(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
26 October 2017 (26.10.2017)

WIPO | PCT

(10) International Publication Number
WO 2017/184832 A1

(51) International Patent Classification:
C07K 16/00 (2006.01) *C12N 15/90* (2006.01)
C07K 16/46 (2006.01)

(21) International Application Number:
PCT/US2017/028555

(22) International Filing Date:
20 April 2017 (20.04.2017)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
62/325,400 20 April 2016 (20.04.2016) US

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(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Declarations under Rule 4.17:
— *as to applicant's entitlement to apply for and be granted a patent (Rule 4.17(ii))*
— *as to the applicant's entitlement to claim the priority of the earlier application (Rule 4.17(iii))*
— *of inventorship (Rule 4.17(iv))*

Published:
— *with international search report (Art. 21(3))*
— *with sequence listing part of description (Rule 5.2(a))*

(54) Title: COMPOSITIONS AND METHODS FOR MAKING ANTIBODIES BASED ON USE OF EXPRESSION-ENHANCING LOCI

(57) Abstract: This invention relates to site-specific integration and expression of recombinant proteins in eukaryotic cells. In particular, the invention includes compositions and methods for improved expression of antigen-binding proteins including monospecific and bispecifc antibodies in eukaryotic cells, particularly Chinese hamster (*Cricetulus griseus*) cell lines, by employing multiple expression-enhancing locus.

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COMPOSITIONS AND METHODS FOR MAKING ANTIBODIES BASED ON USE OF EXPRESSION-ENHANCING LOCI

CROSS REFERENCE TO RELATED APPLICATION

[0001] This application claims the benefit of priority from U.S. Provisional Application No. 62/325,400, filed April 20, 2016, the entire contents of which are incorporated herein by reference.

FIELD OF THE DISCLOSURE

[0002] This disclosure relates to site-specific integration and expression of recombinant proteins in eukaryotic cells. In particular, the disclosure relates to compositions and methods for improved expression of antigen-binding proteins (including monospecific and bispecifc antibodies) in eukaryotic cells, particularly Chinese hamster (*Cricetulus griseus*) cell lines, by employing expression-enhancing loci.

BACKGROUND ART

[0003] Cellular expression systems aim to provide a reliable and efficient source for the manufacture of a given protein, whether for research or therapeutic use. Recombinant protein expression in mammalian cells is a preferred method for manufacturing therapeutic proteins due to, for example, the ability of mammalian expression systems to appropriately post-translationally modify recombinant proteins.

[0004] Despite the availability of various expression systems, the challenge of efficient gene transfer and stability of the integrated gene for expression of a recombinant protein still exists. For long-term expression of a target transgene, one consideration is minimal disruption of cellular genes to avoid changes in the phenotype of the cell line.

[0005] Engineering stable cell lines to accommodate multiple genes for expression, such as multiple antibody chains as in multispecific antibodies, is particularly challenging. Wide variations in expression levels of integrated genes may occur. Integrating additional genes may lead to greater variation in expression and instability due to the local genetic

environment (*i.e.*, position effects). Expression systems for the production of multispecific antigen-binding proteins often requires the expression of two or more different immunoglobulin chains intended to pair as a specific multimeric format, and can often weigh in favor of homodimer production, rather than the desired heterodimer or multimer combination. Accordingly, there is a need in the art for improved mammalian expression systems.

SUMMARY OF THE DISCLOSURE

[0006] In one aspect, this disclosure provides a cell that contains multiple exogenous nucleic acids integrated site-specifically in two expression-enhancing loci wherein the multiple exogenous nucleic acids together encode an antigen-binding protein. The antigen-binding protein can be a bispecific antigen-binding protein, or a conventional monospecific antigen-binding protein.

[0007] In some embodiments, a cell is provided that contains a first exogenous nucleic acid integrated within a first enhanced expression locus, and a second exogenous nucleic acid integrated within a second enhanced expression locus; wherein the first and second exogenous nucleic acids together encode an antigen-binding protein.

[0008] In some embodiments, the first exogenous nucleic acid contains a nucleotide sequence encoding a first heavy chain fragment (HCF), and the second exogenous nucleic acid contains a nucleotide sequence encoding a first light chain fragment (LCF).

[0009] In some embodiments, the second exogenous nucleic acid further includes a nucleotide sequence encoding a second HCF (also HCF*). The first and second HCFs can be the same, or different as in a bispecific antigen-binding protein. Each HCF- or LCF-encoding nucleotide sequence can encode amino acids from a constant region. In some embodiments, the nucleotide sequence encoding the first HCF encodes a first CH3 domain, and the nucleotide sequence encoding the second HCF (HCF*) encodes a second CH3 domain. In some embodiments, the first and second CH3 domains can differ in at least one amino acid position, such as a position that results in different Protein A binding characteristics. In other embodiments, the nucleotide sequences encoding the first and second CH domains differ from each other in that one of the nucleotide sequences has been codon modified.

[0010] In some embodiments, the first exogenous nucleic acid (containing the first HCF-encoding nucleotide sequence) further comprises a nucleotide sequence encoding a second LCF. The second LCF can be the same as or different from the first LCF in the second exogenous nucleic acid.

[0011] In many of the embodiments of a cell provided herein, each of the nucleotide sequences encoding a HCF or LCF is operably linked to a promoter independently so that transcription of each HCF or LCF-encoding sequence is regulated separately.

[0012] In some embodiments, a first RRS and a second RRS are positioned 5' and 3', respectively, relative to the first exogenous nucleic acid, and a third RRS and a fourth RRS are positioned 5' and 3', respectively, relative to the second exogenous nucleic acid, wherein the first and second RRSs are different, and the third and fourth RRSs are different. Generally, the RRSs within a pair of RRSs flanking an exogenous nucleic acid are different to avoid unintended recombination and removal of the exogenous nucleic acid. In some embodiments, the first, second, third and fourth RRS are all different from each other.

[0013] In embodiments where the first exogenous nucleic acid in the first locus includes the first HCF-encoding nucleotide sequence, and the second exogenous nucleic acid in the second locus includes both the first LCF-encoding sequence and the second HCF-encoding sequence, a first additional RRS can be present between the nucleotide sequence encoding the first LCF and the nucleotide sequence encoding the second HCF. The additional RRS can be different from each of the first, second, third and fourth RRSs. In some embodiments, the first additional RRS is included between a promoter to which the selectable marker gene is operably linked, and the selectable marker gene, or the additional RRS may be included in a selectable marker gene, or within an intron of a selectable marker gene, that is present between the first LCF-encoding sequence and an HCF-encoding sequence, or between a first HCF-encoding sequence and a second HCF-encoding sequence.

[0014] In embodiments where the first exogenous nucleic acid in the first locus includes the first HCF-encoding nucleotide sequence and the second HCF-encoding nucleotide sequence, and the second exogenous nucleic acid in the second locus includes both the first LCF-encoding sequence and the second HCF-encoding sequence, a first RRS and a second RRS can be present at 5' and 3', respectively, relative to the first exogenous nucleic acid, and a third

RRS and a fourth RRS can be present at 5' and 3', respectively, relative to the second exogenous nucleic acid, wherein the first and second RRSs are different, and the third and fourth RRSs are different. In some embodiments, the first and second HCFs are the same, and the first and second LCFs are the same, in which instances the RRSs can be engineered such that the first and third RRS are the same, and the second and fourth RRS are the same. In some embodiments, the first and second HCFs are different, and the first and second LCFs are the same, in which instances the RRSs can be engineered such that the first, second, third and fourth RRSs are all different from each other. Irrespective of whether the two HCFs are the same or different, an additional RRS can be present between the first LCF-encoding sequence and the second HCF-encoding sequence, and/or present between the second LCF encoding sequence and the first HCF-encoding sequence. The additional (middle) RRS is different from each of the first, second, third and fourth RRSs. The additional RRS can be included within a selectable marker gene, or within an intron of a selectable marker gene, placed between two HCF/LCF coding sequences.

[0015] In another aspect, cells are provided that contain pairs of RRSs integrated within two expression enhancing loci that can be used for integration of nucleic acids encoding antigen-binding proteins through RMCE.

[0016] In certain embodiments, cells are provided that contain pairs of RRSs integrated within two expression enhancing loci that can be used for simultaneous integration of nucleic acids encoding antigen-binding proteins through RMCE in the presence of a recombinase.

[0017] In some embodiments, a cell is provided that contains, integrated within a first enhanced expression locus, from 5' to 3': a first RRS, a first exogenous nucleic acid, and a second RRS; and integrated within a second enhanced expression locus, from 5' to 3': a third RRS, a second exogenous nucleic acid, and a fourth RRS; wherein the first and second RRS are different, and the third and fourth RRSs are different.

