	Sequence Number [ID]	82	
3-82-2	Molecule Type	AA	
3-82-3	Length	24	
3-82-4	Features	**REGION 1..24**	
	Location/Qualifiers	note=hinge	
		source 1..24	
		mol_type=protein	
		organism=synthetic construct	
	NonEnglishQualifier Value		
3-82-5	Residues	EPKSSGSTHT CPPCPAPELL GGSS	24
3-83	**Sequences**		

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3-83-1	Sequence Number [ID]	83	
3-83-2	Molecule Type	AA	
3-83-3	Length	19	
3-83-4	Features	**REGION 1..19**	
	Location/Qualifiers	note=hinge	
		source 1..19	
		mol_type=protein	
		organism=synthetic construct	
	NonEnglishQualifier Value		
3-83-5	Residues	DKTHTCPPCP APELLGGPS	19
3-84	**Sequences**		
3-84-1	Sequence Number [ID]	84	
3-84-2	Molecule Type	AA	
3-84-3	Length	19	
3-84-4	Features	**REGION 1..19**	
	Location/Qualifiers	note=hinge	
		source 1..19	
		mol_type=protein	
		organism=synthetic construct	
	NonEnglishQualifier Value		
3-84-5	Residues	DKTHTCPPCP APELLGGSS	19
3-85	**Sequences**		
3-85-1	Sequence Number [ID]	85	
3-85-2	Molecule Type	AA	
3-85-3	Length	24	
3-85-4	Features	**REGION 1..24**	
	Location/Qualifiers	note=hinge	
		source 1..24	
		mol_type=protein	
		organism=synthetic construct	
	NonEnglishQualifier Value		
3-85-5	Residues	EPKSSDKTHT CPPCPAPELL GGSS	24
3-86	**Sequences**		
3-86-1	Sequence Number [ID]	86	
3-86-2	Molecule Type	AA	
3-86-3	Length	24	
3-86-4	Features	**REGION 1..24**	
	Location/Qualifiers	note=hinge	
		source 1..24	
		mol_type=protein	
		organism=synthetic construct	
	NonEnglishQualifier Value		
3-86-5	Residues	EPKSSGSTHT CPPCPAPELL GGSS	24
3-87	**Sequences**		
3-87-1	Sequence Number [ID]	87	
3-87-2	Molecule Type	AA	
3-87-3	Length	19	
3-87-4	Features	**REGION 1..19**	
	Location/Qualifiers	note=hinge	
		source 1..19	
		mol_type=protein	
		organism=synthetic construct	
	NonEnglishQualifier Value		
3-87-5	Residues	DKTHTCPPCP APELLGGSS	19
3-88	**Sequences**		
3-88-1	Sequence Number [ID]	88	
3-88-2	Molecule Type	AA	
3-88-3	Length	24	
3-88-4	Features	**REGION 1..24**	
	Location/Qualifiers	note=mutant immunoglobulin	
		source 1..24	
		mol_type=protein	
		organism=synthetic construct	
	NonEnglishQualifier Value		
3-88-5	Residues	EPKSSGSTHT CPPCPAPELL GGSS	24
3-89	**Sequences**		
3-89-1	Sequence Number [ID]	89	
3-89-2	Molecule Type	AA	

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3-89-3	Length	24	
3-89-4	Features	**REGION 1..24**	
	Location/Qualifiers	note=mutant immunoglobulin	
		source 1..24	
		mol_type=protein	
		organism=synthetic construct	
	NonEnglishQualifier Value		
3-89-5	Residues	EPKSSDKTHT CPPCPAPELL GGPS	24
3-90	**Sequences**		
3-90-1	Sequence Number [ID]	90	
3-90-2	Molecule Type	AA	
3-90-3	Length	24	
3-90-4	Features	**REGION 1..24**	
	Location/Qualifiers	note=mutant immunoglobulin	
		source 1..24	
		mol_type=protein	
		organism=synthetic construct	
	NonEnglishQualifier Value		
3-90-5	Residues	EPKSSDKTHT CPPCPAPELL GGSS	24
3-91	**Sequences**		
3-91-1	Sequence Number [ID]	91	
3-91-2	Molecule Type	AA	
3-91-3	Length	24	
3-91-4	Features	**REGION 1..24**	
	Location/Qualifiers	note=mutant immunoglobulin	
		source 1..24	
		mol_type=protein	
		organism=synthetic construct	
	NonEnglishQualifier Value		
3-91-5	Residues	EPKSSGSTHT CPPCPAPELL GGSS	24
3-92	**Sequences**		
3-92-1	Sequence Number [ID]	92	
3-92-2	Molecule Type	AA	
3-92-3	Length	9	
3-92-4	Features	**REGION 1..9**	
	Location/Qualifiers	note=heterologous peptide insertion	
		source 1..9	
		mol_type=protein	
		organism=synthetic construct	
	NonEnglishQualifier Value		
3-92-5	Residues	GGSGGSGGS	9
3-93	**Sequences**		
3-93-1	Sequence Number [ID]	93	
3-93-2	Molecule Type	AA	
3-93-3	Length	97	
3-93-4	Features	**REGION 1..97**	
	Location/Qualifiers	note=cSteA3r2	
		source 1..97	
		mol_type=protein	
		organism=synthetic construct	
	NonEnglishQualifier Value		
3-93-5	Residues	MMPGGLTEAK PATPEVQEIA NEVKPQLEKE TGKTWQEFEA VEYKTQVDAG LNYYIKVRVN	60
		GKYIHLKIFK GLPGQNPTLT LTGYQTDKSK DDELTGF	97
3-94	**Sequences**		
3-94-1	Sequence Number [ID]	94	
3-94-2	Molecule Type	AA	
3-94-3	Length	110	
3-94-4	Features	**REGION 1..110**	
	Location/Qualifiers	note=cSteA3r2 with (GGS)3 heterologous peptide insertions	
		source 1..110	
		mol_type=protein	
		organism=synthetic construct	
	NonEnglishQualifier Value		
3-94-5	Residues	MMPGGLTEAK PATPEVQEIA NEVKPQLEKE TGKTWQEFEA VEYKTQVDGG SGGSGGSGLN	60
		YYIKVRVNGK YIHLKIFKGL GGSGGSGGSP TLTLTGYQTD KSKDDELTGF	110
3-95	**Sequences**		
3-95-1	Sequence Number [ID]	95	
3-95-2	Molecule Type	AA	

