PLASMID SYSTEM FOR MULTIGENE EXPRESSION

This application claims the benefit of U.S. Provisional Patent Application No. 60/519,230; filed November 12, 2003 which is herein incorporated by reference in its entirety.

FIELD OF THE INVENTION

A plasmid system which facilitates construction of a single amplifiable expression plasmid for multi-subunit proteins.

BACKGROUND OF THE INVENTION

Development of any mammalian cell based protein therapeutic requires an efficient expression system. Ideally, if a multi-subunit protein (*e.g.*, an antibody) must be produced, each polypeptide should be expressed from a single plasmid. Construction of expression vectors containing multiple genes, using commercially available expression plasmids, is problematic. Typically, the multiple cloning sites (MCS), of currently available expression plasmids, are inadequate for insertion of multiple expression cassettes. The multiple cloning sites of currently available expression plasmids contain relatively few restriction sites. Ideally, an expression plasmid for expression of multiple polypeptides would contain a large multiple cloning site containing many common and rare restriction sites.

The present invention provides, *inter alia*, an ideal generic plasmid expression system which can help maintain uniformity in vector construction, decrease variability in downstream processing, facilitate running multiple protein therapeutic projects simultaneously, and reduce cycle time significantly. The present invention includes such a generic plasmid platform for mammalian expression and its use for the production of various polypeptides. The platform is flexible enough to be used for expression of simple proteins, such as interferon, as well as large, complex, multi-subunit proteins, such as antibodies.

SUMMARY OF THE INVENTION

The present invention provides a plasmid system comprising in separate containers:

(a) a first universal transfer vector comprising the following, first multiple cloning site: Bss HII, Pme I, Sna B1, Hin dIII, Asp 718, Kpn I, Pae R71, Xho I, Sal I, Acc I, Hinc II, Cla I, Eco RV, Eco RI, Pst I, Eco O109I, Eco O109I, Apa I, Xma I, Bsp EI, Bam H1, Dsa I, Eag I, Ecl XI, Not I, Sac II, Xma III, Xba I, Sac I, Mlu I, Bcl I, Bsr GI, Bss HII; (b) a second universal transfer vector comprising the following, second multiple cloning site: Bss HII, Sgr AI, Xma I, Rsr II, Spe I, Sna B1, Hin dIII, Asp 718, Kpn I, Pae R71, Xho I, Sal I, Acc I, Hinc II, Cla I, Eco RV, Eco RI, Pst I, Eco O109I, Eco O109I, Apa I, Xma I, Bsp EI, Bam H1, Dsa I, Eag I, Ecl XI, Not I, Sac II, Xma III, Xba I, Sac I, Nde I, Msc I, Nru I, Pac I, Bss HII; and (c) an amplifiable vector comprising the following, third multiple cloning site:Sgr AI, Srf I, Xma I, Spe I, Sac II, Rsr II, Pac I, Nru I, Not I, Nde I, Msc I, Mlu I, Kpn I, Fse 1, Bss HII, Bsr GI, Bsp EI, Bcl I, Bbv C1, Pme I, Bss HII, Asc I, Xba I. In an embodiment of the invention, the plasmid system comprises: a first universal transfer vector comprising the plasmid map of Figure 2, a second universal transfer vector comprising the plasmid map of Figure 1 and an amplifiable vector comprising the plasmid map of Figure 3. In another embodiment, the multiple cloning site of the first universal transfer vector comprises the nucleotide sequence set forth in SEQ ID NO: 11; the multiple cloning site of the second universal transfer vector comprises the nucleotide sequence set forth in SEQ ID NO: 12; and the multiple cloning site of the amplifiable vector comprises the nucleotide sequence set forth in SEQ ID NO: 10. In an embodiment of the invention, any of the universal transfer vectors or the amplifiable vector comprises a matrix attachment region (MAR; *e.g.*, chicken lysozyme MAR).

In another embodiment of the invention, the plasmid system comprises only the first and second universal transfer vectors (*supra*).

In an embodiment of the invention, at least one of the plasmids comprises a promoter (*e.g.*, SRα promoter, MMTV LTR, human cytomegalovirus (hCMV) immediate early promoter and murine cytomegalovirus (mCMV) immediate early promoter) located upstream of or within the multiple cloning site. Preferably, in this embodiment, the first universal transfer vector comprises the plasmid map of Figure 10; the second universal transfer vector comprises the plasmid map of Figure 11; and the amplifiable vector comprises the plasmid map of Figure 9. In this embodiment, the first universal transfer vector can comprise the nucleotide sequence set forth in SEQ ID NO: 5; the second universal transfer vector comprise the nucleotide sequence set forth in SEQ ID NO: 4; and the amplifiable vector comprises the nucleotide sequence set forth in SEQ ID NO: 13.

Another embodiment of the present invention includes the plasmid system wherein at least one of the universal transfer vectors comprises a terminator/polyA addition site located in the multiple cloning site wherein the location of the terminator/polyA addition site is such that a gene located in the multiple cloning site would be operably linked to the terminator/polyA addition site.

The amplifiable vector in the plasmid system of the invention may comprise a selectable marker for amplification, such as the *DHFR* gene.

In an embodiment of the invention, the plasmid system of the present invention comprises in separate containers: (a) a first universal transfer vector comprising the nucleotide sequence set forth in SEQ ID NO: 2; (b) a second universal transfer vector comprising the nucleotide sequence set forth in SEQ ID NO: 1; and (c) an amplifiable vector comprising the nucleotide sequence set forth in SEQ ID NO: 3.

An embodiment of the invention includes a plasmid system wherein the first or second universal transfer vector comprises a first set of one or more expression cassettes, the other universal transfer vector comprise a second set of one or more expression cassettes and the amplifiable vector comprises said

first set and second set of expression cassettes; wherein the expression cassettes encode an immunoglobulin heavy chain and an immunogloblin light chain (*e.g.*, anti-IGFR1, anti-IL10 or anti-IL5 immunoglobulin chains); for example wherein (a) the first set of one or more expression cassettes comprises an anti-IL5 immunoglobulin heavy chain gene expression cassette and the second set of one or more expression cassettes comprises an anti-IL5 immunoglobulin light chain gene expression cassette; (b) the first set or one or more expression cassette comprises an anti-IGFR1 immunoglobulin heavy chain gene expression cassette and the second set of one or more expression cassette comprises an anti-IGFR1 immunoglobulin light chain gene expression cassette; (c) the first set of one or more expression cassettes comprises an expression cassette comprising a bicistronic gene expression cassette which bicistronic gene comprises an anti-IGFR1 immunoglobulin light chain gene and an IL2 receptor α gene wherein said genes are linked by an internal ribosome entry sequence (IRES) and the second set of one or more expression cassettes is an anti-IGFR1 immunoglobulin heavy chain gene expression cassette and a hygromycin resistance gene (*Hyg-b*) expression cassette; or (d) the first set of one or more expression cassettes comprises an anti-IL10 immunoglobulin heavy chain gene expression cassette and the second set of one or more expression cassettes comprises an anti-IL10 immunoglobulin light chain gene expression cassette and a hygromycin resistance gene expression cassette. In an embodiment of the invention, the amplifiable vector comprises a plasmid map as set forth in a figure selected from Figures 4-7. For example, the amplifiable vector can comprise a nucleotide sequence selected from SEQ ID NOs: 6-9.

In an embodiment of the present invention, the plasmid system includes the amplifiable vectors pinAIL10/MAR(-); pAIL10V1/puro/MAR(-); pAIGFRLCb2/MAR(-) or pAIGFRLCb2V1/puro/MAR(-). In an embodiment of the invention, the plasmids pinAIL10/MAR(-); pAIL10V1/puro/MAR(-); pAIGFRLCb2/MAR(-) and pAIGFRLCb2V1/puro/MAR(-) are characterized by

figures 13-16, respectively. In another embodiment of the invention, the plasmids pinAIL10/MAR(-); pAIL10V1/puro/MAR(-); pAIGFRLCb2/MAR(-) and pAIGFRLCb2V1/puro/MAR(-) comprise a nucleotide sequence selected from SEQ ID NOs: 24-27.

The present invention also provides a method for expressing a protein comprising two or more types of polypeptide comprising the steps of (a) introducing a set of one or more expression cassettes into a first universal transfer vector; (b) introducing one or more different expression cassettes into a second universal transfer vector; (c) moving the cassettes from the transfer vectors into an amplifiable vector; (d) causing expression of said cassettes; and (e) optionally, isolating/purifying the polypeptide; wherein said vectors are provided in a kit of the present invention. In one embodiment of the invention, the first universal transfer vector comprises the plasmid map of Figure 2 or Figure 10, or the nucleotide sequence set forth in SEQ ID NO: 2 or SEQ ID NO: 5. In another embodiment, the second universal transfer vector comprises the plasmid map of Figure 1 or Figure 11 or the nucleotide sequence set forth in SEQ ID NO: 1 or SEQ ID NO: 4. In another embodiment of the invention, the amplifiable vector comprises the plasmid map of Figure 3 or Figure 9 or the nucleotide sequence set forth in SEQ ID NO: 3 or SEQ ID NO: 13.

In an embodiment of the method, an anti-IGFR heavy chain or anti-IL10 heavy is expressed in an amplifiable vector, comprising a MAR and either the hygromycin resistance gene or the puromycin resistance gene, which selected from pinAIL10/MAR(-); pAIL10V1/puro/MAR(-); pAIGFRLCb2/MAR(-) and pAIGFRLCb2V1/puro/MAR(-). In an embodiment of the invention, the plasmids pinAIL10/MAR(-); pAIL10V1/puro/MAR(-); pAIGFRLCb2/MAR(-) and pAIGFRLCb2V1/puro/MAR(-) are characterized by figures 13-16, respectively. In another embodiment of the invention, the plasmids pinAIL10/MAR(-); pAIL10V1/puro/MAR(-); pAIGFRLCb2/MAR(-) and pAIGFRLCb2V1/puro/MAR(-) comprise a nucleotide sequence selected from SEQ ID NOs: 24-27.

In an embodiment of the method for expressing a protein comprising two or more types of polypeptide, the expression cassettes encode an immunoglobulin heavy or light chain (*e.g.*, anti-IGFR1, anti-IL5 or anti-IL10 immunoglobulin chain); for example: (i) one expression cassette encodes an anti-IL5 immunoglobulin heavy chain and the other expression cassette encodes an anti-IL5 immunoglobulin light chain; (ii) one expression cassette encodes an anti-IGFR1 immunoglobulin heavy chain (*e.g.*, SEQ ID NO: 17 or 21 or any polynucleotide encoding a polypeptide comprising the amino acid sequence of SEQ ID NO: 18 or 22) and the other expression cassette encodes an anti-IGFR1 immunoglobulin light chain (*e.g.*, SEQ ID NO: 15 or 19 or any polynucleotide encoding a polypeptide comprising the amino acid sequence of SEQ ID NO: 16 or 20); (iii) one expression cassette comprises a bicistronic gene encoding an anti-IGFR1 immunoglobulin light chain and an IL2 receptor α-subunit which are linked by an internal ribosome entry sequence (IRES) and the other expression cassette encodes an anti-IGFR1 immunoglobulin heavy chain and HYG-B; or (iv) one expression cassettes encodes an anti-IL10 immunoglobulin heavy chain and the other expression cassette encodes an anti-IL10 immunoglobulin light chain and HYG-B.

In an embodiment of the invention, the amplifiable vector comprises a plasmid map in a figure selected from Figures 4-7. The amplifiable vector may comprise a nucleotide sequence selected from SEQ ID NOs: 6-9.

The scope of the present invention also encompasses any product produced by any of the methods of the invention for producing a polypeptide (*e.g.*, any immunoglobulin chain, such as that of an anti-IGFR1, anti-IL5 or anti-IL10 antibody).

In an embodiment of the method for expressing a protein comprising two or more types of polypeptide, expression is caused in a cell (*e.g.*, a eukaryotic cell such as a CHO cell).