[0018] In some embodiments, the first exogenous nucleic acid includes a first selectable marker gene, and the second exogenous nucleic acid includes a second selectable marker gene, wherein the first and the second selectable marker genes are different.

[0019] In some embodiments, one or both of the first and second exogenous nucleic acids can include an additional RRS, i.e., an additional RRS between the first and second RRSs in the

first locus, and/or an additional RRS between the third and fourth RRS. The additional, middle RRS is different from the RRSs at the 5' and 3'. Where an additional RRS is included between a 5' RRS and a 3' RRS (e.g., between the first and second RRS), one selectable marker gene can be included between the 5' RRS and the additional (middle) RRS, and another, different selectable marker gene can be included between the additional RRS and the 3' RRS.

[0020] In another embodiment, the cell provides a first exogenous nucleic acid that includes a third RRS, i.e., an additional RRS between the first and second RRSs in the first locus, wherein the first and second RRSs flank two selection markers at the 5' and 3' ends of the expression cassette. In other embodiments, the second exogenous nucleic acid can also include an identical third RRS, i.e., an additional RRS between the first and second RRSs in the second locus, wherein the first and second RRSs flank two selection markers at the 5' and 3' ends of the expression cassette. The four selectable marker genes included between the first, third and second RRSs, are different from one another.

[0021] In another embodiment, the cell provides a first exogenous nucleic acid that includes a third RRS, i.e., an additional RRS between the first and second RRSs in the first locus, wherein the first and second RRSs flank two selection markers at the 5' and 3' ends of the expression cassette. In other embodiments, the second exogenous nucleic acid can include an sixth RRS, i.e., an additional RRS between a fourth and fifth RRSs in the second locus, wherein the fourth and fifth RRSs flank two selection markers at the 5' and 3' ends of the expression cassette. The four selectable marker genes included between RRSs, are different from one another.

[0022] In many of the embodiments, the cells provided herein are cells of a CHO cell line.

[0023] In various embodiments, the two enhanced expression loci utilized are selected from the group consisting of a locus containing nucleotide sequence at least 90% identical to SEQ ID NO: 1, a locus containing a nucleotide sequence at least 90% identical to SEQ ID NO: 2, and a locus containing a nucleotide sequence at least 90% identical to SEQ ID NO:3.

[0024] In a further aspect, vector sets are provided for integration and expression of bispecific antigen-binding proteins in a cell.

[0025] In some embodiments, the vector set includes a first vector containing from 5' to 3', a first RRS, a first nucleic acid containing a nucleotide sequence encoding a first HCF, and a second RRS; a second vector containing from 5' to 3', a third RRS, a second nucleic acid containing a nucleotide sequence encoding a second HCF, a fourth RRS; and a nucleotide sequence encoding a first LCF that is either within the first nucleic acid in the first vector, or is in a third vector different from the first and second vectors; wherein the first, second, third, and fourth RRSs are different; and wherein the bispecific antigen-binding protein contains the first HCF, the second HCF and the first LCF, and wherein the first and second HCFs are different.

[0026] In some embodiments, the nucleotide sequence encoding the first LCF is within the first nucleic acid in the first vector. In some embodiments, the first nucleic acid further includes a first selectable marker gene.

[0027] In some embodiments, the nucleotide sequence encoding the first LCF is provided in the third vector and is flanked by a 5' RRS and 3' RRS, wherein (i) the 3' RRS is the same as the first RRS, and the 5' RRS is different from the first and second RRSs, or alternatively (ii) the 5' RRS is the same as the second RRS, and the 3' RRS is different from the first and second RRSs. In some embodiments, the vectors can be designed such that the common RRS shared by the first and third vectors is provided in a split selectable marker gene format (or a split-intron format), e.g., placed at the 3' end of a 5' portion of a selectable marker gene on one of the first and third vectors, and is placed at the 5' end of the remaining 3' portion of the selectable marker gene on the other vector.

[0028] In some embodiments, the vector set further includes a nucleotide sequence encoding a second LCF that is provided either within the second nucleic acid in the second vector, or is in a fourth vector separate from the first, second and third vectors.

[0029] In some embodiments, the first and second LCFs are the same.

[0030] In some embodiments, the nucleotide sequence encoding the first LCF is included within the first nucleic acid in the first vector, and the nucleotide sequence encoding the second VL is provided on the fourth vector. In some embodiments, the nucleotide sequence encoding the second LCF on the fourth vector is flanked by a 5' RRS and 3' RRS, wherein (i) the 3' RRS is the same as the third RRS, and the 5' RRS is different from the third and fourth

RRSs, or (ii) the 5' RRS is the same as the fourth RRS, and the 3' RRS is different from the third and fourth RRSs. In certain embodiments, the vectors are designed such that common RRS shared by the second and fourth vectors is provided in a split marker (e.g., via an intron) format, e.g., placed at the 3' end of a 5' portion of a selectable marker gene on one of the second and fourth vectors, and is placed at the 5' end of the remaining 3' portion of the selectable marker gene on the other vector.

[0031] In some embodiments, the nucleotide sequence encoding the first LCF is within the first nucleic acid in the first vector, and the nucleotide sequence encoding the second VL is within the second nucleic acid on the second vector.

[0032] In some embodiments, the nucleotide sequence encoding the first LCF is on the third vector, and the nucleotide sequence encoding the second VL is on the fourth vector. In some embodiments, the nucleotide sequence encoding the first LCF on the third vector is flanked by a 5' RRS and 3' RRS, wherein (i) the 3' RRS on the third vector is the same as the first RRS, and the 5' RRS on the third vector is different from the first and second RRSs, or (ii) the 5' RRS on the third vector is the same as the second RRS, and the 3' RRS on the third vector is different from the first and second RRSs; and wherein the nucleotide sequence encoding the second LCF on the fourth vector is flanked by a 5' RRS and 3' RRS, wherein (i) the 3' RRS on the fourth vector is the same as the third RRS, and the 5' RRS on the fourth vector is different from the third and fourth RRSs, or (ii) the 5' RRS on the fourth vector is the same as the fourth RRS, and the 3' RRS on the fourth vector is different from the third and fourth RRSs.

[0033] In many embodiments of a vector set provided herein, the nucleotide sequence encoding the first HCF can encode a first CH3 domain, and the nucleotide sequence encoding the second HCF can encode a second CH3 domain. In some embodiments, the first and second CH3 domains differ in at least one amino acid. In some embodiments, the nucleotide sequences encoding the first and second CH3 domains differ in that one of the nucleotide sequences has been codon modified.

[0034] In many embodiments of a vector set provided herein, each of the nucleotide sequences encoding a HCF or LCF is independently linked a promoter.

[0035] In some embodiments, a vector set can further include a nucleotide sequence encoding one or more recombinases that recognize one or more of the RRSs, which can be included in one of the LCF- or HCF--encoding vectors, or provided in a separate vector.

[0036] In still other embodiments, a vector set is provided that includes a first vector containing a first nucleic acid, flanked by a 5' homology arm and a 3' homology arm for integration into a first expression enhancing locus of a cell; and a second vector containing a second nucleic acid, flanked by a 5' homology arm and a 3' homology arm for integration into a second expression enhancing locus of the cell; wherein the first and second nucleic acids together encode an antigen-binding protein.

[0037] In a further aspect, this disclosure provides systems that include a combination of a cell (e.g., a CHO cell) with one or more vectors, and that can be utilized to make cells having integrated within two expression enhancing loci exogenous nucleic acids that together encode an antigen binding protein, either a monospecific protein or a bispecific protein. The systems can be provided in the form of a kit, for example.

[0038] In certain embodiments, a system is provided that includes a cell and a set of vectors, wherein the cell contains, integrated within two separate enhanced expression loci of its genome a set of RRSs that are different from one another and spaced between one or more exogenous nucleic acids, such as selection markers, for recombinant exchange with genes of interest in a set of vectors; and wherein the RRSs in the set of vectors comprise the same arrangement as the RRSs in the cell.

[0039] In some embodiments, a system is provided that includes a cell and a set of vectors, wherein the cell contains, integrated within a first enhanced expression locus: from 5' to 3', a first RRS, a first exogenous nucleic acid, and a second RRS, and integrated within a second enhanced expression locus: from 5' to 3', a third RRS, a second exogenous nucleic acid, and a fourth RRS; wherein the first and second RRSs are different, and the third and fourth RRSs are different; and wherein the first and second enhanced expression loci are different; wherein the vector set includes (i) a first vector containing from 5' to 3', a first vector 5' RRS, a first nucleic acid, and a first vector 3' RRS, wherein the first vector 5' and 3' RRSs are different; (ii) a second vector containing from 5' to 3', a second vector 5' RRS, a second nucleic acid, and a second vector 3' RRS, wherein the second vector 5' and 3' RRSs are different; and (iii) a

nucleotide sequence encoding a first HCF and a nucleotide sequence encoding a first LCF, wherein one of the two heavy chain-encoding nucleotide sequences is in the first nucleic acid and the other nucleotide sequences is in the second nucleic acid; wherein the first HCF and the first LCF are regions of an antigen-binding protein; and wherein upon introduction of the vectors into the cell, the first and second nucleic acids in the vectors integrate into the first enhanced expression locus and the second enhanced expression locus, respectively, through recombination mediated by the RRSs.