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3-95-3	Length	98
3-95-4	Features	**REGION 1..98**
	Location/Qualifiers	note=cSteAtA
		source 1..98
		mol_type=protein
		organism=synthetic construct
	NonEnglishQualifier Value	
3-95-5	Residues	MIPGGLTEAK PATPEVQEIA NEVKPQLEEK TGETYQEFEA VEYKTQVDAG INYYIKVRVG 60 DNSYIHLKIF KGLPGQNPTL TLTGYQTDKS KDDELTGF 98
3-96	**Sequences**	
3-96-1	Sequence Number [ID]	96
3-96-2	Molecule Type	AA
3-96-3	Length	98
3-96-4	Features	**REGION 1..98**
	Location/Qualifiers	note=cSteAtB
		source 1..98
		mol_type=protein
		organism=synthetic construct
	NonEnglishQualifier Value	
3-96-5	Residues	MIPGGLTEAK PATPEVQEIA NEVKPQLEEK TGETYQEFEA VEYKTQVDAG INYYIKVRVG 60 DNSYIHLKIF KSLPGQNEDL TLTGYQTDKS KDDELTGF 98
3-97	**Sequences**	
3-97-1	Sequence Number [ID]	97
3-97-2	Molecule Type	AA
3-97-3	Length	98
3-97-4	Features	**REGION 1..98**
	Location/Qualifiers	note=cSteAtC
		source 1..98
		mol_type=protein
		organism=synthetic construct
	NonEnglishQualifier Value	
3-97-5	Residues	MIPGGLTEAK PATPEVQEIV NEVKPQLEEK TGETYQELEA VEYKTQVDAG INYYIKVRVG 60 DNSYIHLKIF KGLPGQNPTL TLTGYQTDKS KDDELTGF 98
3-98	**Sequences**	
3-98-1	Sequence Number [ID]	98
3-98-2	Molecule Type	AA
3-98-3	Length	98
3-98-4	Features	**REGION 1..98**
	Location/Qualifiers	note=cSteAtD
		source 1..98
		mol_type=protein
		organism=synthetic construct
	NonEnglishQualifier Value	
3-98-5	Residues	MIPGGLTEAK PATPEVQEIV NEVKPQLEEK TGETYQELEA VEYKTQVDAG INYYIKVRVG 60 DNSYIHLKIF KSLPGQNEDL TLTGYQTDKS KDDELTGF 98
3-99	**Sequences**	
3-99-1	Sequence Number [ID]	99
3-99-2	Molecule Type	AA
3-99-3	Length	111
3-99-4	Features	**REGION 1..111**
	Location/Qualifiers	note=cSteAt1AL
		source 1..111
		mol_type=protein
		organism=synthetic construct
	NonEnglishQualifier Value	
3-99-5	Residues	MIPGGLTEAK PATPEVQEIA NEVKPQLEEK TGETYQEFEA VEYKTQVDGG SGGSGGSGIN 60 YYIKVRVGDN SYIHLKIFKG LGGSGGSGGS PTLTLTGYQT DKSKDDELTG F 111
3-100	**Sequences**	
3-100-1	Sequence Number [ID]	100
3-100-2	Molecule Type	AA
3-100-3	Length	111
3-100-4	Features	**REGION 1..111**
	Location/Qualifiers	note=cSteAt2BL
		source 1..111
		mol_type=protein
		organism=synthetic construct
	NonEnglishQualifier Value	
3-100-5	Residues	MIPGGLTEAK PATPEVQEIA NEVKPQLEEK TGETYQEFEA VEYKTQVDGG SGGSGGSGIN 60 YYIKVRVGDN SYIHLKIFKS LGGSGGSGGS EDLTLTGYQT DKSKDDELTG F 111