The present invention also comprises a method for producing an anti-IGFR1 antibody comprising the steps of (a) introducing an expression cassette comprising a polynucleotide encoding a polypeptide comprising an amino acid sequence selected from SEQ ID NOs: 18 and 22 or an expression cassette comprising a polynucleotide encoding a polypeptide comprising an amino acid sequence selected from SEQ ID NOs: 16 and 20 into a first universal transfer vector comprising the following, first multiple cloning site: Bss HII, Pme I, Sna B1, Hin dIII, Asp 718, Kpn I, Pae R71, Xho I, Sal I, Acc I, Hinc II, Cla I, Eco RV, Eco RI, Pst I, Eco O109I, Eco O109I, Apa I, Xma I, Bsp EI, Bam H1, Dsa I, Eag I, Ecl XI, Not I, Sac II, Xma III, Xba I, Sac I, Mlu I, Bcl I, Bsr GI, Bss HII (*e.g.*, pUHLS or PUHSRstopLS); (b) introducing the other expression cassette, not introduced into said first vector, into a second universal transfer vector comprising the following, second multiple cloning site: Bss HII, Sgr AI, Xma I, Rsr II, Spe I, Sna B1, Hin dIII, Asp 718, Kpn I, Pae R71, Xho I, Sal I, Acc I, Hinc II, Cla I, Eco RV, Eco RI, Pst I, Eco O109I, Eco O109I, Apa I, Xma I, Bsp EI, Bam H1, Dsa I, Eag I, Ecl XI, Not I, Sac II, Xma III, Xba I, Sac I, Nde I, Msc I, Nru I, Pac I, Bss HII (*e.g.*, pULLS or PULSRstopLS); (c) optionally, moving the cassettes from the transfer vectors into an amplifiable vector comprising the following, third multiple cloning site: Sgr AI, Srf I, Xma I, Spe I, Sac II, Rsr II, Pac I, Nru I, Not I, Nde I, Msc I, Mlu I, Kpn I, Fse 1, Bss HII, Bsr GI, Bsp EI, Bcl I, Bbv C1, Pme I, Bss HII, Asc I, Xba I (*e.g.*, pXBLS or pSRXBLS); (d) causing expression of said cassettes; and (e) optionally isolating/purifying the antibody. The polynucleotide encoding a polypeptide comprising an amino acid sequence selected from SEQ ID NOs: 18 and 22 can comprise a nucleotide sequence selected from SEQ ID NOs: 17 and 21. The polynucleotide encoding a polypeptide comprising an amino acid sequence selected from SEQ ID NOs: 16 and 20 can comprise a nucleotide sequence selected from SEQ ID NOs: 15 and 19. In one embodiment of the invention, the expression cassettes are operably linked to a human cytomegalovirus (*hCMV*) promoter. The scope of the present invention includes embodiments wherein the

expression cassettes mentioned above are linked to an immunoglobulin constant region such as that of any one of κ or γ1 or γ2 or γ3 or γ4.

The present invention also provides a kit comprising the plasmid system of the invention and one or more components selected from: (i) sterile, distilled water; (ii) calcium phosphate transformation reagents $CaCl_2$ and 2X HEPES buffered saline; (iii) DEAE-dextran transformation reagents chloroquine in Phosphate buffered saline and phosphate buffered saline; (iv) DOTAP/cholesterol extruded liposomes; (v) transformation competent *E.coli*; (vi) Dulbecco/Vogt modified Eagle's minimal essential medium (DMEM); (vii) Fetal calf serum; (viii) luria broth media; and (ix) paper instructions for usage of the plasmid system.

One embodiment of the present invention includes a single stranded or double stranded polynucleotide (*e.g.*, an oligonucleotide primer) comprising a nucleotide sequence of SEQ ID NO: 10, 11 or 12.

The present invention also includes a plasmid comprising a nucleotide sequence selected from the group consisting of SEQ ID NOs: 6-9.

BRIEF DESCRIPTION OF THE FIGURES

The scope of the present invention includes any plasmid or plasmid system containing a plasmid that comprises a plasmid map substantially identical to any of the following plasmid maps:

Figure 1. Plasmid map of universal transfer vector pULLS.

Amp: Start: 1955 End: 2812
Col E1 origin: Start: 1012 End: 1952
Multiple Cloning Site (MCS): Start: 620 End: 772
f1 (+) origin: Start: 3 End: 459

Figure 2. Plasmid map of universal transfer vector pUHLS.

Amp: Start: 1949 End: 2806
MCS: Start: 620 End: 766
f1 (+) origin: Start: 3 End: 459
Col E1 origin: Start: 1006 End: 1946

Figure 3. Plasmid map of amplifiable vector pXBLS.

SV40 T-antigen (t Ag) Intron: Start: 5431 End: 600
SV40 POLY A signal: Start: 5184 End: 5432
MCS: Start: 5037 End: 5183
Ampicillin resistance (Amp): Start: 3965 End: 4828
pBR ORI: Start: 3207 End: 3207
pBR322 sequences: Start: 3020 End: 5033
pBR322 sequences: Start: 2811 End: 3019
MMTV-LTR promoter: Start: 1348 End: 2810
DHFR cDNA: Start: 601 End: 1347.

Figure 4. Plasmid map of pAIL5V1.

The anti-IL-5 antibody heavy (VDJ-IgG4) and light (VDJ-IgK) chain expression cassettes, driven by the *CMV* promoter, are inserted into the multiple cloning site of pXBLS along with the hygromycin B expression cassette driven by the TK promoter (TK/Hyg).

SV40 t Ag Intron: Start: 12177 End: 600
SV40 POLYA signal: Start: 11930 End: 12178
CMV Promoter: Start: 11238 End: 11892
T 7 promoter/priming site: Start: 11219 End: 11238

VDJ (Anti-IL-5 light chain): Start: 10718 End: 11148
IGκ (Anti-IL-5 light chain): Start: 10382 End: 10717
Beta Globin Poly A signal: Start: 10126 End: 10374
TK/Hyg: Start: 8161 End: 10033
Beta Globin Poly A signal: Start: 7877 End: 8115
IGG4-CH3 (Anti-IL-5 antibody heavy chain): Start: 7517 End: 7834
IGG4-CH2 (Anti-IL-5 antibody heavy chain): Start: 7087 End: 7419
IGG4-HINGE (Anti-IL-5 antibody heavy chain): Start: 6933 End: 6968
IGG4-CH1 (Anti-IL-5 antibody heavy chain): Start: 6247 End: 6540
VDJ (Anti-IL-5 antibody heavy chain): Start: 5813 End: 6247
T 7 promoter/priming site: Start: 5723 End: 5742
CMV Promoter: Start: 5069 End: 5723
AP^r (Ampicillin resistance): Start: 3965 End: 4828
PBR ORI: Start: 3207 End: 3207
pBR322 sequences: Start: 3020 End: 5033
pBR322 sequences: Start: 2811 End: 3019
MMTV-LTR promoter: Start: 1348 End: 2810
DHFR cDNA: Start: 601 End: 1347

Figure 5. Plasmid map of pAIGFRV3.

The anti-IGFR1 antibody heavy (VDJ-IgG4) and light (VDJ-IgK) chain expression cassettes, driven by the *CMV* promoter, are inserted into the multiple cloning site of pXBLS along with the hygromycin B expression cassette driven by the TK promoter (TK-Hygromycin). The *DHFR* cDNA, along with its promoter (MMTV-LTR) for plasmid amplification and the hygromycin B coding sequence, along with its TK promoter for selection in mammalian cells, are shown.

AP(R): Start: 3965 End: 4828
IgG1 non genomic region: Start: 7234 End: 8214
VDJ of IGFR1 of 11D8 hybridoma: Start: 8214 End: 8641

DHFR cDNA: Start: 601 End: 1347
SV40 t Ag Intron: Start: 11603 End: 600
Kappa chain of hu-antiIGFR gene: Start: 9761 End: 10096
VDJ Domain of hu-anti IGFR gene for light chain: Start: 10097 End: 10477
pBR322 sequence: Start: 2811 End: 3019
pBR322 sequence: Start: 3020 End: 5033
TK-Hygromycin: Start: 5053 End: 6925
Beta Globin Poly A signal: Start: 6971 End: 7209
Beta globin pA signal: Start: 9505 End: 9753
SV40 POLYA: Start: 11356 End: 11604
MMTV-LTR: Start: 1348 End: 2810
CMV promoter: Start: 10664 End: 11318
T 7 promoter/priming site: Start: 8723 End: 8742
CMV promoter: Start: 8742 End: 9396
T 7 promoter/priming site: Start: 10645 End: 10664
pBR ORI: Start: 3207 End: 3207

Figure 6. Plasmid map of pAIG1FR(-)IL2LS.

This plasmid drives expression of the anti-IGFR1 antibody and the membrane domain of the IL2 receptor. Three independent expression cassettes containing four genes including heavy and light chain anti-IGFR1, truncated IL2 receptor and hygromycin B are incorporated into the multiple cloning site of pXBLS.

SV40 t Ag Intron: Start: 13066 End: 600
SV40 POLYA: Start: 12819 End: 13067
CMV: Start: 12115 End: 12769
T7 promoter/priming site: Start: 12096 End: 12115
VDJ (Anti-IGFR1 light chain): Start: 11548 End: 11928
Kappa (Kap; Anti-IGFR1 light chain): Start: 11212 End: 11547

IRES: Start: 10621 End: 11195

IL-2R alpha: Start: 9787 End: 10615

Beta Globin Poly A signal (β-globin pA)Start: 9505 End: 9753

CMV: Start: 8742 End: 9396

T7 promoter/priming site: Start: 8723 End: 8742

VDJ (Anti-IGFR1 heavy chain of 11D8 hybridoma): Start: 8214 End: 8641

IgG1(Anti-IGFR1 heavy chain of 11D8 hybridoma): Start: 7234 End: 8214

Beta Globin Poly A signal (b-globin pA): Start: 6971 End: 7209

TK-Hygromycin: Start: 5053 End: 6925

AP^r: Start: 3965 End: 4828

pBR ORI: Start: 3207 End: 3207

pBR322 sequences: Start: 3020 End: 5033

pBR322 sequences: Start: 2811 End: 3019

MMTV-LTR: Start: 1348 End: 2810

DHFR cDNA: Start: 601 End: 1347

Figure 7. Plasmid map of pAIL10V3.

The plasmid drives expression of anti-IL10. The anti-IL10 antibody heavy (VDJ-IgG4) and light (VDJ-IgK) chain expression cassettes, driven by the CMV promoter, are inserted into the multiple cloning site of pXBLS along with the hygromycin B expression cassette driven by the TK promoter (TK-Hygromycin). The *dhfr* cDNA, along with its promoter (MMTV-LTR) for plasmid amplification and the hygromycin B coding sequence, along with its TK promoter for selection in mammalian cells, are shown.

SV40 t Ag Intron: Start: 11507 End: 600

SV40 POLYA signal: Start: 11260 End: 11508

CMV: Start: 10568 End: 11222

T7 promoter/priming site: Start: 10549 End: 10568

VDJ-IgK (anti-IL10 rat antibody 12G8 light chain): Start: 9739 End: 10468
Beta globin Poly A signal: Start: 9478 End: 9726
CMV promoter: Start: 8715 End: 9369
T7 promoter/priming site: Start: 8696 End: 8715
VDJ (anti-IL10 rat antibody 12G8 heavy chain): Start: 8214 End: 8644
IgG1 non genomic region (anti-IL10 rat antibody 12G8 heavy chain): Start: 7234 End: 8214
Beta Globin Poly A signal: Start: 6971 End: 7209
TK promoter driving Hygromycin gene (TK-Hygromycin): Start: 5053 End: 6925
AP^r: Start: 3965 End: 4828
pBR ORI: Start: 3207 End: 3207
pBR322 sequences: Start: 3020 End: 5033
pBR322 sequences: Start: 2811 End: 3019
MMTV-LTR promoter: Start: 1348 End: 2810
DHFR cDNA: Start: 601 End: 1347

Figure 8. Plasmid map of pDSRG.

This plasmid is deposited at the American Type Culture Collection (10801 University Boulevard; Manassas, Virginia 20110-2209), under catalogue number 68233. The plasmid includes the SRα promoter, a strong SV40-based promoter and the dihydrofolate reductase (*DHFR*) cDNA for plasmid amplification in the presence of methotrexate in *dhfr*(-) Chinese hamster ovary (CHO) cells.

SV40 t Ag Intron: Start: 6371 End: 600
SV40 POLYA signal: Start: 6124 End: 6372
SRα promoter: Start: 5486 End: 6123
Beta Globin Poly A signal: Start: 5038 End: 5298
AP^r: Start: 3965 End: 4828
pBR322 sequences: Start: 3020 End: 5033

pBR322 sequences: Start: 2811 End: 3019
MMTV-LTR promoter: Start: 1348 End: 2810
DHFR cDNA: Start: 601 End: 1347

Figure 9. Plasmid map of pSRXBLS.

pSRXBLS is the direct descendent of pDSRG replacing its own multiple cloning site with a large multiple cloning site. pSRXBLS is the progenitor plasmid of pXBLS.