[0040] In some embodiments, the antigen-binding protein is a monospecific antigen-binding protein.

[0041] In some embodiments, the first and third RRSs are the same, and the second and fourth RRSs are the same. In certain embodiments, a first additional RRS is present between the first and second RRS in the first locus. In some embodiments, the first vector 5' RRS is the same as the first and third RRS; the first vector 3' RRS, the second vector 5' RRS, and the first additional RRS are the same; and the second vector 3' RRS is the same as the second and fourth RRS. In some embodiments, the LCF-encoding nucleotide sequence is in the first vector, and the HCF-encoding nucleotide sequence is in the second vector. In some embodiments, the first vector 3' RRS is placed at the 3' end of a 5' portion of a selectable marker gene, and the second vector 5' RRS is placed at the 5' end of the remaining 3' portion of the selectable marker gene. In other embodiments, the first vector 5' RRS is the same as the first RRS, and the first vector 3' RRS is the same as the second RRS; and wherein the second vector 5' RRS is the same as the third RRS, and the second vector 3' RRS is the same as the fourth RRS.

[0042] In various embodiments, the antigen-binding protein is a bispecific antigen-binding protein.

[0043] In some embodiments, the vector set in the system further includes a nucleotide sequence encoding a second HCF that is different from the first HCF.

[0044] In some embodiments, the nucleotide sequence encoding the first LCF and the nucleotide sequence encoding the second HCF are both included in the first nucleic acid in the first vector, and the nucleotide sequence encoding the first HCF is in the second vector. In some embodiments, the first vector 5' RRS is the same as the first RRS, the first vector 3'

RRS is the same as the second RRS, the second vector 5' RRS is the same as the third RRS, and the second vector 3' RRS is the same as the fourth RRS.

[0045] In some embodiments, the nucleotide sequence encoding the second HCF is on a third, separate vector, flanked by a third vector 5' RRS and a third vector 3' RRS. In some embodiments, the nucleotide sequence encoding the first LC is in the first vector, the nucleotide sequence encoding the first HCF is in the second vector, the first vector 5' RRS is the same as the first RRS, the first vector 3' RRS is the same as the second vector 5' RRS and as a first additional RRS, and the second vector 3' RRS is the same as the second RRS, the third vector 5' RRS is the same as the third RRS, and the third vector 3' RRS is the same as the fourth RRS, wherein the first additional RRS is included in the first locus between the first and second RRSs. In some embodiments, the vectors are designed to provide the common RRS in a split marker format, e.g., the first vector 3' RRS is placed at the 3' end of a 5' portion of a selectable marker gene included in the first vector, and the second vector 5' RRS is placed at the 5' end of the remaining selectable marker gene included in the second vector.

[0046] In some embodiments, the vector set of the system further includes a nucleotide sequence encoding a second LCF, which can be the same or different from the first LCF.

[0047] In some embodiments, the nucleotide sequence encoding the second LCF is in the second nucleic acid of the second vector, wherein the first vector 5' RRS is the same as the first RRS, the first vector 3' RRS is the same as the second RRS, the second vector 5' RRS is the same as the third RRS, and the second vector 3' RRS is the same as the fourth RRS.

[0048] In some embodiments, the nucleotide sequence encoding the second LCF is in a third, separate vector, flanked by a third vector 5' RRS and a third vector 3' RRS. In some embodiments, the first vector 5' and 3' RRS are identical to the first and second RRS in the first locus, respectively; the third vector 5' RRS is the same as the third RRS, the third vector 3' RRS is the same as the second vector '5 RRS and as an additional RRS present between the third and fourth RRSs in the second locus, the second vector 3' RRS is the same as the fourth RRS. In some embodiments, the common RRS is designed in a split marker format, e.g., the third vector 3' RRS is placed at the 3' end of a 5' portion of a selectable marker gene included in the third vector, and the second vector '5 RRS is placed at the 5' end of the remaining 3' portion of the selectable marker gene included in the second vector.

[0049] In many embodiments of a system provided herein, the nucleotide sequence encoding a HCF or LCF can encode amino acids from a constant region. In some embodiments, the nucleotide sequence encoding the first HCF can encode a first CH3 domain, and the nucleotide sequence encoding the second HCF can encode a second CH3 domain. In some embodiments, the first and second CH3 domains differ in at least one amino acid. In some embodiments, the nucleotide sequences encoding the first and second CH3 domains differ in that one of the nucleotide sequences has been codon modified.

[0050] In many embodiments of a system provided herein, each of the nucleotide sequences encoding a HCF or LCF is independently linked to a promoter.

[0051] In some embodiments, the vector set of a system can further include a nucleotide sequence encoding one or more recombinases that recognize one or more of the RRSs, which can be included in one of the HCF- or LCF- encoding vectors, or provided in a separate vector.

[0052] In various embodiments, the cell in a system provided herein is a CHO cell.

[0053] In various embodiments, the two enhanced expression loci are selected from the group consisting of a locus comprising the nucleotide sequence of SEQ ID NO: 1, a locus comprising the nucleotide sequence of SEQ ID NO: 2, and a locus comprising the nucleotide sequence of SEQ ID NO: 3.

[0054] In another aspect, this disclosure also provides methods of making bispecific antigen-binding proteins.In one embodiment, the method utilizes a system disclosed herein and introduces the vectors of the system into the cell of the system by transfection. Transfected cells where the exogenous nucleic acids have been properly integrated into two enhanced expression loci of the cell through RMCE can be screened and identified. HCF-containing polypeptides and LCF-containing polypeptides can be expressed from the integrated nucleic acids, and the antigen-binding protein of interest can be obtained from the identified transfected cell, and purified using known methods.

[0055] In another embodiment, the method simply utilizes a cell described hereinabove, which contains exogenous nucleic acids integrated at two enhanced expression loci that together encode an antigen-binding protein, and expresses the antigen-binding protein from the cell.

BRIEF DESCRIPTION OF THE DRAWINGS

[0056] Figure 1. An exemplary antibody cloning strategy for integration within one expression enhancing locus compared to multiple expression enhancing loci for a conventional monospecific antibody. Two vectors were transfected into the host cell, the first vector carrying a nucleic acid encoding antibody chain 1 (AbC1), such as a light chain, and a second vector carrying a nucleic acid encoding antibody chains 2 (AbC2), such as a heavy chain, and a selection marker different from the markers integrated within the targeted locus of the host cell. From 5′ to 3′: RRS1, middle RRS3 and RRS2 sites of the vector constructs match the RRS sites flanking selection markers within the loci of the host cell. An additional vector transfected into the host cells encodes for a recombinase. When the selection marker of the second vector is an antibiotic resistance gene, and since the two vectors are engineered to combine and allow expression of the marker, positive recombinant clones are selected for growth in the antibiotic. Alternatively, fluorescent marker enables positive clone selection by fluorescent activated cell sorting (FACS) analysis. The same vectors may be utilized for site-specific integration at a single locus, such as the EESYR® locus (Locus 1).

[0057] Figure 2. Using the two vector cloning strategy for integration within one expression enhancing locus versus two loci, antibody A and antibody B were cloned into the loci as depicted in Figure 1. Cells expressing antibody were isolated and subjected to 12 day fed batch culture, followed by harvest and an Octet titer assay using Protein A sensors. The cells were also observed to be isogenic and stable. Overall titer was observed to increase of 0.5 to 0.9 fold from utilization of the two site integration method.

[0058] Figure 3. An exemplary antibody cloning strategy for integration within one expression enhancing locus compared to integration within two separate expression enhancing loci for a bispecific antibody encoded by three antibody chains. Three vectors were utilized for this bispecific strategy, a first vector carrying a nucleic acid encoding antibody chains 1 (AbC1), for example a common light chain, flanked by RRS1 and RRS3, for integration into EESYR® (SEQ ID NO:1; Locus 1); a second vector carrying antibody chain 2 (AbC2), for example a heavy chain, having an upstream selection marker, flanked by RRS4 and RRS6, for integration into the SEQ ID NO:2 locus; and additionally a third vector carrying a nucleic acid encoding a second copy AbC1 linked to a different selection marker than in the second

vector and linked to antibody chain 3 (AbC3), for example a second (different) heavy chain, flanked 5′ by RRS4 and 3′ by RRS5 in the vector cassette (5′ and 3′ RRSs matched to the RRS sites in the host cell at the locus comprising SEQ ID NO:2). The two vector system as shown may be utilized for site-specific integration at a single locus, such as the EESYR® locus (comprising SEQ ID NO:1; Locus 1). Titers from the respective production cell lines were analyzed, see Figure 5.