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3-101	**Sequences**	
3-101-1	Sequence Number [ID]	101
3-101-2	Molecule Type	AA
3-101-3	Length	111
3-101-4	Features	**REGION 1..111**
	Location/Qualifiers	note=cSteAt3CL
		source 1..111
		mol_type=protein
		organism=synthetic construct
	NonEnglishQualifier Value	
3-101-5	Residues	MIPGGLTEAK PATPEVQEIV NEVKPQLEEK TGETYQELEA VEYKTQVDGG SGGSGGSGIN 60 YYIKVRVGDN SYIHLKIFKG LGGSGGSGGS PTLTLTGYQT DKSKDDELTG F 111
3-102	**Sequences**	
3-102-1	Sequence Number [ID]	102
3-102-2	Molecule Type	AA
3-102-3	Length	111
3-102-4	Features	**REGION 1..111**
	Location/Qualifiers	note=cSteAt4DL
		source 1..111
		mol_type=protein
		organism=synthetic construct
	NonEnglishQualifier Value	
3-102-5	Residues	MIPGGLTEAK PATPEVQEIV NEVKPQLEEK TGETYQELEA VEYKTQVDGG SGGSGGSGIN 60 YYIKVRVGDN SYIHLKIFKS LGGSGGSGGS EDLTLTGYQT DKSKDDELTG F 111
3-103	**Sequences**	
3-103-1	Sequence Number [ID]	103
3-103-2	Molecule Type	AA
3-103-3	Length	97
3-103-4	Features	**REGION 1..97**
	Location/Qualifiers	note=cSteAr1
		source 1..97
		mol_type=protein
		organism=synthetic construct
	NonEnglishQualifier Value	
3-103-5	Residues	MMPGGLTEAK PATPEVQEIA NEVKPQLEKE TGKTWQEFEA VEYKTQVDAG LNYYIKVRVG 60 NKYIHLKIFK GLPGQNPTLT LTGYQTDKSK DDELTGF 97
3-104	**Sequences**	
3-104-1	Sequence Number [ID]	104
3-104-2	Molecule Type	AA
3-104-3	Length	7
3-104-4	Features	**REGION 1..7**
	Location/Qualifiers	note=tag
		source 1..7
		mol_type=protein
		organism=synthetic construct
	NonEnglishQualifier Value	
3-104-5	Residues	GHHHHHH 7
3-105	**Sequences**	
3-105-1	Sequence Number [ID]	105
3-105-2	Molecule Type	AA
3-105-3	Length	116
3-105-4	Features	**REGION 1..116**
	Location/Qualifiers	note=Mouse IgG2b G12 adhiron scaffold
		source 1..116
		mol_type=protein
		organism=synthetic construct
	NonEnglishQualifier Value	
3-105-5	Residues	MSAATGVRAV PGNENSLEIE ELARFAVDEH NKKENALLEF VRVVKAKEQX XXXXXXXXTM 60 YYLTLEAKDG GKKKLYEAKV WVKXXXXXXX XXNFKELQEF KPVGDAAAAH HHHHHG 116
3-106	**Sequences**	
3-106-1	Sequence Number [ID]	106
3-106-2	Molecule Type	AA
3-106-3	Length	98
3-106-4	Features	**REGION 1..98**
	Location/Qualifiers	note=canine Affimers with the mutations M2I, N32G and V48D from wildt ype cSteA
		source 1..98
		mol_type=protein
		organism=synthetic construct
	NonEnglishQualifier Value	

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3-106-5	Residues	MIPGGLTEAK PATPEVQEIA NEVKPQLEEK TGETYQEFEA VEYKTQVDAG INYYIKVRVG 60 DNSYIHLKIF KGLPGQNPTL TLTGYQTDKS KDDELTGF 98
3-107	**Sequences**	
3-107-1	Sequence Number [ID]	107
3-107-2	Molecule Type	AA
3-107-3	Length	106
3-107-4	Features	**REGION 1..106**
	Location/Qualifiers	note=Zika NS1 C12 based on the adhiron scaffold
		source 1..106
		mol_type=protein
		organism=synthetic construct
	NonEnglishQualifier Value	
3-107-5	Residues	MSAATGVRAV PGNENSLEIE ELARFAVDEH NKKENALLEF VRVVKAKEQX XXXXXXXXTM 60 YYLTLEAKDG GKKKLYEAKV WVKXXXXXXX XXNFKELQEF KPVGDG 106