SV40 t Ag Intron: Start: 6065 End: 600
SV40 POLYA signal: Start: 5818 End: 6066
SRα promoter: Start: 5180 End: 5817
MCS: Start: 5038 End: 5179
Amp(R): Start: 3965 End: 4828
pBR ORI: Start: 3207 End: 3207
pBR322 sequences: Start: 3020 End: 5033
pBR322 sequences: Start: 2811 End: 3019
MMTV-LTR promoter: Start: 1348 End: 2810
DHFR cDNA: Start: 601 End: 1347.

Figure 10. Plasmid map of pUHSRstopLS.

pUHSRstopLS is the descendent plasmid to pUHLS carrying the SRα promoter and 249 bp of chicken β-globin terminator. This plasmid alone can be used to express any gene of interest. Also, it can be used as a transfer vector to transfer a complete expression cassette of part of a complex protein to pXBLS where all the expression cassettes can be assembled on a single plasmid.

Amp: Start: 2975 End: 3832
Col E1 origin: Start: 2032 End: 2972

Srα promoter with Intron: Start: 955 End: 1764
Beta Globin Poly A signal: Start: 673 End: 911
f1 (+) origin: Start: 3 End: 459

Figure 11. Plasmid map of pULSRstopLS.

pULSRstopLS is the descendent plasmid to pULLS carrying the SRα promoter and a 249 bp of chicken β-globin terminator. This plasmid alone can be used to express any gene of interest. Also, it can be used as a transfer vector to transfer a complete expression cassette of part of a complex protein to pXBLS where all the expression cassettes can be assembled on a single plasmid.

Amp: Start: 2981 End: 3838
Col E1 origin: Start: 2038 End: 2978
Beta globin poly A signal: Start: 1512 End: 1760
Srα promoter: Start: 665 End: 1474
f1 (+) origin: Start: 3 End: 459

Figure 12. Plasmid map of pPAG01.

This plasmid contains Selexis's (Geneva, Switzerland) ~3kb chicken lysozyme MAR, flanked by Xba1 and BamH1 site.
AP(R) (*bla* gene- Ap(r) determinant): Start: 4165 End: 5022
Selexis Inc. 5' lys MAR: Start: 1 End: 2960
P(LAC): Start: 3043 End: 3043
P(BLA) (*bla* gene promoter): Start: 5057 End: 5057
Replication Origin ORI (RNaseH cleavage point): Start: 3403 End: 3403

Figure 13. Plasmid map of pinAIL10/MAR(-).

The figure describes the map of plasmid, pinAIL10/MAR(-), that has the chicken lysozyme MAR element juxtaposed to the heavy chain expression cassette of the anti-IL10 gene containing the hygromycin resistance marker.

AP(R): Start: 3965 End: 4828
MAR-lys (MAR-lys is Matrix Attachment Region): Start: 5087 End: 8045
VDJ (VDJ region of anti-IL10 (12G8)): Start: 8928 End: 9369
IgG1 (IgG1 non genomic region): Start: 9374 End: 10354
DHFR cDNA: Start: 601 End: 1347
SV40 t Ag Intron: Start: 14897 End: 600
VDJ-IgK (VDJ-IgK for 12G8 light chain (anti-IL10)): Start: 13026 End: 13755
pBR322: Start: 2811 End: 3019
pBR322: Start: 3020 End: 5033
TK-Hygromycin: Start: 10663 End: 12672
Beta Globin Poly A signal: Start: 10379 End: 10617
Beta globin pA signal: Start: 12765 End: 13013
SV40 POLYA: Start: 14650 End: 14898
MMTV-LTR: Start: 1348 End: 2810
hCMV/intron (Human CMV promoter with hybrid intron): Start: 8077 End: 8918
hCMV/intron (Human CMV promoter and hybrid intron): Start: 13771 End: 14612
pBR ORI: Start: 3207 End: 3207

Figure 14. Plasmid map of pAIL10V1/puro/MAR(-).

The figure describes the map of plasmid, pAIL10/puro/MAR(-), that has chicken lysozyme MAR element juxtaposed to the heavy chain expression cassette of the anti-IL10 gene containing the puromycin instead of the hygromycin resistance marker.

AP: Start: 3965 End: 4828

MAR-lys (MAR-lys is Matrix Attachment Region): Start: 5087 End: 8045
VDJ: Start (VDJ region of anti-IL10 (12G8)): 8928 End: 9369
IgG1 (IgG1 non genomic region): Start: 9374 End: 10354
PURO: Start: 11674 End: 12905
DHFR cDNA: Start: 601 End: 1347
SV40 t Ag Intron: Start: 15070 End: 600
VDJ-IgK (VDJ-IgK for 12G8 light chain (anti-IL10)): Start: 13199 End: 13928 (Complementary)
pBR322: Start: 2811 End: 3019
pBR322: Start: 3020 End: 5033
Beta Globin Poly A signal: Start: 10379 End: 10617
SV40 POLYA: Start: 10784 End: 10789
Beta globin pA signal: Start: 12938 End: 13186
SV40 POLYA: Start: 14823 End: 15071
MMTV-LTR: Start: 1348 End: 2810
hCMV/intron (Human CMV promoter with hybrid intron): Start: 8077 End: 8918
HCMV-MIE: Start: 10902 End: 11660
hCMV/intron (Human CMV promoter and hybrid intron): Start: 13944 End: 14785 (Complementary)
pBR ORI: Start: 3207 End: 3207

Figure 15. Plasmid map of pAIGFRLCb2/MAR(-).

The figure describes the map of plasmid, that has chicken lysozyme MAR element juxtaposed to the heavy chain expression cassette of the anti-IGFR1 gene containing the hygromycin resistance marker.

AP(R): Start: 3965 End: 4828
MAR-lys (MAR-lys is Matrix Attachment Region): Start: 5087 End: 8045
VDJ (VDJ of IGFR1 of 11D8 hybridoma): Start: 8974 End: 9401

IgG1 (IgG1 non genomic region): Start: 9401 End: 10381
DHFR cDNA: Start: 601 End: 1347
SV40 t Ag Intron: Start: 14924 End: 600
Kappa (Kappa Chain): Start: 13063 End: 13386
VDJ (VDJ of IGFR1 (LCb, human germline sequence)): Start: 13387 End: 13764
pBR322: Start: 2811 End: 3019
pBR322: Start: 3020 End: 5033
TK-Hygromycin: Start: 10690 End: 12699
Beta Globin Poly A signal: Start: 10406 End: 10644
Beta globin pA signal: Start: 12792 End: 13040
SV40 POLYA: Start: 14677 End: 14925
MMTV-LTR: Start: 1348 End: 2810
hCMV/intron (Human CMV promoter with hybrid intron): Start: 8077 End: 8918
hCMV/intron (Human CMV promoter and hybrid intron): Start: 13786 End: 14627
pBR ORI: Start: 3207 End: 3207

Figure 16. Plasmid map of pAIGFRLCb2V1/puro/MAR(-).

The figure describes the map of plasmid, that has chicken lysozyme MAR element juxtaposed to the heavy chain expression cassette of the anti-IGFR1 gene containing the puromycin instead of the hygromycin resistance marker.

AP(R): Start: 3965 End: 4828
MAR-lys (MAR-lys is Matrix Attachment Region): Start: 5087 End: 8045
VDJ (VDJ of IGFR1 of 11D8 hybridoma): Start: 8974 End: 9401
IgG1 (IgG1 non genomic region): Start: 9401 End: 10381
PURO(R): Start: 11701 End: 12932
DHFR cDNA: Start: 601 End: 1347
SV40 t Ag Intron: Start: 15097 End: 600
Kappa (Kappa Chain): Start: 13236 End: 13559

VDJ (VDJ of IGFR1 (LCb, human germline sequence)): Start: 13560 End: 13937
pBR322: Start: 2811 End: 3019
pBR322: Start: 3020 End: 5033
Beta Globin Poly A signal: Start: 10406 End: 10644
SV40 POLYA: Start: 10811 End: 10816
Beta globin pA signal: Start: 12965 End: 13213
SV40 POLYA: Start: 14850 End: 15098
MMTV-LTR: Start: 1348 End: 2810
hCMV/intron (Human CMV promoter with hybrid intron): Start: 8077 End: 8918
HCMV-MIE: Start: 10929 End: 11687
hCMV/intron (Human CMV promoter and hybrid intron): Start: 13959 End: 14800
pBR ORI: Start: 3207 End: 3207

DETAILED DESCRIPTION

The present invention provides a plasmid system useful for recombinant protein expression in any cell, for example in a mammalian cell, a bacterial cell, a yeast cell or an insect cell. The plasmid system is amenable to any cell based expression of a broad range of recombinant proteins, ranging from simple proteins, such as interferon, to complex proteins, such as antibodies. The system offers many common and rare restriction sites to accommodate a variety of expression cassettes. It also provides flexibility in the choice of various elements of an expression cassette, such as a promoter, enhancer, and terminator, as well as an antibiotic resistance marker. The plasmids can also be used as simple transfer vectors. The system offers potential for both transient as well as stable expression. The pXBLS vector carries the dihydrofolate reductase (*DHFR*) coding region for selection and amplification of the plasmid in *DHFR* deficient mammalian cells, *e.g.* CHO DXB-11 and CHO DG44. Thus, the system can be used for isolating stable clones, harnessing gene amplification and selection. The plasmid system includes two universal transfer plasmids, pUHLS

and pULLS, which are useful for carrying out expression of the parts of a complex protein such as an antibody. Thus, the system offers options of co-transfection with universal vectors and single transfection with pXBLS. The ability of the plasmid system to cause such segregated expression of various parts is advantageous since it is sometimes necessary to take a deeper insight into the expression of individual units of a multi-subunit protein in order to analyze the overall expression of the complex protein. The system can also be used to address the effect of directional variability, resulting from the orientation of the multiple genes in the plasmid for the expression of multi-subunit proteins. Thus, a strategy in placing multiple expression cassettes can be arrived at for optimal expression of a complex protein.

The plasmid system of the invention has been demonstrated to direct high levels of expression of multiple polypeptides including anti-IL5 antibody, anti-IGFR1 antibody, IL2 receptor membrane domain, and anti-IL10 antibody. Other proteins may also be expressed in the plasmid system of the invention including interferon, fibrinogen, ion channels, bacterial porins (*e.g.*, ompF), and the nicotinic acetylcholine receptor (nAChR).

In one embodiment of the invention, the plasmid system comprises the light and heavy chain of the fully human, monoclonal anti-IGFR1 antibody 15H12/19D12 which may also be referred to as 15H12 or as 19D12.

The parts to the plasmid system can be provided separately or, conveniently, together as part of a kit.

The present invention includes any of the polynucleotide comprising or consisting of a nucleotide sequence set forth, below, in Table 1, individually or as part of a plasmid system or kit. Polynucleotides of the invention can be in any form, including circular, linear, double-stranded or single-stranded.

Table 1. Polynucleotides of the invention.

Polynucleotide	Sequence Identifier

pULLS	SEQ ID NO: 1
pUHLS	SEQ ID NO: 2
pXBLS	SEQ ID NO: 3
pULSRstopLS	SEQ ID NO: 4
pUHSRstopLS	SEQ ID NO: 5
pAIL5V1	SEQ ID NO: 6
pAIGFRV3	SEQ ID NO: 7
pAIG1FR(-)IL2LS	SEQ ID NO: 8
pAIL10V3	SEQ ID NO: 9
pXBLS multiple cloning site	SEQ ID NO: 10
pUHLS multiple cloning site	SEQ ID NO: 11
pULLS multiple cloning site	SEQ ID NO: 12
pSRXBLS	SEQ ID NO: 13
pDSRG	SEQ ID NO: 14
Nucleotide sequence encoding the 15H12 and 19D12 light chain variable region-including signal peptide (15H12/19D12 LC)	SEQ ID NO: 15
Amino acid sequence of the15H12 and 19D12 light chain variable region-including signal peptide	SEQ ID NO: 16
Nucleotide sequence encoding the15H12 and 19D12 heavy chain variable region including signal peptide (15H12/19D12 HC)	SEQ ID NO: 17
Amino acid sequence of the 15H12 and 19D12 heavy chain variable region including signal peptide	SEQ ID NO: 18
Nucleotide sequence encoding the 15H12/19D12 light chain F (LCF)	SEQ ID NO: 19
Amino acid sequence of the 15H12/19D12 light chain F	SEQ ID NO: 20
Nucleotide sequence encoding the 15H12/19D12 heavy chain A (HCA)	SEQ ID NO:21
Amino acid sequence of the	SEQ ID NO:22

15H12/19D12 heavy chain A	
Nucleotide sequence of the chicken lysozyme MAR element	SEQ ID NO: 23
pinAIL10/MAR(-)	SEQ ID NO: 24
pAIL10V1/puro/MAR(-)	SEQ ID NO: 25
pAIGFRLCb2/MAR(-)	SEQ ID NO: 26
PAIGFRLCb2/puro/MAR(-)	SEQ ID NO: 27

Molecular Biology

In accordance with the present invention there may be employed conventional molecular biology, microbiology, and recombinant DNA techniques within the skill of the art. Such techniques are explained in the literature. See, *e.g.*, Sambrook, Fritsch & Maniatis, Molecular Cloning: A Laboratory Manual, Second Edition (1989) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (herein "Sambrook, *et al.*, 1989"); DNA Cloning: A Practical Approach, Volumes I and II (D.N. Glover ed. 1985); Oligonucleotide Synthesis (M.J. Gait ed. 1984); Nucleic Acid Hybridization (B.D. Hames & S.J. Higgins eds. (1985)); Transcription And Translation (B.D. Hames & S.J. Higgins, eds. (1984)); Animal Cell Culture (R.I. Freshney, ed. (1986)); Immobilized Cells And Enzymes (IRL Press, (1986)); B. Perbal, A Practical Guide To Molecular Cloning (1984); F.M. Ausubel, *et al.* (eds.), Current Protocols in Molecular Biology, John Wiley & Sons, Inc. (1994).