[0059] Figure 4. An exemplary antibody cloning strategy for integration within one expression enhancing locus compared to integration within two separate expression enhancing loci for a bispecific antibody encoded by three or four antibody chains. Four vectors are utilized for this bispecific strategy, a first vector carrying a nucleic acid encoding antibody chain 1 (AbC1), for example a first light chain, flanked by RRS1 and RRS3, for integration into EESYR® (SEQ ID NO:1; Locus 1); a second vector carrying antibody chain 2 (AbC2), for example a heavy chain, having an upstream selection marker, flanked 5′ by RRS3 and 3′ by RRS2, for integration also into the EESYR® locus (comprising (SEQ ID NO:1; Locus 1); and a third vector carrying a nucleic acid encoding a different selection marker than in the second vector and linked to antibody chain 3 (AbC3), for example a second (different) heavy chain, flanked 5′ by RRS6 and 3′ by RRS5 in the vector cassette (5′ and 3′ RRSs matched to the RRS sites in the host cell at the locus comprising SEQ ID NO: 2; Locus 2); and additionally a fourth vector carrying a nucleic acid encoding a second light chain, for example antibody chain 1 (AbC1) (however may be the same or different than first light chain). The four vector system as shown may be utilized for site-specific integration at two loci, such as the EESYR® locus (comprising SEQ ID NO:1; Locus 1) and a locus comprising SEQ ID NO:2 or 3. The four vector system is compared to a two vector system integration at one locus (the EESYR® locus; Locus 1).

[0060] Figure 5. Using the two vector cloning strategy for integration within one expression enhancing locus versus two loci, bispecific antibody C and bispecific antibody D were cloned, cells isolated and subjected to 12 day fed batch culture, followed by harvest and an Octet titer assay using immobilized anti-Fc, and a second anti-Fc* (modified Fc detection antibody, see US 2014-0134719 A1, published May 15, 2014). The cells were also observed to be isogenic and stable. Total bispecific titer (heterodimer formation) was observed to increase of 1.75 to

2 fold utilizing the two site integration method, compared to expression of the bispecific antibody (heterodimer) in one integration site.

[0061] Figures 6A and 6B. Bispecific antibody E (Ab E), bispecific antibody F (Ab F), bispecific antibody G (Ab G), and bispecific antibody H (Ab H) were cloned in RSX or RSX^{2BP}. Each bispecific antibody-expressing cell comprises a (common) light chain nucleotide, heavy chain nucleotide (wild-type Fc) and modified heavy chain nucleotide (Fc*) in either one expression-enhancing locus (RSX) or two expression-enhancing loci (RSX^{2BP}). Cells were isolated and subjected to 13 day fed batch culture in bioreactors, followed by harvest and HPLC elution methods to determine overall antibody and bispecific antibody titers (Figure 6A). Ratio of bispecific antibody species titer (purified away from the homodimeric species) per total antibody titer was determined as a percentage total Ab (Figure 6B).

[0062] Figure 7. Monospecifc antibodies J and K (Ab J and Ab K, respectively) were cloned in RSX or RSX^2. Cells were isolated and subjected to 13 day fed batch culture in bioreactors, followed by harvest and HPLC methods to determine overall IgG titers.

DETAILED DESCRIPTION

Definitions

[0063] The term "antibody", as used herein, includes immunoglobulin molecules comprised of four polypeptide chains, two heavy (H) chains and two light (L) chains inter-connected by disulfide bonds. Each heavy chain may comprise a heavy chain variable region (abbreviated herein as HCVR or VH) and a heavy chain constant region. The heavy chain constant region comprises three domains, CH1, CH2 and CH3 and a hinge. Each light chain comprises a light chain variable region (abbreviated herein as LCVR or VL) and a light chain constant region. The light chain constant region comprises one domain, CL. The VH and VL regions can be further subdivided into regions of hypervariability, termed complementarity determining regions (CDR), interspersed with regions that are more conserved, termed framework regions (FR). Each VH and VL is composed of three CDRs and four FRs, arranged from amino-terminus to carboxy-terminus in the following order: FR1, CDR1, FR2, CDR2, FR3, CDR3,

FR4 (heavy chain CDRs may be abbreviated as HCDR1, HCDR2 and HCDR3; light chain CDRs may be abbreviated as LCDR1, LCDR2 and LCDR3).

[0064] The phrase "antigen-binding protein" includes a protein that has at least one CDR and is capable of selectively recognizing an antigen, i.e., is capable of binding an antigen with a $_{KD}$ that is at least in the micromolar range. Therapeutic antigen-binding proteins (e.g., therapeutic antibodies) frequently require a $_{KD}$ that is in the nanomolar or the picomolar range. Typically, an antigen-binding protein includes two or more CDRs, e.g., 2, 3, 4, 5, or 6 CDRs. Examples of antigen binding proteins include antibodies, antigen-binding fragments of antibodies such as polypeptides containing the variable regions of heavy chains and light chains of an antibody (e.g., Fab fragment, F(ab')$_2$ fragment), and proteins containing the variable regions of heavy chains and light chains of an antibody and containing additional amino acids from the constant regions of heavy and/or light chains (such as one or more constant domains, i.e., one or more of CL, CH1, hinge, CH2, and CH3 domains).

[0065] The phrase "bispecific antigen-binding protein" includes antigen-binding proteins capable of selectively binding, or having different specificities to, two or more epitopes - either on two different molecules (e.g., antigens) or on the same molecule (e.g., on the same antigen). The antigen binding portion, or fragment antigen binding (Fab) portion of such protein renders specificity to a particular antigen, and is typically comprised of a heavy chain variable region and a light chain variable region of an immunoglobulin. In some circumstances, the heavy chain variable region and light chain variable region may not be a cognate pair, in other words, have a different binding specificities.

[0066] An example of a bispecific antigen-binding protein is a "bispecific antibody", which includes an antibody capable of selectively binding two or more epitopes. Bispecific antibodies generally comprise two different heavy chains, with each heavy chain specifically binding a different epitope - either on two different molecules (e.g., antigens) or on the same molecule (e.g., on the same antigen). If a bispecific antigen-binding protein is capable of selectively binding two different epitopes (a first epitope and a second epitope), the affinity of the variable region of the first heavy chain for the first epitope will generally be at least one to two or three or four orders of magnitude lower than the affinity of the variable region of the first heavy chain for the second epitope, and vice versa. Bispecific antigen-binding proteins

such as bispecific antibodies can include the variable regions of heavy chains that recognize different epitopes of the same antigen. A typical bispecific antibody has two heavy chains each having three heavy chain CDRs, followed by (N-terminal to C-terminal) a CH1 domain, a hinge, a CH2 domain, and a CH3 domain, and an immunoglobulin light chain that either does not confer antigen-binding specificity but that can associate with each heavy chain, or that can associate with each heavy chain and that can bind one or more of the epitopes bound by the heavy chain antigen-binding regions, or that can associate with each heavy chain and enable binding of one or both of the heavy chains to one or both epitopes. In one embodiment, an Fc domain includes at least CH2 and CH3. An Fc domain may include a hinge, a CH2 domain and CH3 domain.

[0067] One embodied bispecific format includes, a first heavy chain (HC), a second heavy chain which has a modified CH3 (HC*), and a common light chain (LC) (two copies of the same light chain). Another embodiment includes a first heavy chain (HC), a common LC and a HC-ScFv fusion polypeptide (wherein the second HC is fused to the N-terminus of the ScFv). Another embodiment includes a first HC, a cognate LC, an HC-ScFv fusion polypeptide (wherein the second HC is fused to the N-terminus of the ScFv). Another embodiment includes a first heavy chain (HC), a LC and an Fc domain. Another embodiment includes a first HC, an LC, an ScFv-Fc fusion polypeptide (wherein the Fc is fused to the C-terminus of the ScFv). Another embodiment includes a first HC, a common LC, and an Fc-ScFv fusion polypeptide (wherein the Fc is fused to the N-terminus of the ScFv). Another embodiment includes a first HC, a LC and an ScFv-HC (wherein the second HC is fused to the C-terminus of the ScFv).