A "polynucleotide", "nucleic acid " or "nucleic acid molecule" includes the polymeric form of ribonucleosides (adenosine, guanosine, uridine or cytidine; "RNA molecules") or deoxyribonucleosides (deoxyadenosine, deoxyguanosine, deoxythymidine, or deoxycytidine; "DNA molecules"), or any phosphoester analogs thereof, such as phosphorothioates and thioesters, in single stranded form, double-stranded form or otherwise.

A "polynucleotide sequence", "nucleic acid sequence" or "nucleotide sequence" is a series of nucleotide bases (also called "nucleotides") in a nucleic acid, such as DNA or RNA, and means any chain of two or more nucleotides.

A "coding sequence" or a sequence "encoding" an expression product, such as a RNA or peptide, is a nucleotide sequence that, when expressed, results in production of the product.

The term "gene" means a DNA sequence that codes for or corresponds to a particular sequence of ribonucleotides or amino acids which comprise all or part of one or more RNA molecules, proteins or enzymes, and may or may not include regulatory DNA sequences, such as promoter sequences, which determine, for example, the conditions under which the gene is expressed. Genes may be transcribed from DNA to RNA which may or may not be translated into an amino acid sequence.

As used herein, the term "oligonucleotide" refers to a nucleic acid, generally of no more than about 300 nucleotides (*e.g.*, 30, 40, 50, 60, 70, 80, 90, 150, 175, 200, 250, 300), that may be hybridizable to a genomic DNA molecule, a cDNA molecule, or an mRNA molecule encoding a gene, mRNA, cDNA, or other nucleic acid of interest. Oligonucleotides are usually single-stranded, but may be double-stranded. Oligonucleotides can be labeled, *e.g.*, by incorporation of ^{32}P-nucleotides, ^{3}H-nucleotides, ^{14}C-nucleotides, ^{35}S-nucleotides or nucleotides to which a label, such as biotin, has been covalently conjugated. In one embodiment, a labeled oligonucleotide can be used as a probe to detect the presence of a nucleic acid. In another embodiment, oligonucleotides (one or both of which may be labeled) can be used as PCR primers, either for cloning full length or a fragment of the gene, or to detect the presence of nucleic acids. Generally, oligonucleotides are prepared synthetically, preferably on a nucleic acid synthesizer.

A "protein sequence", "peptide sequence" or "polypeptide sequence" or "amino acid sequence" refers to a series of two or more amino acids in a protein, peptide or polypeptide.

"Protein", "peptide" or "polypeptide" includes a contiguous string of two or more amino acids.

The term "isolated polynucleotide" or "isolated polypeptide" includes a polynucleotide (*e.g.*, RNA or DNA molecule, or a mixed polymer) or a polypeptide, respectively, which is partially or fully separated from other components that are normally found in cells or in recombinant DNA expression systems or any other contaminant. These components include, but are not limited to, cell membranes, cell walls, ribosomes, polymerases, serum components and extraneous genomic sequences.

An isolated polynucleotide or polypeptide will, preferably, be an essentially homogeneous composition of molecules but may contain some heterogeneity.

"PCR amplification" of DNA as used herein includes the use of polymerase chain reaction (PCR) to increase the concentration of a particular DNA sequence within a mixture of DNA sequences. For a description of PCR see Saiki, *et al.*, Science (1988) 239:487. Genes can be amplified, for example, in a plasmid in a cell. Cells harboring a plasmid containing an amplifiable, selectable marker, but lacking an endogenous marker gene, such as *DHFR*, can be selected with increasing amounts of a selecting agent, such as methotrexate (*e.g.*, if the *DHFR* gene is on the plasmid). Typically, this procedure will cause the copy number of the plasmid containing the amplifiable, selectable marker in the cell to increase.

The term "host cell" includes any cell of any organism that is selected, modified, transfected, transformed, grown, or used or manipulated in any way, for the production of a substance by the cell, for example the expression or replication, by the cell, of a gene, a DNA or RNA or a protein. For example, a host cell may be a bacteria such as *E.coli* or an eukaryotic cell such as a CHO cell.

A "cassette" or an "expression cassette" refers to a DNA coding sequence or segment of DNA that codes for an expression product (*e.g.*, peptide or RNA) that can be inserted into a vector at defined restriction sites. The DNA

coding sequence can be operably linked to a promoter and/or to a terminator and/or polyA signal.

The sequence of a nucleic acid may be determined by any method known in the art (*e.g.*, chemical sequencing or enzymatic sequencing). "Chemical sequencing" of DNA includes methods such as that of Maxam and Gilbert (Proc. Natl. Acad. Sci. USA (1977) 74:560), in which DNA is randomly cleaved using individual base-specific reactions. "Enzymatic sequencing" of DNA includes methods such as that of Sanger (Sanger, *et al.*, Proc. Natl. Acad. Sci. USA (1977) 74:5463).

The present invention includes nucleic acids of the invention flanked by natural regulatory (expression control) sequences, which may be associated with heterologous sequences, including promoters, internal ribosome entry sites (IRES) and other ribosome binding site sequences, enhancers, response elements, suppressors, signal sequences, polyadenylation sequences, introns, 5'- and 3'- non-coding regions, and the like.

"Internal ribosome entry sites" "IRES" are commonly known in the art. Internal ribosome entry sites have been identified in a several genes including eIF4G (Johannes *et al.*, RNA 4: 1500-1513 (1998)), DAP5 (Henis-Korenblit *et al.*, Molecular and Cellular Biology 20: 496-506 (2000)), c-Myc (Stoneley *et al.*, Molecular and Cellular Biology 20: 1162-1169 (2000)), NF-κ-b repressing factor (Oumard *et al.*, Molecular and Cellular Biology 20: 2755-2759 (2000)), VEGF (Huez *et al.*, Molecular and Cellular Biology 18: 6178-6190 (1998)), FGF-2 (Creancier *et al.*, Journal of Cell Biology 150: 275-281 (2000)), PDGF-B (Bernstein *et al.*, Journal of Biological Chemistry 272: 9356-9362 (1997)), X-linked inhibitor of apoptosis (XIAP) (Holcik *et al.*, Oncogene 19: 4174-4177 (2000)) , Apaf-1 (Coldwell *et al.*, Oncogene 19: 899-905 (2000)) and BiP (Macejak *et al.*, Nature 353: 90-94 (1991)).

In general, a "promoter" or "promoter sequence" is a DNA regulatory region capable of binding an RNA polymerase in a cell (*e.g.*, directly or through

other promoter-bound proteins or substances) and initiating transcription of a coding sequence. A promoter sequence is, in general, bounded at its 3' terminus by the transcription initiation site and extends upstream (5' direction) to include the minimum number of bases or elements necessary to initiate transcription at any level. Within the promoter sequence may be found a transcription initiation site (conveniently defined, for example, by mapping with nuclease S1), as well as protein binding domains (consensus sequences) responsible for the binding of RNA polymerase. The promoter may be operably associated with other expression control sequences, including enhancer and repressor sequences or with a nucleic acid of the invention.

A coding sequence is "under the control of", "functionally associated with", "operably linked to" or "operably associated with" transcriptional and translational control sequences in a cell when the sequences direct or regulate expression of the sequence. For example, a promoter operably linked to a gene will direct RNA polymerase mediated transcription of the coding sequence into RNA, preferably mRNA, which then may be RNA spliced (if it contains introns) and, optionally, translated into a protein encoded by the coding sequence. A terminator/polyA signal operably linked to a gene terminates transcription of the gene into RNA and directs addition of a poly A signal onto the RNA.

The terms "express" and "expression" mean allowing or causing the information in a gene, RNA or DNA sequence to become manifest; for example, producing a protein by activating the cellular functions involved in transcription and translation of a corresponding gene. "Express" and "expression" include transcription of DNA to RNA and RNA to protein. A DNA sequence is expressed in or by a cell to form an "expression product" such as an RNA (*e.g.*, mRNA) or a protein. The expression product itself may also be said to be "expressed" by the cell.

The term "transformation" means the introduction of a nucleic acid into a cell. The introduced gene or sequence may be called a "clone". A host cell that

receives the introduced DNA or RNA has been "transformed" and is a "transformant" or a "clone." The DNA or RNA introduced to a host cell can come from any source, including cells of the same genus or species as the host cell, or from cells of a different genus or species. Examples of transformation methods which are very well known in the art include liposome delivery, electroporation, $CaPO_4$ transformation, DEAE-Dextran transformation, microinjection and viral infection.

The present invention includes vectors which comprise polynucleotides of the invention. The term "vector" may refer to a vehicle (*e.g.*, a plasmid) by which a DNA or RNA sequence can be introduced into a host cell, so as to transform the host and, optionally, promote expression and/or replication of the introduced sequence.

The polynucleotides of the invention may be expressed in an expression system. The term "expression system" means a host cell and compatible vector which, under suitable conditions, can express a protein or nucleic acid which is carried by the vector and introduced to the host cell. Common expression systems include *E.coli* host cells and plasmid vectors, insect host cells and baculovirus vectors, and mammalian host cells and vectors such as plasmids, cosmids, BACs, YACs and viruses such as adenovirus and adenovirus associated virus (AAV).

Plasmids

In one embodiment, the present invention comprises a kit comprising a first universal transfer vector comprising a multiple cloning site, a replication origin, and a selectable marker; a second universal transfer vector comprising a multiple cloning site, a replication origin, and a selectable marker and an amplifiable vector comprising a multiple cloning site, a promoter, a replication origin or a chromosomal integration site, a poly-adenylation site and an

amplifiable selectable marker. Generally, the multiple cloning sites comprise about 20, 25 or 30 restriction sites.

Plasmids of the present invention may include any of several amplifiable markers known in the art. Use of amplifiable markers is discussed in Maniatis, Molecular Biology: A Laboratory Manual, Cold Spring Harbor Laboratory, NY (1989)). Useful selectable markers for gene amplification in drug-resistant mammalian cells include *DHFR* (MTX (methotrexate) resistance) (Alt *et al.*, J. Biol. Chem. 253:1357 (1978); Wigler *et al.*, Proc. Natl. Acad. Sci. USA 77:3567 (1980)); metallothionein (cadmium resistance) (Beach *et al.*, Proc Natl. Acad. Sci. USA 78:210 (1981)); *CAD* (N-(phosphonoacetyl)-l-aspartate (PALA) resistance) (Wahl *et al.*, J. Biol. Chem. 254: 8679 (1979)); adenylate deaminase (coformycin resistance) (Debatisse *et al.*, Mol. Cell. Biol. 6:1776 (1986)); IMP 5'-dehydrogenase (mycophenolic acid resistance) (Huberman *et al.*, Proc. Natl. Acad. Sci. USA 78:3151 (1981)) and other markers known in the art (as reviewed, for example, in Kaufman *et al.*, Meth. Enzymology 185:537-566 (1988)).

In one embodiment, the metallothionein II A gene under the control of a metallothionein promoter is an amplifiable marker in cell lines such as CHO-K1. Amplification can be induced by addition of Cd^{2+} or Zn^{2+} to the cell culture.

Plasmids of the invention may include other eukaryotic, non-amplifiable selectable markers known in the art. In an embodiment of the invention, the drug-resistance marker is the hygromycin B gene which confers resistance to hygromycin. Other markers include the G418 resistance gene. The plasmids of the invention may also include a prokaryotic antibiotic resistance marker such as the ampicillin resistance gene or the kanamycin resistance gene.