[0068] In certain embodiments, one heavy chain (HC) may be native or "wild-type" sequence and the second heavy chain may be modified in the Fc domain. In other embodiments, one heavy chain (HC) may be native or "wild-type" sequence and the second heavy chain may be codon-modified.

[0069] The term "cell" includes any cell that is suitable for expressing a recombinant nucleic acid sequence, and has a locus that allows for stable integration and enhanced expression of an exogenous nucleic acid. Cells include mammalian cells, such as non-human animal cells, human cells, or cell fusions such as, for example, hybridomas or quadromas. In some

embodiments, the cell is a human, monkey, ape, hamster, rat, or mouse cell. In some embodiments, the cell is a mammalian cell selected from the following cells: CHO (e.g., CHO K1, DXB-11 CHO, Veggie-CHO), COS (e.g., COS-7), retinal cell, Vero, CV1, kidney (e.g., HEK293, 293 EBNA, MSR 293, MDCK, HaK, BHK), HeLa, HepG2, WI38, MRC 5, Colo205, HB 8065, HL-60, (e.g., BHK21), Jurkat, Daudi, A431 (epidermal), CV-1, U937, 3T3, L cell, C127 cell, SP2/0, NS-0, MMT 060562, Sertoli cell, BRL 3A cell, HT1080 cell, myeloma cell, tumor cell, and a cell line derived from an aforementioned cell. In some embodiments, the cell comprises one or more viral genes, e.g. a retinal cell that expresses a viral gene (e.g., a PER.C6™ cell).

[0070] "Cell density" refers to the number of cells per volume of sample, for example as number of total (viable and dead) cells per mL. The number of cells may be counted manually or by automation, such as with a flow cytometer. Automated cell counters have been adapted to count the number of viable or dead or both viable/dead cells using for example a standard tryptan blue uptake technique. The phrase "viable cell density" or "viable cell concentration" refers to the number of viable cells per volume of sample (also referred to as "viable cell count"). Any number of well-known manual or automated techniques may be used to determine cell density. Online biomass measurements of the culture may be measured, where the capacitance or optical density is correlated to the number of cells per volume. Final cell density in a cell culture, such as in a production culture, varies depending on the starting cell line, for example in the range of about 1.0 to 10 x 10^6 cells/mL. In some embodiments, final cell density reaches 1.0 to 10 x 10^6 cells/mL prior to harvest of protein of interest from a production cell culture. In other embodiments, final cell density reaches greater than 5.0 x 10^6 cells/mL, greater than 6 x 10^6 cells/mL greater than 7 x 10^6 cells/mL greater than 8 x 10^6 cells/mL, greater than 9 x 10^6 cells/mL, or greater than 10 x 10^6 cells/mL.

[0071] The term "codon modified" means that a protein-coding nucleotide sequence has been modified in one or more nucleotides, i.e., one or more codons, without changing the amino acids encoded by the codons, resulting in a codon-modified version of the nucleotide sequence. Codon modification of a nucleotide sequence can provide a convenient basis to differentiate a nucleotide sequence from its codon-modified version in a nucleic acid-based assay (e.g., a hybridization based assays, PCR, among others). In some instances, codons of a

nucleotide sequence are modified to provide improved or optimized expression of the encoded protein in a host cell by employing codon optimization techniques well known in the art (Gustafsson, C., et al., 2004, *Trends in Biotechnology*, 22:346–353; Chung, B.K.-S., et al., 2013, Journal of Biotechnology, 167:326–333; Gustafsson, C., et al., 2012, *Protein Expr Purif*, 83(1): 37–46). Sequence design software tools using such techniques are also well-known in the art, including but not limited to Codon optimizer (Fuglsang A. 2003, *Protein Expr Purif*, 31:247–249), Gene Designer (Villalobos A, et al., 2006, *BMC Bioinforma*, 7:285), and OPTIMIZER (Puigbò P, et al. 2007, *Nucleic Acids Research*, 35:W126-W131), among others..

[0072] The phrase "complementarity determining region," or the term "CDR," includes an amino acid sequence encoded by a nucleic acid sequence of an organism's immunoglobulin genes that normally (i.e., in a wild-type animal) appears between two framework regions in a variable region of a light or a heavy chain of an immunoglobulin molecule (e.g., an antibody or a T cell receptor). A CDR can be encoded by, for example, a germline sequence or a rearranged or unrearranged sequence, and, for example, by a naive or a mature B cell or a T cell. In some circumstances (e.g., for a CDR3), CDRs can be encoded by two or more sequences (e.g., germline sequences) that are not contiguous (e.g., in an unrearranged nucleic acid sequence) but are contiguous in a B cell nucleic acid sequence, e.g., as the result of splicing or connecting the sequences (e.g., V-D-J recombination to form a heavy chain CDR3).

[0073] The term "expression enhancing locus" refers to a locus in the genome of a cell that contains a sequence or sequences and exhibits a higher level expression as compared to other regions or sequences in the genome when a suitable gene or construct is exogenously added (i.e., integrated) in or near the sequence or sequences, or "operably linked" to the sequence or sequences.

[0074] The term "enhanced" when used to describe enhanced expression includes an enhancement of at least about 1.5-fold to at least about 3-fold enhancement in expression over what is typically observed by random integration of an exogenous sequence into a genome or by integration at a different locus, for example, as compared to a pool of random integrants of a single copy of the same expression construct. Fold-expression enhancement observed

employing the sequences of the invention is in comparison to an expression level of the same gene, measured under substantially the same conditions, in the absence of a sequence of the invention, for example in comparison to integration at another locus into the same species genome. Enhanced recombination efficiency includes an enhancement of the ability of a locus to recombine (for example, employing recombinase-recognition sites ("RRS")). Enhancement refers to an efficiency of recombination over random recombination for example, without employing recombinase-recognition sites or the like, which is typically 0.1%. A preferred enhanced recombination efficiency is about 10-fold over random, or about 1%. Unless specified, the claimed invention is not limited to a specific recombination efficiency. Enhanced expression loci typically support high productivity of the protein of interest by the host cell. Hence, enhanced expression includes high production of the protein of interest (elevated titer in grams of protein) per cell, rather than attaining high titers simply by high copy number of cells in culture. Specific productivity Qp (pg/cell/day, i.e. pcd) is considered a measure of sustainable productivity. Recombinant host cells exhibiting Qp greater than 5 pcd, or greater than 10 pcd, or greater than 15 pcd, or greater than 20 pcd, or greater than 25 pcd, or even greater than 30 pcd are desirable. Host cells with a gene of interest inserted into an expression-enhancing locus, or "hotspot", exhibit high specific productivity.

[0075] Where the phrase "exogenously added gene", "exogenously added nucleic acid", or simply "exogenous nucleic acid", is employed with reference to a locus of interest, the phrase refers to any DNA sequence or gene not present within the locus of interest as the locus is found in nature. For example, an "exogenous nucleic acid" within a CHO locus (*e.g.*, a locus comprising a sequence of SEQ ID NO: 1 or SEQ ID NO: 2), can be a hamster gene not found within the particular CHO locus in nature (*i.e.*, a hamster gene from another locus in the hamster genome), a gene from any other species (*e.g.*, a human gene), a chimeric gene (*e.g.*, human/mouse), or any other gene not found in nature to exist within the CHO locus of interest.

[0076] The phrase "heavy chain," or "immunoglobulin heavy chain" includes an immunoglobulin heavy chain constant region sequence from any organism, and unless otherwise specified includes a heavy chain variable domain. Heavy chain variable domains

include three heavy chain CDRs and four FR regions, unless otherwise specified. A typical heavy chain has, following the variable domain (from N-terminal to C-terminal), a CH1 domain, a hinge, a CH2 domain, and a CH3 domain. The term "a fragment of a heavy chain" includes a peptide of at least 10, 20, 30, 40, 50, 60, 70, 80, 90, 100 or more amino acids of a heavy chain, and may include one or more CDRs, one or more CDRs combined with one or more FRs, one or more of CH1, hinge, CH2, or CH3, the variable region, the constant region, fragments of the constant region (*e.g.* CH1, CH2 CH3), or combinations thereof. Examples of an HCF include VHs, and full or parts of Fc regions. The phrase "a nucleotide sequence encoding an HCF" includes nucleotide sequences encoding a polypeptide consisting of an HCF and nucleotide sequences encoding a polypeptide containing an HCF, e.g., polypeptides that may contain additional amino acids in addition to a specified HCF. For example, a nucleotide sequence encoding an HCF includes nucleotide sequences encoding polypeptides consisting of a VH, consisting of a VH linked to a CH3, consisting of a full heavy chain, among others.

[0077] A "homologous sequence" in the context of nucleic acid sequences refers to a sequence that is substantially homologous to a reference nucleic acid sequence. In some embodiments, two sequences are considered to be substantially homologous if at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more of their corresponding nucleotides are identical over a relevant stretch of residues. In some embodiments, the relevant stretch is a complete (i.e., full) sequence.

[0078] The phrase "light chain" includes an immunoglobulin light chain constant region sequence from any organism, and unless otherwise specified includes human kappa and lambda light chains. Light chain variable (VL) domains typically include three light chain CDRs and four framework (FR) regions, unless otherwise specified. Generally, a full-length light chain includes, from amino terminus to carboxyl terminus, a VL domain that includes FR1-CDR1-FR2-CDR2-FR3-CDR3-FR4, and a light chain constant domain. Light chains that can be used with this invention include those, e.g., that do not selectively bind either the first or second epitope selectively bound by a bispecific antibody. Suitable light chains also include those that can bind or contribute to the binding of, one or both epitopes that are bound by the antigen-binding regions of an antibody. The term "a fragment of a light chain" or " a

light chain fragment" (or "LCF") includes a peptide of at least 10, 20, 30, 40, 50, 60, 70, 80, 90, 100 or more amino acids of a light chain, and may include one or more CDRs, one or more CDRs combined with one or more FRs, the variable region, the constant region, fragments of the constant region, or combinations thereof. Examples of an LCF include VLs and full or parts of light chain constant regions ("CLs"). The phrase "a nucleotide sequence encoding an LCF" includes nucleotide sequences encoding a polypeptide consisting of an LCF and nucleotide sequences encoding a polypeptide containing an LCF, e.g., polypeptides that may contain additional amino acids in addition to a specified LCF. For example, a nucleotide sequence encoding an LCF includes nucleotide sequences encoding polypeptides consisting of a VL, or consisting of a full light chain, among others.

[0079] The phrase "operably linked" refers to linkage of nucleic acids or proteins in a manner that the linked molecules function as intended. DNA regions are operably linked when they are functionally related to each other. For example, a promoter is operably linked to a coding sequence if the promoter is capable of participating in the transcription of the sequence; a ribosome-binding site is operably linked to a coding sequence if it is positioned so as to permit translation. Generally, operably linked can include, but does not require, contiguity. In the case of sequences such as secretory leaders, contiguity and proper placement in a reading frame are typical features. An expression-enhancing sequence of the locus of interest is operably linked to a gene of interest (GOI) where it is functionally related to the GOI, for example, where its presence results in enhanced expression of the GOI.

[0080] "Percent identity", when describing a locus of interest, such as SEQ ID NO: 1 or SEQ ID NO: 2, or a fragment thereof, is meant to include homologous sequences that display the recited identity along regions of contiguous homology, but the presence of gaps, deletions, or insertions that have no homolog in the compared sequence are not taken into account in calculating percent identity.

[0081] As used herein, a "percent identity" determination between, *e.g.*, SEQ ID NO: 1, or fragment thereof, with a species homolog, would not include a comparison of sequences where the species homolog has no homologous sequence to compare in an alignment (*i.e.*, SEQ ID NO: 1 or the fragment thereof has an insertion at that point, or the species homolog

has a gap or deletion, as the case may be). Thus, "percent identity" does not include penalties for gaps, deletions, and insertions.

[0082] "Recognition site" or "recognition sequence" is a specific DNA sequence recognized by a nuclease or other enzyme to bind and direct site-specific cleavage of the DNA backbone. Endonucleases cleave DNA within a DNA molecule. Recognition sites are also referred to in the art as recognition target sites.

[0083] "Recombinase recognition site" (or "RRS") is the specific DNA sequence recognized by a recombinase, such as Cre recombinase (Cre) or flippase (flp). Site-specific recombinases can perform DNA rearrangements, including deletions, inversions and translocations when one or more of their target recognition sequences are placed strategically into the genome of an organism. In one example, Cre specifically mediates recombination events at its DNA target recognition site *loxP*, which is composed of two 13-bp inverted repeats separated by an 8-bp spacer. More than one recombinase recognition site may be employed, for example, to facilitate a recombination-mediated exchange of DNA. Variants or mutants of recombinase recognition sites, for example lox sites, may also be employed (Araki, N. et al, 2002, *Nucleic Acids Research*, 30:19, e103).

[0084] "Recombinase-mediated cassette exchange" or "RMCE" relates to a process for precisely replacing a genomic target cassette with a donor cassette. The molecular compositions typically provided in order to perform this process include 1) a genomic target cassette flanked both 5' and 3' by recognition target sites specific to a particular recombinase, 2) a donor cassette flanked by matching recognition target sites, and 3) the site-specific recombinase. Recombinase proteins are well known in the art (Turan, S. and Bode J., 2011, *FASEB J.*, 25, pp. 4088–4107) and enable precise cleavage of DNA within a specific recognition target site (sequence of DNA) without gain or loss of nucleotides. Common recombinase/site combinations include, but are not limited to, Cre/*lox* and Flp/*frt*. Commercially available kits also provide vectors containing the R4-*attP* site and a vector encoding the phiC31 integrase for RMCE. (See also, *e.g.* U.S. Published Application No. US20130004946.)

[0085] "Site-specific integration" or "targeted insertion" refers to gene targeting methods employed to direct insertion or integration of a gene or nucleic acid sequence to a specific

location in the genome, *i.e.*, to direct the DNA to a specific site between two nucleotides in a contiguous polynucleotide chain. Site-specific integration or targeted insertion may also be done for a particular nucleic acid that includes multiple expression units or cassettes, such as multiple genes, each having their own regulatory elements (such as promoters, enhancers, and/or transcriptional termination sequences). "Insertion" and "integration" are used interchangeably. It is understood that insertion of a gene or nucleic acid sequence (for example a nucleic acid sequence comprising an expression cassette) may result in (or may be engineered for) the replacement or deletion of one or more nucleic acids depending on the gene editing technique being utilized.

[0086] "Stable integration" means that an exogenous nucleic acid integrated in the genome of a host cell remains integrated for an extended period of time in cell culture, for example, at least 7 days, at least 10 days, at least 15 days, at least 20 days, at least 25 days, at least 30 days, at least 35 days, at least 40 days, at least 45 days, at least 50 days, at least 55 days, at least 60 days, or longer. It is understood that making bispecific antigen-binding proteins for manufacturing and purification at large-scale is a challenging task. Stability and clonality are essential to the reproducibility of any biomolecule, especially one to be used therapeutically. The stable clones expressing bispecific antibodies made by the methods of this disclosure provide a consistent and reproducible way to generate therapeutic biomolecules.

General Description

[0087] This disclosure provides for compositions and methods for improved expression of multiple polypeptides in a host cell particularly Chinese hamster (*Cricetulus griseus*) cell lines, by employing multiple (e.g., two) expression-enhancing loci in the host cell. More specifically, the disclosure provides compositions and methods designed to integrate multiple exogenous nucleic acids that together encode an antigen-binding protein into multiple expression-enhancing loci in a host cell such as a CHO cell in a site-specific manner. In particular, this disclosure provides cells containing multiple exogenous nucleic acids integrated within multiple expression-enhancing loci wherein the multiple exogenous nucleic acids together encode an antigen-binding protein. This disclosure further provides nucleic acid vectors designed for site-specific integration of multiple exogenous nucleic acids into multiple expression-enhancing loci. This disclosure additionally provides systems that

include a host cell containing multiple recombinase recognition sites (RRSs) at each of multiple expression-enhancing loci, and a set of vectors containing matching RRSs and multiple exogenous nucleic acids, for site-specific integration of the multiple exogenous nucleic acids from the vectors into the multiple expression-enhancing loci. Further, this disclosure provides methods for making an antigen-binding protein using the cells, vectors and systems disclosed herein.

Cells Having Multiple Exogenous Nucleic Acids Integrated Site-Specifically Within Multiple Expression Enhancing Loci

[0088] In one aspect, this disclosure provides a cell that contains multiple exogenous nucleic acids integrated site-specifically in two expression-enhancing loci wherein the multiple exogenous nucleic acids together encode an antigen-binding protein. The antigen-binding protein can be a bispecific antigen-binding protein, or a conventional (i.e., monospecific) antigen-binding protein.

[0089] The cells provided herein are capable of producing a desired antigen-binding protein with high titers and/or high specific productivity (pg/cell/day). In some embodiments, a cell produces an antigen-binding protein at a titer of at least 1 g/L, 1.5 g/L, 2.0 g/L. 2.5 g/L, 3.0 g/L, 3.5 g/L, 4.0 g/L, 4.5 g/L, 5.0 g/L, 10 g/L, or greater. In some embodiments, a cell that produces an antigen-binding protein has a specific productivity of at least 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15 picogram/cell/day, or higher, determined based on total antigen-binding proteins (in pg) produced per cell per day.