Plasmids of the invention may also include a matrix attachment region (MAR). Generally, MARs are DNA sequences capable of specific binding to nuclear proteins that are part of a fibrillar nuclear matrix analogous to the cytoskeleton. In one embodiment, the MAR is the chicken lysozyme MAR.

Promoters which may be used to control gene expression include, but are not limited to, SRα promoter (Takebe *et al.*, Molec. and Cell. Bio. 8:466-472 (1988)), the human CMV immediate early promoter (Boshart *et al.*, Cell 41:521-530 (1985); Foecking *et al.*, Gene 45:101-105 (1986)), the mouse CMV immediate early promoter, the SV40 early promoter region (Benoist, *et al.*, Nature 290:304-310 (1981)), the *Orgyia pseudotsugata* immediate early promoter, the herpes thymidine kinase promoter (Wagner, *et al.*, Proc. Natl. Acad. Sci. USA 78:1441-1445 (1981)), the regulatory sequences of the metallothionein gene (Brinster, *et al.*, Nature 296:39-42 (1982)); prokaryotic expression vectors such as the β-lactamase promoter (Villa-Komaroff, *et al.*, Proc. Natl. Acad. Sci. USA 75:3727-3731 (1978)), or the *tac* promoter (DeBoer, *et al.*, Proc. Natl. Acad. Sci. USA 80:21-25 (1983)); and promoter elements from yeast or other fungi such as the *GAL1, GAL4 or GAL10* promoter, the *ADH* (alcohol dehydrogenase) promoter, *PGK* (phosphoglycerol kinase) promoter or the alkaline phosphatase promoter.

Viral long terminal repeat promoters such as the mouse mammary tumor virus long terminal repeat (MMTV-LTR) (Fasel *et al.*, EMBO J. 1(1):3-7 (1982)), the moloney murine sarcoma virus long terminal repeat (Reddy *et al.*, Proc. Natl. Acad. Sci. USA 77(9): 5234-5238 (1980)), the moloney murine leukemia virus long terminal repeat (Van Beveren *et al.*, Proc. Natl. Acad. Sci. USA 77(6): 3307-3311 (1980)), the HIV LTR (Genbank Accession No. AB100245), the bovine foamy virus LTR (Genbank Accession No. NC_001831), RSV 5'-LTR (Genbank Accession No. K00087), the HIV-2 LTR (Genbank Accession No. NC_001722), an avian retroviral LTR (Ju *et al.*, Cell 22: 379-386 (1980)) and the human herpesvirus LTR (Genbank Accession No. NC_001806) may be included in the plasmids of the present invention.

Other acceptable promoters include the human CMV promoter, the human CMV5 promoter, the murine CMV promoter, the EF1α promoter, the SV40 promoter, a hybrid CMV promoter for liver specific expression (*e.g.*, made by

conjugating CMV immediate early promoter with the transcriptional promoter elements of either human α1-antitrypsin (HAT) or albumin (HAL) promoter), or promoters for hepatoma specific expression (*e.g.*, wherein the transcriptional promoter elements of either human albumin (HAL; about 1000 bp) or human α1-antitrypsin (HAT, about 2000 bp) are combined with a 145 bp long enhancer element of human α1-microglobulin and bikunin precursor gene (AMBP); HAL-AMBP and HAT-AMBP).

In addition, bacterial promoters, such as the T7 RNA Polymerase promoter or the *tac* promoter, may be used to control expression.

A promoter (*e.g.*, SRα promoter) may be linked to the cassette and then moved into a transfer vector (*e.g.*, pULLS or pUHLS). In another embodiment, the transfer vector can contain a promoter upstream of the multiple cloning site (*e.g.*, pULSRstopLS or pUHSRstopLS). When a gene, not linked to a promoter, is inserted into the multiple cloning site, it will be operably linked to the upstream promoter.

In yet another embodiment of the invention, a gene in a transfer vector, not linked to a promoter, can be moved into the amplifiable vector comprising a promoter (*e.g.*, SRα promoter) upstream of the multiple cloning site (*e.g.*, pSRXBLS). When the unlinked gene is placed in the multiple cloning site, it will become operably linked to the promoter.

Plasmids of the invention may also include a polyadenylation signal/terminator for termination of the transcription of a gene in the plasmid and for the addition of a polyA tail to the transcript. For example, the chicken β-globin terminator/polyA signal may be included in a plasmid of the invention. Other acceptable poly A signals include the SV40 Poly A signal and the bovine growth hormone poly A signal.

In one embodiment of the invention, the amplifiable vector comprises a multiple cloning site including the following restriction sites: Sgr AI, Srf I, Xma I, Spe I, Sac II, Rsr II, Pac I, Nru I, Not I, Nde I, Msc I, Mlu I, Kpn I, Fse 1, Bss HII,

Bsr GI, Bsp EI, Bcl I, Bbv C1, Pme I, Bss HII, Asc I, Xba I ; for example, wherein the amplifiable vector multiple cloning site is that of pXBLS:

```
                  AscI                        BclI        BsrGI
                 ~~~~~~~~~                   ~~~~~~      ~~~~~~
                          PmeI
                         ~~~~~~~~~
            XbaI   BssHII           BbvC1         BspEI
           ~~~~~~  ~~~~~~          ~~~~~~~~      ~~~~~~~
     1   AAATCTAGAG GCGCGCCGTT TAAACCCTCA GCTGATCATC CGGATGTACA
         TTTAGATCTC CGCGCGGCAA ATTTGGGAGT CGACTAGTAG GCCTACATGT
                Fse1          MluI         NdeI          NruI
              ~~~~~~~~~      ~~~~~~      ~~~~~~        ~~~~
         BssHII          KpnI         MscI          NotI
         ~~~~~~         ~~~~~~~      ~~~~~~~      ~~~~~~~~~
    51   GCGCGCGGCC GGCCGGTACC ACGCGTTGGC CACATATGGC GGCCGCTCGC
         CGCGCGCCGG CCGGCCATGG TGCGCAACCG GTGTATACCG CCGGCGAGCG
            PacI          SacII          Srf1            XhoI
           ~~~~~~~~~     ~~~~~~~     ~~~~~~~~~         ~~~~~
         NruI        RsrII         SpeI    XmaI   SgrAI
         ~~         ~~~~~~~      ~~~~~~  ~~~~~~ ~~~~~~~~~
   101   GATTAATTAA CGGACCGCCG CGGACTAGTG CCCGGGCCAC CGGTGCTCGA
         CTAATTAATT GCCTGGCGGC GCCTGATCAC GGGCCCGGTG GCCACGAGCT
         XhoI
         ~
   151   GAAAA
         CTTTT (SEQ ID NO: 10).
```

In an embodiment of the invention, a universal transfer vector comprises a multiple cloning site including the following restriction sites: Bss HII, Pme I, Sna B1, Hin dIII, Asp 718, Kpn I, Pae R71, Xho I, Sal I, Acc I, Hinc II, Cla I, Eco RV, Eco RI, Pst I, Eco O109I, Eco O109I, Apa I, Xma I, Bsp EI, Bam H1, Dsa I, Eag I, Ecl XI, Not I, Sac II, Xma III, Xba I, Sac I, Mlu I, Bcl I, Bsr GI, Bss HII; for example, wherein the transfer vector multiple cloning site is that of pUHLS:

```
          AscI                        HindIII      PaeR7I
         ~~~~~~~~~                    ~~~~~~       ~~~~~~
                    PmeI          SnaBI          KpnI
                  ~~~~~~~~~      ~~~~~~~       ~~~~~~~
         XmaI                                      XhoI
         ~~~                                      ~~~~~~
             BssHII          BbvC1              Asp718
```

```
          ~~~~~~~          ~~~~~~~~             ~~~~~~~
     1    GGGGGCGCGC CGTTTAAACC CTCAGCTACG TAAAGCTTGG TACCCTCGAG
          CCCCCGCGCG GCAAATTTGG GAGTCGATGC ATTTCGAACC ATGGGAGCTC
                 ClaI                                   BspEI
                ~~~~~~~                                 ~~~~~~
          HincII       EcoRV         PstI
          ~~~~~~       ~~~~~~       ~~~~~~~
           SalI                                  XmaI
          ~~~~~~                                 ~~~~~~~
           AccI             EcoRI          ApaI              BamHI
          ~~~~~~            ~~~~~~~       ~~~~~~             ~~
    51    GTCGACATCG ATGATATCGA ATTCCTGCAG GGGCCCCCCG GGTCCGGAGG
          CAGCTGTAGC TACTATAGCT TAAGGACGTC CCCGGGGGGC CCAGGCCTCC
                NotI                 MluI        BsrGI
               ~~~~~~~~~             ~~~~~~~     ~~~~~~~
                              SacI
                             ~~~~~~~
          BamHI         XbaI                BclI          Fse1
          ~~~~          ~~~~~~              ~~~~~~       ~~~~~~~~
    101   ATCCGCGGCC GCTCTAGAGA GCTCACGCGT TGATCATGTA CAGGCCGGCC
          TAGGCGCCGG CGAGATCTCT CGAGTGCGCA ACTAGTACAT GTCCGGCCGG
                 XmaI
                 ~~~
           BssHII
           ~~~~~~
    151   AGCGCGCCCC
          TCGCGCGGGG (SEQ ID NO: 11).
```

A universal transfer vector may comprise a multiple cloning site including the following restriction sites: Bss HII, Sgr AI, Xma I, Rsr II, Spe I, Sna B1, Hin dIII, Asp 718, Kpn I, Pae R71, Xho I, Sal I, Acc I, Hinc II, Cla I, Eco RV, Eco RI, Pst I, Eco O109I, Eco O109I, Apa I, Xma I, Bsp EI, Bam H1, Dsa I, Eag I, Ecl XI, Not I, Sac II, Xma III, Xba I, Sac I, Nde I, Msc I, Nru I, Pac I, Bss HII; for example, wherein the transfer vector multiple cloning site is that of pULLS:

```
                  SgrAI                          SnaBI
                 ~~~~~~~~                        ~~~~~~~
          XmaI                Srf1             SpeI
          ~~~                 ~~~~~~~~~        ~~~~~~
              BssHII           XmaI    RsrII              HindIII
              ~~~~~~~          ~~~~~~~ ~~~~~~~~           ~~~~~
     1    GGGGGCGCGC CACCGGTGGC CCGGGCCGGT CCGACTAGTT ACGTAAAGCT
          CCCCCGCGCG GTGGCCACCG GGCCCGGCCA GGCTGATCAA TGCATTTCGA
                 PaeR7I        ClaI                          BspEI
```

```
                ~~~~~~~      ~~~~~~~                              ~
          KpnI         HincII        EcoRV         PstI
         ~~~~~~       ~~~~~~        ~~~~~~~      ~~~~~~~
                       SalI
                      ~~~~~~
              XhoI
             ~~~~~~~
        HindIII         AccI               EcoRI          ApaI
        ~               ~~~~~~             ~~~~~~         ~~~~~~
  51    TGGTACCCTC GAGGTCGACA TCGATGATAT CGAATTCCTG CAGGGGCCCT
        ACCATGGGAG CTCCAGCTGT AGCTACTATA GCTTAAGGAC GTCCCCGGGA
        BspEI          NotI                NdeI          NruI
        ~~~~~        ~~~~~~~~              ~~~~~~        ~~~~~~
                                  SacI
                                 ~~~~~~~
             BamHI           XbaI                MscI          PacI
             ~~~~~~~         ~~~~~~~             ~~~~~~~       ~
 101    CCGGAGGATC CGCGGCCGCT CTAGAGAGCT CCATATGTGG CCATCGCGAT
        GGCCTCCTAG GCGCCGGCGA GATCTCTCGA GGTATACACC GGTAGCGCTA
         PacI          XmaI                                   -
        ~~~~~~~        ~~~
               BssHII
               ~~~~~~~
 151    TAATTAAGCG CGCCCC
        ATTAATTCGC GCGGGG (SEQ ID NO: 12).
```

The present invention contemplates amplifiable vectors or universal transfer vectors comprising the above–referenced multiple cloning sites in the orientation shown or in the opposite orientation.