[0090] The host cells comprising exogenous nucleic acids which together encode an antigen-binding protein and are integrated within two enhanced expression loci exhibit high cell density in a production culture, e.g. 1 to 10 x 10^6 cells/mL. In other embodiments, the antigen-binding protein-encoding host cell reaches a final cell density of at least 5 x 10^6 cells/mL, 6 x 10^6 cells/mL, 7 x 10^6 cells/mL, 8 x 10^6 cells/mL, 9 x 10^6 cells/mL, or 10 x 10^6 cells/mL (in production culture).

[0091] In some embodiments, a cell is provided that is capable of producing a bispecific antigen-binding protein at a ratio of the bispecific antigen-binding protein titer versus the total antigen-binding protein titer of at least 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 50%, 60%, or higher. In some embodiments, a cell is provided that is capable of producing a

bispecific antigen-binding protein, wherein the ratio of the bispecific antigen-binding protein titer is at least 50% of the total antigen-binding protein titer produced by the cell.

[0092] In other embodiments, a cell is provided that is capable of producing an antigen-binding protein wherein the total antigen-binding protein titer produced by expression in two loci compared with the total antigen-binding protein titer produced by expression in one loci is at least 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 50%, 60%, or higher. In certain embodiments, a cell is provided that is capable of producing an antigen-binding protein wherein the total antigen-binding protein titer produced by expression in two loci compared with the total antigen-binding protein titer produced by expression in one loci is at least 0.5 fold, 0.75 fold, 1 fold, 1.5 fold, 1.75 fold, 2 fold, or higher.

[0093] In some embodiments, the cell contains a first exogenous nucleic acid integrated at a specific site within a first enhanced expression locus; and a second exogenous nucleic acid integrated at a specific site within a second enhanced expression locus; wherein the first and second exogenous nucleic acids together encode an antigen-binding protein. The first and second exogenous nucleic acids together include multiple nucleotide sequences encoding HCF(s) or LCF(s) (e.g., variable regions) of the antigen-binding protein. For instance, for monospecific antibodies, there can be one nucleotide sequence encoding a HCF (e.g., a VH) and one nucleotide sequence encoding a LCF (e.g., a VL) for a monospecific antigen-binding protein, or multiple copies (e.g., two copies) of each. For bispecific antibodies, there can be two nucleotide sequences each encoding a HCF (typically the two HCFs being different from each other), one or two copies of a nucleotide sequence encoding a LCF, or two nucleotide sequences encoding two different LCFs. Depending on whether the antigen-binding protein is monospecific or bispecific, nucleotide sequences encoding HCF(s) or LCF(s) (e.g., variable regions) can be integrated in different variations or combinations at two enhanced expression loci. For example, for monospecific antigen-binding proteins, in one instance, a nucleotide sequence encoding a HCF and a nucleotide sequence encoding a LCF can be integrated separately in two loci, one at each locus; whereas in another instance, a nucleic acid encoding a HCF and a LCF is integrated at one locus, and a separate nucleic acid encoding the same HCF and the same LCFs is integrated at another locus. For bispecific antigen-binding proteins, in one instance, a nucleotide sequence encoding a first HCF and a LCF can be

integrated in a first locus, and a nucleotide sequence encoding a second HCF is integrated in a second locus, wherein the two HCFs are different, and the LCF is the common LCF of the bispecific antigen-binding protein; and in another instance, a nucleotide sequence encoding a first HCF and a LCF is integrated in a first locus, and a nucleotide sequence encoding a second HCF and the same LCF is integrated in a second locus.

[0094] In some embodiments, the nucleotide sequence encoding a HCF or a LCF can encode amino acids from a constant region. For example, the nucleotide sequence encoding a HCF or LCF can encode one or more of CL, CH1, CH2, CH3, or a combination of CH1, CH2 or CH3, or can encode an entire constant region. In some embodiments, a nucleotide sequence encoding a HCF encodes a CH3 domain. In specific embodiments, a nucleotide sequence encoding a HHCF encodes a heavy chain. In some embodiments, a nucleotide sequence encoding a LCF encodes a light chain.

[0095] In embodiments where two HCFs are involved, the nucleotide sequence encoding a first HCF can encode amino acids from a first constant region, and the nucleotide sequence encoding a second HCF can encode amino acids from a second constant region, wherein the amino acids from the two constant regions can be the same or different in at least one position (such as positions resulting in a different Protein A binding characteristics, or other positions described hereinbelow for various bispecific antigen-binding proteins). Independent of the differences in amino acids, the two nucleotide sequences encoding amino acids from two constant regions can be differentiated by modifying one or more codons of one nucleotide sequence, which provides a convenient basis to differentiate the two nucleotide sequences in a nucleic acid-based assay.

[0096] In some embodiments, each HCF- or LCF-coding nucleotide sequence is independently and operably linked to a transcriptional regulatory sequence that contains a promoter. By "independently", it means that each coding sequence is operably linked to a separate transcriptional regulatory sequence such as a promoter, so that transcription of the coding sequences is under separate regulation and control. In some embodiments, the promoters directing transcription of the two HCF-containing polypeptides are the same. In some embodiments, the promoters directing transcription of the two HCF-containing polypeptides, as well as the promoter directing transcription of the VL-containing

polypeptide, are all the same, e.g., a CMV promoter. In some embodiments, each HCF- or LCF-coding nucleotide sequence is independently and operably linked to an inducible or repressible promoter. Inducible and repressible promoters allow production to occur only in production phase (fed-batch culture) and not during growth phase (seed train culture); or to differentially control expression of antibody components (HCF and LCF) in different loci with precision. Fine control of production (expression) of each gene product may be achieved by way of different promoters.

[0097] In one such example, cells are first engineered to express the tetracycline repressor protein (TetR) and each HCF- and LCF-coding nucleotide sequence is placed under transcriptional control of a promoter whose activity is regulated by TetR. Two tandem TetR operators (TetO) are placed immediately downstream of the CMV promoter. In some embodiments, each HCF- and/or LCF-coding nucleotide sequence is independently and operably linked to a promoter upstream of at least one TetR operator (TetO) or Arc operator (ArcO). In other embodiments, each HCF- and/or LCF-coding nucleotide sequence is independently and operably linked to a CMV/TetO or CMV/ArcO hybrid promoter. Additional suitable promoters are described herein below.

[0098] In some embodiments, the multiple exogenous nucleic acids integrated at two loci are flanked by RRSs. For example, a first RRS and a second RRS are positioned 5' and 3', respectively, relative to a first exogenous nucleic acid integrated at a first locus, and a third RRS and a fourth RRS are positioned 5' and 3', respectively, relative to a second exogenous nucleic acid integrated at a second locus, wherein the first and second RRSs are different, and the third and fourth RRSs are different. In some embodiments, the first, second, third and fourth RRS are all different from each other. In other embodiments, the first and third RRSs are the same, and the second and fourth RRS ' are the same, where the first exogenous nucleic acid encodes a HCF and a LCF, and the second exogenous nucleic acid encodes the same HCF and LCF.

[0099] In some embodiments, where an exogenous nucleic acid integrated at a locus includes two HCF or LCF-coding nucleotide sequences, an additional RRS can be included between the two nucleotide sequences. Such additional RRS should be different from the two RRSs flanking the exogenous nucleic acid. In some embodiments, the additional RRS is inserted

into an intron of a selectable marker gene that is included in the integrated exogenous nucleic acid and positioned between the two HCF or LCF-coding nucleotide sequences. After transcription and post transcriptional processing, the intron will be excised giving rise to mRNA encoding the selectable marker. In embodiments where both the exogenous nucleic acid integrated at a first locus and the exogenous nucleic acid integrated at a second locus include two HCF or LCF-coding sequences (e.g., LCF-HCF1 and LCF-HCF2), an additional RRS can be included in only one, or both, of the first and second exogenous nucleic acids between the two HCF or LCF-coding sequences at each locus. The additional RRS in the first locus can be the same or different from the additional RRS in the second locus. Each additional RRS should be different from the two RRSs flanking the exogenous nucleic acid integrated at that locus. Each additional RRS can be optionally inserted within an intron of a selectable marker gene, and the selectable marker genes having an intron in the two loci can be different.

[00100] Where multiple HCF or LCF-coding sequences are included within an exogenous nucleic acid integrated at a locus, the relative positions of the multiple coding sequences within the locus can vary. For example, in embodiments where an integrated exogenous nucleic acid includes a LCF-encoding nucleotide sequence and a HCF-encoding nucleotide sequence, the LCF-encoding nucleotide sequence can be located upstream or downstream relative to the HCF-encoding nucleotide sequence. In specific embodiments, the LCF-encoding nucleotide sequence is located upstream relative to the HCF-encoding nucleotide sequence. Where both loci include a LCF-encoding nucleotide sequence and a HCF-encoding nucleotide sequence, in specific embodiments, the LCF-encoding nucleotide sequence is located upstream relative to the HCF-encoding nucleotide sequence in both loci.