The plasmids of the present invention can be introduced into any cell line for expression of the target polypeptides. In one embodiment of the invention, the plasmids are introduced into a mammalian cell line, preferably a Chinese hamster ovary (CHO) cell line. A commonly used cell line is DHFR-CHO cell line which can be transformed to the DHFR$^+$ phenotype using *DHFR* cDNA as an amplifiable dominant marker. One such known DHFR-CHO cell line is DX-B11 or DG-44. In another embodiment, the plasmids of the invention can be introduced into a lower eukaryotic cell line, such as from *S.cerevisiae*, *K.lactis*, *P.pastoris*, *C.albicans* or *A.fumigatus*. Further, the plasmids of the invention may also be introduced into higher eukaryotic non-mammalian cell lines such as from insect

cells (*e.g.*, *Drosophila melanogaster*, sf9 cells, sf21 cells), amphibian cells (*e.g.*, *X.laevis*), plant cells (*e.g.*, *A.thaliana*) and avian cells.

Plasmids of the invention can also be introduced into a bacterial cell. In one embodiment, competent *E.coli* are transformed. Examples of suitable *E.coli* include DH1, DH5, DH5α, XL1-Blue, SURE, SCS110, OneShot Top 10, and HB101.

Plasmids may be introduced into a cell by any of the many methods which are commonly known in the art. For example, a plasmid of the invention can be used to transform a cell by the calcium phosphate method, electroporation, the DEAE-dextran method or the liposome method.

The plasmids of the invention can include any gene or combination of genes. In an embodiment of the invention the plasmids include heavy and light chain immunoglobulin genes. The immunoglobulin chains may be part of antibodies which specifically recognize any antigen such as IL-5, IGFR1 or IL-10. Receptors or receptor subunits may also be expressed. For example, a gene encoding the IL-2 receptor or a portion thereof (*e.g.*, membrane domain) can be included in a plasmid of the invention.

U.S. Patent Application No. 10/443,466; filed May 22, 2003, which is herein incorporated by reference in its entirety, sets forth the nucleotide and amino acid sequences of immunoglobulin light chain and heavy chain variable regions of anti-IGFR1 antibodies. Any of the light and heavy chain variable regions disclosed therein can be incorporated into the plasmid system of the invention and expressed. In one embodiment, the anti-IGFR1 antibody light chain variable region is encoded by the nucleotide sequence set forth in SEQ ID NO: 15 or 19 or is any polynucleotide encoding a polypeptide comprising the amino acid sequence of SEQ ID NO: 16 or 20 and/or the anti-IGFR1 antibody heavy chain variable region is encoded by the nucleotide sequence set forth in SEQ ID NO: 17 or 21 or is any polynucleotide encoding a polypeptide comprising the amino acid sequence of SEQ ID NO: 18 or 22.

Expression cassettes encoding the immunoglobulin heavy and light chain of the anti-IGFR1 antibody can each be introduced into the multiple cloning site of either pULLS or pUHLS. Preferably, the immunoglobulin heavy and light chains, in the expression cassettes, are linked to an immunoglobulin constant region such as γ1, γ4 or κ. Preferably, the expression cassettes are then inserted into the amplifiable vector pXBLS which is then introduced into a cell suitable for causing the expression of the light and heavy chains. For example, the plasmid ,pAIGFRV3, which contains the immunoglobulin heavy and light chains of an anti-IGFR1 antibody, can be introduced into a *dhfr*⁻ mammalian cell line (*e.g.*, CHO-DXB11) wherein the chains are expressed.

Kits

The plasmid system of the invention may be provided in a kit. The kits of the invention may include, in addition to the plasmid system, any reagent which may be employed in the use of the plasmid system. In one embodiment, the kit includes reagents necessary for transformation of the plasmids into mammalian cells. For example, the kit may include reagents for a calcium phosphate transformation procedure: calcium chloride, buffer (*e.g.*, 2× HEPES buffered saline), and sterile, distilled water. In another embodiment, the kit includes reagents for a DEAE-Dextran transformation: Chloroquine in PBS, DEAE-dextran in PBS and Phosphate buffered saline. In yet another embodiment, reagents for a liposome transformation are included in the kit: Liposomes extruded from DOTAP/cholesterol extruded liposomes. For example, the kit may include the cationic lipid-based transfection reagent Lipofectamine™ (Invitrogen Life Technologies; Carlsbad, CA).

The kit may include reagents required for bacterial transformation of the plasmids of the invention. For example, the kit may include transformation competent bacteria (*e.g.*, DH1, DH5, DH5α, XL1-Blue, SURE, SCS110, OneShot Top 10, or HB101).

The kit may include growth media or reagents required for making growth media. For example, in one embodiment, the kit can include fetal calf serum or DMEM (Dulbecco/Vogt modified Eagle's (Harry Eagle) minimal essential medium) for growth of mammalian cells. In another embodiment, the kit can contain powdered luria broth media or luria broth plates containing an appropriate antibiotic (*e.g.*, ampicillin or kanamycin) for growing bacteria.

Components supplied in the kit may be provided in appropriate vials or containers (*e.g.*, plastic or glass vials). The kit can include appropriate label directions for storage, and appropriate instructions for usage.

Protein Expression and Purification

Polypeptides produced in the plasmid system of the invention can be purified by standard methods, including, but not limited to, salt or alcohol precipitation, affinity chromatography (*e.g.*, used in conjunction with a purification tag), preparative disc-gel electrophoresis, isoelectric focusing, high pressure liquid chromatography (HPLC), reversed-phase HPLC, gel filtration, cation and anion exchange and partition chromatography, and countercurrent distribution. Such purification methods are well known in the art and are disclosed, *e.g.*, in *"Guide to Protein Purification"*, Methods in Enzymology, Vol. 182, M. Deutscher, Ed., 1990, Academic Press, New York, NY.

Particularly where a polypeptide is being isolated from a cellular or tissue source, it is preferable to include one or more inhibitors of proteolytic enzymes in the assay system, such as phenylmethanesulfonyl fluoride (PMSF), Pefabloc SC, pepstatin, leupeptin, chymostatin and EDTA.

Polypeptides of the invention may be fused with a second polypeptide or polynucleotide moiety, which may be referred to as a "tag". A tag may be used, for example, to facilitate purification or detection of the polypeptide after expression. A fused polypeptide may be constructed, for example, by in-frame insertion of a polynucleotide encoding the tag on the 5' or 3' end of the

polynucleotide encoding the polypeptide to be expressed. The fused polynucleotide may then be expressed in the plasmid system of the invention. Such tags include glutathione-S-transferase (GST), hexahistidine (His6) tags, maltose binding protein (MBP) tags, haemagglutinin (HA) tags, cellulose binding protein (CBP) tags and myc tags. Detectable tags such as ^{32}P, ^{35}S, ^{3}H, ^{99m}Tc, ^{123}I, ^{111}In, ^{68}Ga, ^{18}F, ^{125}I, ^{131}I, ^{113m}In, ^{76}Br, ^{67}Ga, ^{99m}Tc, ^{123}I, ^{111}In and ^{68}Ga may also be used to label the polypeptides and polynucleotides of the invention. Methods for constructing and using such fusions are very conventional and well known in the art.

EXAMPLES

The following examples are provided to further describe the present invention and should not be construed as a limitation thereof. The scope of the present invention includes any and all plasmids set forth, below, in the following examples either individually or as part of a kit. Also included within the scope of the invention are any and all of the methods which are set forth below in the following examples.

Example 1: Construction of Amplifiable Cloning Vectors, pSRXBLS and pXBLS.

This example describes the construction of the mammalian expression vectors, pSRXBLS and pXBLS. A large multicloning site was inserted in the plasmid pDSRG downstream of the SRα promoter, to generate pSRXBLS. pXBLS, a derivative of pSRXBLS, is devoid of any promoter. Both of the plasmids can serve as amplifiable vectors into which more than one expression cassette, *e.g.*, for the heavy and light chain cDNAs of an antibody gene, can be easily inserted.

A multiple cloning site of 155 bp, pDSRG-xba-xho, was designed, synthesized by PCR and cloned initially in the TA Cloning Vector (Invitrogen;

Carlsbad, CA). It was later cloned at the XhoI and XbaI sites of pDSRG, resulting in pSRXBLS. The SRα promoter was retained in the pSRXBLS vector.

pSRXBLS was further digested with XhoI and HindIII to remove the SRα promoter. The ends were then filled in by Klenow and religated, regenerating the HindIII site, to construct pXBLS.

Example 2: Construction of Universal Transfer Vectors pUHLS, pULLS and Their Descendents.

This example describes the construction of universal transfer vectors, each having a large multiple cloning site, and their descendents, each carrying a promoter and a terminator/poly A addition site. pUHLS and pULLS are the universal transfer vectors, and pUHSRstopLS and pULSRstopLS are their corresponding descendents that carry the SRα promoter and the chicken β-globin terminator. The plasmid system is so constructed that different subunits of a large, complex protein can be expressed in these vectors separately. Later, the expression cassettes for each subunit can be transferred to a single vector, such as pXBLS or pSRXBLS, to facilitate transfection, integration and equimolar production of a multi-subunit protein.

Two multiple cloning sites, Universal Plasmid Primer 1 of 160 bp and Universal Plasmid Primer 2 of 166 bp, were designed to construct pUHLS and pULLS. Both of the cloning sites were synthesized by PCR, cloned in the TA Cloning Vector (Invitrogen) and later cloned at the BssHII sites of the pCRScript vector (Stratagene). Thus, the original multiple cloning site of pCRScript was replaced with newly synthesized multiple cloning sites. The new vectors, pUHLS and pULLS, were derived from the Universal Plasmid Primer 1 and Universal Plasmid Primer 2, respectively.

A 249 bp region of the chicken β-globin terminator, derived from pDSRG by digestion with BamHI and XbaI, was inserted in both pUHLS and pULLS at the BamHI and XbaI sites to generate pUHstopLS and pULstopLS, respectively. The

SRα promoter and its accompanying intron, derived from pDSRG by digestion with HindIII and SalI, was inserted in pUHstopLS and pULstopLS at the HindIII and XhoI sites to generate pUHSRstopLS and pULSRstopLS, respectively.

Example 3: Construction of pAIL5V1.

The construction of pAIL5V1 for the expression of heavy and light antibody chains in a single vector is described here. Aside from variations in their orientations, two types of plasmids have been constructed. The first carries only the *dhfr* marker for selection with amplification. The second type of expression plasmid carries the *dhfr* marker, along with the gene for hygromycin resistance (*Hyg*). This adds versatility, allowing selection with or without amplification.

The heavy chain gene of the anti-huIL5 human monoclonal antibody, (MAb) was isolated and inserted in the pUHSRstopLS vector (*supra*) at the EcoRI and XmaI sites to generate pUSRHLS. The light chain gene of the anti-huIL5 MAb was isolated and inserted in pULSRstopLS at the EcoRI and ApaI sites to generate pUSRLLS.

The *hCMV* minimal promoter, derived from pcDNA3.1 (Invitrogen; Carlsbad, CA) by digestion with NruI and EcoRI, replaced the *SRα* promoter, which was removed by digestion with SnaBI and EcoRI, in pUSRHLS and pUSRLLS to generate pUhCMVHLS and pUhCMVLLS. The TK-hygromycin gene (TK/Hyg) was inserted in pUhCMVHLS at the FseI sites to construct pUHhyg(+)hCMVLS and pUHhyg(-)hCMVLS. The light chain antibody cassette was transferred from pUhCMVLLS, by digestion with PacI and SrfI, to pXBLS at the PacI and SrfI sites to construct pAIL5L(-)hCMVLS and pAIL5L(+)hCMVLS. The heavy chain antibody cassette was transferred from pUHhyg(-)hCMVLS to pAIL5L(-)hCMVLS at the BssHII sites to generate pAIL5V1.

Example 4: Construction of pAIGFRV3.

cDNAs encoding the variable regions from a hybridoma expressing an anti-IGFR1 monoclonal antibody 19D12/15H12 were isolated and cloned in TA cloning vectors (Invitrogen; Carlsbad, CA). The light and heavy chain amino acid and nucleotide sequences of antibody 19D12/15H12 are set forth in U.S. Patent Application No. 10/443,466; filed May 22, 2003 which is herein incorporated by reference in its entirety. The heavy chain was transferred from the EcoRI and ApaI sites of the TA vector containing cDNA for heavy chain of variable region of anti-IGFR1 to the same sites of pUhCMVHLS (*supra*) to construct pUhCMVIGFRHLS containing cDNA for light chain of anti-IGFR1. For selection, a TK-hygromycin resistance cassette was inserted at the FseI site of pUhCMVIGFRHLS to construct pUhCMVHyg(-)IGFRHLS. The light chain was transferred from the EcoRI and BbsI sites of the TA plasmid to the same sites of pUhCMVLLS (*supra*) to construct pUhCMVIGFRLLS. The entire light chain expression cassette was then transferred from pUhCMVIGFRLLS to pXBLS at the PacI and SrfI sites to construct pAIGFRLLS. The heavy chain expression cassette, along with the hygromycin expression cassette, was transferred to pAIGFRLLS at the BssHII sites to construct pAIGFRV1 and pAIGFRV3 (pIAGFRV1 is essentially identical to pAIGFRV3 except that the orientation of the heavy chain and the TK-Hyg genes are opposite).

Example 5: Construction of pAIL10V3.

cDNAs encoding the variable regions of 12G8, a rat antibody which recognizes IL-10 were isolated. The heavy chain variable region of 12G8 was transferred to KpnI and ApaI site of pUhyg(-)IG1FRhuH plasmid to construct pUIL10H. The pUhyg(-)IG1FrhuH plasmid carries the modified cDNA for variable region of IGFR1 along with IgG1 cDNA and TK-Hygromycin cassette. The light chain variable region of 12G8 was transferred to the EcoRI and ApaI sites of pAIL5(-)hCMVLS to construct pAIL10(-)L. The heavy chain expression cassette

from the pUIL10H was transferred to pAIL10(-)L at BssHII restriction sites to construct pAIL10V3.

Example 6: Construction of pAIG1FR(-)IL2LS.

pAIG1FR(-)IL2LS was constructed in a three step process. The construction process started with transfer of an IRES-IL2Rα element to pULstopLS. The plasmid containing the IRES-IL2Rα, pme18IRES, was digested with SpeI and NotI restriction enzymes and the NotI site was completely filled in using Klenow enzyme to derive the IRES-IL2Rα element. Simultaneously, pULstopLS was digested with EcoRV and SpeI enzymes and the SpeI site was filled in, using the Klenow enzyme, and ligated with the IRES-IL2Rα element to construct pULstopIRESIL2R. pULstopIRESIL2R was further digested with SpeI and XbaI enzyme and SpeI site was completely filled in with Klenow enzyme. Also pUhCMVIGFRLLS was digested with XbaI and BspEI enzymes and the BspEI site was completely filled in using Klenow enzyme and ligated with the XbaI-SpeI fragment that was generated from pULstopIRESIL2R to construct pUIGFRL-IRESIL2R. The heavy chain expression cassette of IGFR1 was transferred from pUhyg(-)IG1FRhuH to pUIGFRL-IRESIL2R at BssHII restriction sites to construct pAIG1FR(-)IL2LS.

Example 7: Development of Cell Lines for Expressing Anti-IGFR1 Monoclonal Antibody 19D12.

In this example, the development and growth of cell lines for expressing the 19D12 antibody (LCF/HCA) are presented.

DXB11 Cell Culture. Cells were grown in MEM Alpha Medium with ribonucleosides and deoxyribonucleosides (GIBCO Cat. # 12571-063; Gibco-Invitrogen Corp; Carlsbad, CA) plus 10% FBS (HyClone Cat. # SH30071.03; Hyclone; Logan, UT).

Hygromycin selection media. Cells were split at 48 hours post-transfection. Cells were grown in MEM Alpha Medium without ribonucleosides and deoxyribonucleosides (GIBCO Cat. # 12561-056) plus 10% Dialyzed FBS (HyClone Cat. # SH30079.03) plus Hygromycin B (CLONTECH Cat. # 8057-1; BD Biosciences-Clontech; Palo Alto, CA) at 300 μg/mL.

Subcloning media. Subcloning was performed in MEM Alpha Medium without ribonucleosides and deoxyribonucleosides (GIBCO Cat. # 12561-056) plus 10% Dialyzed FBS (HyClone Cat. # SH30079.03).

Methotrexate (MTX) amplification media. Methotrexate amplification was carried out in MEM Alpha Medium without ribonucleosides and deoxyribonucleosides (GIBCO Cat. # 12561-056) plus 10% Dialyzed FBS (HyClone Cat. # SH30079.03) plus MTX (Sigma Cat. # M8407; Sigma-Aldrich Corp; St.Louis, MO) at 20, 80 and 320 nM, respectively.

Media for adaptation to serum free suspension. Adaptation to serum free suspension was performed in CHO Protein-Free Medium (Sigma Cat. # C5467) supplemented with 20 ml/L L-Glutamine-200 mM (GIBCO Cat. # 25030-081) and 10 ml/L Lipids (Cholesterol rich) (Sigma Cat. # L4646).

Feed medium for 3L production batch. L-Glutamine-200 mM (GIBCO Cat. # 25030-081) and Glucose Solution (Sigma Cat. # G8769) were served as the feed during production runs.

Transfection and Subcloning method. DXB11 cells were trypsinized, counted and plated @ 2 x 10^6 cells / T25 flask on the day before transfection, so that they became 50-90% confluent on the day of transfection. Transfections were performed using 2.5 μg DNA (pAIGFRV3) / T25 flask and LipofectAMINE PLUS™ reagent (GIBCO, cat. # 10964-013). As per the vendor's instructions, the DNA was first complexed with PLUS reagent, the DNA-PLUS complex was mixed with LipofectAMINE reagent and the DNA-PLUS-LipofectAMINE complex was then used to transfect the cells. The cells were incubated at 37^{0}C at 5% CO_2 for 3 hours. Following incubation, DXB11 cell culture medium was added to the

desired volume, the cells and medium were transferred to a T75 flask, and the cells were grown for 2 days. The medium was exchanged with hygromycin selection medium, and the cells were grown for 10 days to 2 weeks. Some cells were banked at this stage, and the remaining cells were subcloned in 96 well plates.

Subcloning was initiated in 96 well plates with subcloning media. Single clones were successively grown in 24 well plates, 6 well plates, T-25 flasks and T-75 flasks, following detection of satisfactory expression by ELISA at each stage. Methotrexate media was added on 20-30% confluent cultures for amplification. Amplification was carried out at 20, 40, 80, and 320 nM methotrexate for 10 days to 2 weeks. Following amplification, the media was exchanged with the subcloning media and the cells were allowed to grow to ~10% confluence. The cells were subjected to another round of subcloning at this stage. Following the second round of subcloning, the cells were subjected to adaptation to serum free suspension culture with the designated media at the T-25 flask stage. Serum was sequentially eliminated from the media by dilution with serum free adaptation medium, and the cells were finally transferred to shake flasks with 2.5% serum. The remaining serum was eliminated by subsequent dilution (splitting) of the cultures. The serum free culture was scaled to 3 liters.

Example 8: Propagation of cells expressing anti-IL5 antibody.

Cells carrying pAIL5V1 from a frozen vial are thawed and propagated in suspension using Sigma CHO protein-free medium (C-5467 supplemented with 0.57 g/L L-glutamine). All cultures are maintained in a 37°C, 7.5% CO_2 incubator or on a rocker bag platform set at 37°C and supplying 7.5% CO_2. The inoculum train begins in a shake flask and is continuously passaged and scaled-up until there is enough culture to start a 20-Liter bag with a 2-liter working volume. When the bag reaches the predetermined split criteria, it is scaled up to a 10-liter

working volume. When the bag reaches the predetermined split criteria, it is split and the remaining culture will be used to start another 20-liter rocker bag (10-liter working volume) in parallel. When the two rocker bags reach the appropriate split density, they are used to seed the production bioreactor. Shake flasks and rocker bags are typically split at 1:4 dilutions when the viable cell density reaches 1-1.5 x 10^6 viable cells/mL. The inoculum pool is diluted 1:4 going into the bioreactor.

Flow diagram illustrating the propagation process:

Vial

↓

Thaw

SF-250V (C-5467 CHO media supplemented with L-glutamine)

↓

20mL, then to 60mL (2 passages)

SF-1000V (C-5467 CHO media supplemented with L-glutamine)
250mL (1 passage), then create another SF-1000V with a 250mL working volume.

↓

20-liter rocker bag (C-5467 CHO media supplemented with L-glutamine)
2L (1passage), then to 10L (1passage), then create another 20-liter bag with 10L working volume.

↓

Production Bioreactor at quarter volume (C-5467 CHO media supplemented with L-glutamine)
60L (1passage), then to full volume (200L)

Example 9: Process for purifying anti-IL10 antibody.

This example describes the process for purifying the anti-IL10 antibody encoded by pAIL10V3 from a 200 liter CHO cell fermentation. The steps include:

- Harvesting of cell culture supernatant by filtration with a positively charged CUNO filter in series with a 0.2 µm filter.
- Affinity chromatography on Amersham rProtein-A Sepharose™ Fast Flow (4L) eluted by a pH 3.0 step.
- Viral Inactivation by incubation at pH 3.5 for 1 hour at 20-22°C, followed by pH adjustment to 5.5.
- Cation exchange chromatography on EMD Fractogel® SE HiCap (4L) at pH 5.5 eluted with a 20 BV gradient to 250mM NaCl.
- Concentration (2x) / Diafiltration (10x) into 20mM Tris, pH 8.0.
- Anion exchange chromatography on Amersham Q Sepharose™ Fast Flow (4L) in flow-through mode. The unretained peak is pooled and adjusted to pH 5.5.
- Viral Filtration with Planova filters: one 0.1m^2 Planova 35 in series with 2-4 0.1m^2 Planova 20 filters.
- Final concentration (6-10x) and diafiltration (10x) into 20mM sodium acetate followed by filtration (0.2 µm).

This process yields material that is >99% pure by RP-HPLC. Overall yield is 70%.

Example 10: Expression of anti-IGFR1 and anti-IL-10 antibody.

In this example, expression plasmids including the anti-IGFR and anti-IL-10 antibody chains were constructed wherein the antibody chain genes were situated, in the plasmids, adjacent to a MAR element (Selexis; Geneva, Switzerland; Kim *et al.*, J. Biotechnol. 107(2): 95-105 (2004); Stief *et al.*, Nature 341: 343–345 (1989); Phi-Van *et al.*, Mol. Cell. Biol. 10: 2302–2307 (1990); Kalos

et al., Mol. Cell. Biol. 15: 198–207 (1995)). The MAR element is a ~3 kb DNA element that aids the expression of a recombinant gene which is stably integrated in the host chromosome following incorporation into the cell.

The MAR element was inserted into the mammalian expression plasmids, pAIL10Vi, having anti-IL10 along with hygromycin expression cassette, pAIL10V1/puro, having anti-IL10 along with puromycin instead of hygromycin expression cassette, pAIGFRLCb2V1, having anti-IGFR1 along with hygromycin expression cassette, and pAIGFRLCb2V1/puro, having anti-IGFR1 along with puromycin instead of hygromycin expression cassette. Each plasmid already contained four independent mammalian expression cassettes.

The vector, pPAG01 contained the ~3 kb chicken lysozyme matrix attachment region (MAR) DNA element. One of the universal vectors, pULLS was digested by restriction enzymes, Age1 and BamH1 and was religated, following end filling by Klenow enzyme, to construct vector pULLSmod. The pPAG01 plasmid was digested by BamH1 and Xba1 to transfer the MAR element over to pULLSmod at the same sites to construct the plasmid pULMAR. The MAR element was finally transferred to the plasmids expressing anti-IL10 and anti-IGFR1. pULMAR was digested with BssHII and the fragment containing MAR element was transferred to the Asc1 sites of the plasmids pAIL10Vi, pAIL10V1/puro, pAIGFRLCb2V1 and pAIGFRLCb2V1/puro to construct pinAIL10/MAR(-), pAIL10V1/puro/MAR(-), pAIGFRLCb2/MAR(-) and pAIGFRLCb2/puro/MAR(-), respectively.

The MAR containing plasmids were introduced into the CHO cell line, DXB11 cells and the antibody chains were expressed. Expression of the antibody chains were confirmed by ELISA as well as HPLC analysis. In the HPLC analysis, the proteins isolated from the CHO cells was fractionated using a reverse-phase column or a protein-A column. Eluted protein was detected spectrophotometrically at A_{280nm}.

****

The present invention is not to be limited in scope by the specific embodiments described herein. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description. Such modifications are intended to fall within the scope of the appended claims.

Patents, patent applications, publications, product descriptions, and protocols are cited throughout this application, the disclosures of which are incorporated herein by reference in their entireties for all purposes.

WE CLAIM:

1. A plasmid system comprising in separate containers:

(a) a first universal transfer vector comprising the following, first multiple cloning site:

Bss HII, Pme I, Sna B1, Hin dIII, Asp 718, Kpn I, Pae R71, Xho I, Sal I, Acc I, Hinc II, Cla I, Eco RV, Eco RI, Pst I, Eco O109I, Eco O109I, Apa I, Xma I, Bsp EI, Bam H1, Dsa I, Eag I, Ecl XI, Not I, Sac II, Xma III, Xba I, Sac I, Mlu I, Bcl I, Bsr GI, Bss HII;

(b) a second universal transfer vector comprising the following, second multiple cloning site:

Bss HII, Sgr AI, Xma I, Rsr II, Spe I, Sna B1, Hin dIII, Asp 718, Kpn I, Pae R71, Xho I, Sal I, Acc I, Hinc II, Cla I, Eco RV, Eco RI, Pst I, Eco O109I, Eco O109I, Apa I, Xma I, Bsp EI, Bam H1, Dsa I, Eag I, Ecl XI, Not I, Sac II, Xma III, Xba I, Sac I, Nde I, Msc I, Nru I, Pac I, Bss HII; and

(c) an amplifiable vector comprising the following, third multiple cloning site:

Sgr AI, Srf I, Xma I, Spe I, Sac II, Rsr II, Pac I, Nru I, Not I, Nde I, Msc I, Mlu I, Kpn I, Fse 1, Bss HII, Bsr GI, Bsp EI, Bcl I, Bbv C1, Pme I, Bss HII, Asc I, Xba I.

2. The plasmid system of claim 1 wherein the first universal transfer vector comprises the plasmid map of Figure 2, the second universal transfer vector comprises the plasmid map of Figure 1 and the amplifiable vector comprises the plasmid map of Figure 3.

3. The plasmid system of claim 1 wherein the multiple cloning site of the first universal transfer vector comprises the nucleotide sequence set forth in SEQ ID NO: 11.

4. The plasmid system of claim 1 wherein the multiple cloning site of the second universal transfer vector comprises the nucleotide sequence set forth in SEQ ID NO: 12.

5. The plasmid system of claim 1 wherein the multiple cloning site of the amplifiable vector comprises the nucleotide sequence set forth in SEQ ID NO: 10.

6. The plasmid system of claim 1 wherein at least one of the plasmids comprises a promoter located upstream of or within the multiple cloning site.

7. The plasmid system of claim 6 wherein the promoter is selected from SRα promoter, mouse mammary tumor virus long terminal repeat (MMTV LTR), human cytomegalovirus (hCMV) immediate early promoter and murine cytomegalovirus (mCMV) immediate early promoter.

8. The plasmid system of claim 7 wherein the first universal transfer vector comprises the plasmid map of Figure 10.

9. The plasmid system of claim 7 wherein the second universal transfer vector comprises the plasmid map of Figure 11.

10. The plasmid system of claim 7 wherein the amplifiable vector comprises the plasmid map of Figure 9.

11. The plasmid system of claim 7 wherein the first universal transfer vector comprises the nucleotide sequence set forth in SEQ ID NO: 5.

12. The plasmid system of claim 7 wherein the second universal transfer vector comprises the nucleotide sequence set forth in SEQ ID NO: 4.

13. The plasmid system of claim 7 wherein the amplifiable vector comprises the nucleotide sequence set forth in SEQ ID NO: 13.

14. The plasmid system of claim 1 wherein at least one of the universal transfer vectors comprises a terminator/polyA addition site.

15. The plasmid system of claim 1 wherein the amplifiable vector comprises a *DHFR* gene.

16. A plasmid system comprising in separate containers:
(a) a first universal transfer vector comprising the nucleotide sequence set forth in SEQ ID NO: 2;
(b) a second universal transfer vector comprising the nucleotide sequence set forth in SEQ ID NO: 1; and
(c) an amplifiable vector comprising the nucleotide sequence set forth in SEQ ID NO: 3.

17. The plasmid system of claim 1 wherein the cassettes encode an immunoglobulin light chain or an immunoglobulin heavy chain.

18. A plasmid system according to claim 1 wherein the first or second universal transfer vector comprises a first set of one or more expression cassettes, the other universal transfer vector comprises a second set of one or more expression cassettes and the amplifiable vector comprises said first set and second set of expression cassettes; wherein:
(a) the first set of one or more expression cassettes comprises an anti-IL5 immunoglobulin heavy chain gene expression cassette and the second set of

one or more expression cassettes comprises an anti-IL5 immunoglobulin light chain gene expression cassette;

(b) the first set of one or more expression cassette comprises an anti-IGFR1 immunoglobulin heavy chain gene expression cassette and the second set of one or more expression cassette comprises an anti-IGFR1 immunoglobulin light chain gene expression cassette;

(c) the first set of one or more expression cassettes comprises an expression cassette comprising a bicistronic gene expression cassette which bicistronic gene comprises an anti-IGFR1 immunoglobulin light chain gene and an IL-2 receptor α gene wherein said genes are linked by an internal ribosome entry sequence (IRES) and the second set of one or more expression cassettes an anti-IGFR1 immunoglobulin heavy chain gene expression cassette and a hygromycin resistance gene expression cassette; or

(d) the first set of one or more expression cassettes comprises an anti-IL10 immunoglobulin heavy chain gene expression cassette and the second set of one or more expression cassettes comprises an anti-IL10 immunoglobulin light chain gene expression cassette and a hygromycin resistance gene expression cassette.

19. The plasmid system according to claim 18 wherein the amplifiable vector comprises a plasmid map as set forth in a figure selected from Figures 4-7.

20. The plasmid system of claim 19 wherein the amplifiable vector comprises a nucleotide sequence selected from SEQ ID NOs: 6-9.

21. The plasmid system of claim 1 wherein the first universal transfer vector, second universal transfer vector or the amplifiable vector comprises a matrix attachment region.

22. The plasmid system of claim 21 wherein the amplifiable vector comprises (a) an anti-IGFR antibody heavy chain or an anti-IL10 antibody heavy chain and (b) a puromycin resistance marker or a hygromycin resistance marker.

23. The plasmid system of claim 21 wherein the amplifiable vector is characterized by a plasmid map selected from the group consisting of figures 13-16.

24. The plasmid system of claim 21 wherein the amplifiable vector comprises a nucleotide sequence selected from the group consisting of SEQ ID NOs: 24-27.

25. A method for producing a protein comprising two or more types of polypeptide comprising the steps of:
(a) introducing a set of one or more expression cassettes into a first universal transfer vector comprising the following, first multiple cloning site: Bss HII, Pme I, Sna B1, Hin dIII, Asp 718, Kpn I, Pae R71, Xho I, Sal I, Acc I, Hinc II, Cla I, Eco RV, Eco RI, Pst I, Eco O109I, Eco O109I, Apa I, Xma I, Bsp EI, Bam H1, Dsa I, Eag I, Ecl XI, Not I, Sac II, Xma III, Xba I, Sac I, Mlu I, Bcl I, Bsr GI, Bss HII;
(b) introducing a set of one or more different expression cassettes into a second universal transfer vector comprising the following, second multiple cloning site: Bss HII, Sgr AI, Xma I, Rsr II, Spe I, Sna B1, Hin dIII, Asp 718, Kpn I, Pae R71, Xho I, Sal I, Acc I, Hinc II, Cla I, Eco RV, Eco RI, Pst I, Eco O109I, Eco O109I, Apa I, Xma I, Bsp EI, Bam H1, Dsa I, Eag I, Ecl XI, Not I, Sac II, Xma III, Xba I, Sac I, Nde I, Msc I, Nru I, Pac I, Bss HII;
(c) moving the cassettes from the transfer vectors into an amplifiable vector comprising the following, third multiple cloning site:
Sgr AI, Srf I, Xma I, Spe I, Sac II, Rsr II, Pac I, Nru I, Not I, Nde I, Msc I, Mlu I, Kpn I, Fse 1, Bss HII, Bsr GI, Bsp EI, Bcl I, Bbv C1, Pme I, Bss HII, Asc I, Xba I; and

(d) causing expression of said cassettes.

26. The method of claim 25 wherein the first universal transfer vector, second universal transfer vector or the amplifiable vector comprises a matrix attachment region.

27. The method of claim 26 wherein the amplifiable vector comprises (a) an anti-IGFR antibody heavy chain or an anti-IL10 antibody heavy chain and (b) a puromycin resistance marker or a hygromycin resistance marker.

28. The method of claim 26 wherein the amplifiable vector is characterized by a plasmid map selected from the group consisting of figures 13-16.

29. The method of claim 26 wherein the amplifiable vector comprises a nucleotide sequence selected from the group consisting of SEQ ID NOs: 24-27.

30. The method of claim 25 further comprising purifying the protein.

31. The method of claim 25 wherein:

(a) one expression cassette encodes an anti-IL5 immunoglobulin heavy chain and the other expression cassette encodes an anti-IL5 immunoglobulin light chain;

(b) one expression cassette encodes an anti-IGFR1 immunoglobulin heavy chain and the other expression cassette encodes an anti-IGFR1 immunoglobulin light chain;

(c) one expression cassette comprises a bicistronic gene encoding an anti-IGFR1 immunoglobulin light chain and an IL-2 receptor α-subunit which are linked by an internal ribosome entry sequence (IRES) and the other

expression cassette encodes an anti-IGFR1 immunoglobulin heavy chain and HYG-B; or

(d) one expression cassettes encodes an anti-IL10 immunoglobulin heavy chain and the other expression cassette encodes an anti-IL10 immunoglobulin light chain and HYG-B.

32. A method for producing an anti-IGFR1 antibody comprising the steps of:

(a) introducing an expression cassette comprising a polynucleotide encoding a polypeptide comprising an amino acid sequence selected from SEQ ID NOs: 18 and 22 or an expression cassette comprising a polynucleotide encoding a polypeptide comprising an amino acid sequence selected from SEQ ID NOs: 16 and 20 into a first universal transfer vector comprising the following, first multiple cloning site: Bss HII, Pme I, Sna B1, Hin dIII, Asp 718, Kpn I, Pae R71, Xho I, Sal I, Acc I, Hinc II, Cla I, Eco RV, Eco RI, Pst I, Eco O109I, Eco O109I, Apa I, Xma I, Bsp EI, Bam H1, Dsa I, Eag I, Ecl XI, Not I, Sac II, Xma III, Xba I, Sac I, Mlu I, Bcl I, Bsr GI, Bss HII;

(b) introducing the other expression cassette, not introduced into said first vector, into a second universal transfer vector comprising the following, second multiple cloning site:

Bss HII, Sgr AI, Xma I, Rsr II, Spe I, Sna B1, Hin dIII, Asp 718, Kpn I, Pae R71, Xho I, Sal I, Acc I, Hinc II, Cla I, Eco RV, Eco RI, Pst I, Eco O109I, Eco O109I, Apa I, Xma I, Bsp EI, Bam H1, Dsa I, Eag I, Ecl XI, Not I, Sac II, Xma III, Xba I, Sac I, Nde I, Msc I, Nru I, Pac I, Bss HII;

(c) moving the cassettes from the transfer vectors into an amplifiable vector comprising the following, third multiple cloning site:

Sgr AI, Srf I, Xma I, Spe I, Sac II, Rsr II, Pac I, Nru I, Not I, Nde I, Msc I, Mlu I, Kpn I, Fse 1, Bss HII, Bsr GI, Bsp EI, Bcl I, Bbv C1, Pme I, Bss HII, Asc I, Xba I; and

(d) causing expression of said cassettes.

33. The method of claim 32 further comprising purifying the antibody.

34. The method of claim 32 wherein the polynucleotide encoding a polypeptide comprising an amino acid sequence selected from SEQ ID NOs: 18 and 22 comprises a nucleotide sequence selected from SEQ ID NOs: 17 and 21.

35. The method of claim 32 wherein the polynucleotide encoding a polypeptide comprising an amino acid sequence selected from SEQ ID NOs: 16 and 20 comprises a nucleotide sequence selected from SEQ ID NOs: 15 and 19.

36. The method of claim 32 wherein the expression cassettes are operably linked to a human cytomegalovirus (*hCMV*) promoter.

37. A kit comprising the plasmid system of claim 1 and one or more components selected from

(i) sterile, distilled water;

(ii) calcium phosphate transformation reagents $CaCl_2$ and 2X HEPES buffered saline;

(iii) DEAE-dextran transformation reagents chloroquine in Phosphate buffered saline and phosphate buffered saline;

(iv) DOTAP/cholesterol extruded liposomes;

(v) Transformation competent *E.coli*;

(vi) Dulbecco/Vogt modified Eagle's minimal essential medium (DMEM);

(vii) Fetal calf serum;

(viii) luria broth media; and

(ix) paper instructions for usage of the plasmid system.

38. An oligonucleotide primer comprising a nucleotide sequence selected from SEQ ID NOs: 10, 11 and 12.

39. A plasmid comprising a nucleotide sequence selected from the group consisting of SEQ ID NOs: 6-9.