[00101] In further embodiments, cells are provide that contains a first pair of RRSs integrated within a first enhanced expression locus, and a second pair of RRSs integrated within a second enhanced expression locus, wherein the two RRSs within each pair are different. Such cells are useful for receiving multiple exogenous nucleic acids to be integrated that together encode an antigen-binding protein.

[00102] In some embodiments, a first exogenous nucleic acid is present between the two RRSs at the first locus, and a second exogenous nucleic acid is present between the two RRSs

at the second locus. The first and second exogenous nucleic acids can each encode one or more selectable marker genes. The selectable marker genes can differ from each other.

[00103] In some embodiments, an additional RRS is present between the two RRSs in the pair (i.e., the 5' RRS and the 3' RRS) at a locus, wherein the additional RRS is different from both the 5' RRS and the 3' RRS at the locus. In some embodiments, an additional RRS is present between the 5' RRS and the 3' RRS at one of the two loci; and in other embodiments, an additional RRS is present between the 5' RRS and the 3' RRS at each of the two loci. Where an additional RRS is present between the 5' RRS and the 3' RRS, a selectable marker gene can be included between the 5' RRS and the additional RRS, and another selectable marker gene can be included between the additional RRS and the 3' RRS, and the two selectable markers are different.

[00104] In many of the embodiments described, the cell is a CHO cell, wherein one of two enhanced expression loci is selected from the group consisting of a nucleotide sequence at least 90% identical to SEQ ID NO: 1, a nucleotide sequence at least 90% identical to SEQ ID NO: 2, and a nucleotide sequence at least 90% identical to SEQ ID NO: 3.

Bispecific Antigen-Binding Proteins

[00105] Bispecific antigen-binding proteins, such as bispecific antibodies, suitable for cloning and production in the cells, vectors, and systems described in this disclosure are not limited to any particular format of bispecific antigen-binding proteins.

[00106] In various embodiments, the bispecific antigen-binding protein includes two polypeptides, each containing an antigen-binding moeity (e.g., a HC) and a CH3 domain, wherein the antigen-binding moeity of the two polypeptides have different antigen specificities, and wherein the two CH3 domains are heterodimeric in respect to each other in that one of the CH3 domains has been modified in at least one amino acid position to give rise to differential Protein A binding characteristics between the two polypeptides. See, e.g., the bispecific antibodies described in U.S. Patent 8,586,713. In this way, a differential protein A isolation scheme can be employed to readily isolate the heterodimeric bispecific antigen-binding proteins from homodimers.

[00107] In some embodiments, the bispecific antigen-binding protein includes two heavy chains having different antigen specificities and differing in at least one amino acid position

in the CH3 domain to give rise to differential Protein A binding characteristics between the two heavy chains.

[00108] In some embodiments, the two polypeptides contain CH3 domains of human IgG, wherein one of the two polypeptides contains the CH3 domain of a human IgG selected from IgG1, IgG2 and IgG4, and the other one of the two polypeptides contains a modified CH3 domain of a human IgG selected from IgG1, IgG2 and IgG4 wherein the modification reduces or eliminates the binding of the modified CH3 region to Protein A. In specific embodiments, one of the two polypeptides contains the CH3 domain of human IgG1, and the other one of the two polypeptides contains a modified CH3 domain of human IgG1 wherein the modification is selected from the group consisting of (i) 95R and (ii) 95R and 96F in the IMGT exon numbering system. In other specific embodiments, the modified CH3 domain comprises one to five additional modifications selected from the group consisting of 16E, 18M, 44S, 52N, 57M, and 82I in the IMGT exon numbering system.

[00109] In other various embodiments, the two polypeptides contain CH3 domains of mouse IgG, wherein one of the two polypeptides contains the CH3 domain of an unmodified mouse IgG, and the other one of the two polypeptides contains a modified CH3 domain of the mouse IgG wherein the modification reduces or eliminates the binding of the modified CH3 region to Protein A. In various embodiments, a mouse IgG CH3 region is modified to comprise particular amino acids at particular positions (EU numbering), selected from the group consisting of: 252T, 254T, and 256T; 252T, 254T, 256T, and 258K; 247P, 252T, 254T, 256T, and 258K; 435R and 436F; 252T, 254T, 256T, 435R, and 436F; 252T, 254T, 256T, 258K, 435R, and 436F; 24tP, 252T, 254T, 256T, 258K, 435R, and 436F; and, 435R. In a specific embodiment, a particular group of modifications is made, selected from the groups consisting of: M252T, S254T, S256T; M252T, S254T, S256T, I258K; I247P, M252T, S254T, S256T, I258K; H435R, H436F; M252T, S254T, S256T, H435R, H436F; M252T, S254T, S256T, I258K, H435R, H436F; I247P, M252T, S254T, S256T, I258K, H435R, H436F; and, H435R.

[00110] In various embodiments, a bispecific antigen-binding protein is a hybrid of a mouse and a rat monoclonal antibody or antigen-binding protein, e.g., a hybrid of mouse IgG2a and rat IgG2b. According to these embodiments, a bispecific antibody is composed of a heterodimer of the two antibodies comprising one heavy/light chain pair of each, associating

via their Fc portions. The desired heterodimer can be easily purified from a mixture of two parental antibody homodimers and the bispecific heterodimer, because the binding properties of the bispecific antibody to Protein A are different from those of the parental antibodies: rat IgG2b does not bind to protein A, whereas the mouse IgG2a does. Consequently, the mouse-rat heterodimer binds to Protein A but elutes at a higher pH than the mouse IgG2a homodimer, and this makes selective purification of the bispecific heterodimer possible.

[00111] In other various embodiments, a bispecific antigen-binding protein is of a format that is referred to as "knobs-into-holes" in the art (see, e.g., U.S. Patent No. 7,183,076). In these embodiments, the Fc portions of two antibodies are engineered to give one a protruding "knob", and the other a complementary "hole." When produced in the same cell, the heavy chains are said to preferentially form heterodimers rather than homodimers, by association of the engineered "knobs" with the engineered "holes."

[00112] In another embodiment, the first heavy chain and the second heavy chain comprises one or more amino acid modifications in the CH3 domain to enable interaction between two heavy chains. CH3-CH3 interface amino acid residues can be replaced with charged amino acid to provide electrostatically unfavorable homodimer formation. (See, *e.g.* PCT Publication No. WO2009089004; and European Publication No. EP1870459.)

[00113] In other embodiments, the first heavy chain comprises a CH3 domain of the isotype IgA and the second heavy chain comprises a CH3 domain of IgG (or vice versa) to promote preferential formation of heterodimers. (See *e.g.* PCT Publication No. WO2007110205.)

[00114] In other embodiments, various formats can be incorporated with immunoglobulin chains by engineering methods to foster formation of heterodimers, such as Fab-arm exchange (PCT Publication No. PCT Publication No. WO2008119353; PCT Publication No. WO2011131746), coiled-coil domain interaction (PCT Publication No. WO2011034605) or leucine zipper peptides (Kostelny, et al. *J. Immunol.* 1992, 148(5):1547-1553).

[00115] Immunoglobulin heavy chain fragments (e.g., variable regions) that can be used to generate bispecific antigen binding proteins can be generated using any method known in the art. For example, a first heavy chain comprises a variable region that is encoded by a nucleic acid that is derived from the genome of a mature B cell of a first animal that has been immunized with a first antigen, and the first heavy chain specifically recognizes the first

antigen; and a second heavy chain comprises a variable region that is encoded by a nucleic acid that is derived from the genome of a mature B cell of a second animal that has been immunized with a second antigen, and the second heavy chain specifically recognizes the second antigen. Immunoglobulin heavy chain variable region sequences can also be obtained by any other method known in the art, e.g., by phage display. In other examples, nucleic acids encoding the heavy chain variable regions include those of antibodies that have been described or otherwise available in the art. In some embodiments, one of the two heavy chain coding sequences have been codon modified in order to provide a convenient basis to differentiate the two coding sequences in nucleic acid based assays.

[00116] Bispecific antibodies comprising two heavy chains that recognize two different epitopes (or two different antigens) are more easily isolated where they can pair with the same light chain (i.e., light chains having identical variable and constant domains). A variety of methods are known in the art for generating light chains that can pair with two heavy chains of differing specificity, while not interfering or not substantially interfering with the selectivity and/or affinity of the heavy chain variable domain with its target antigen, as described in e.g., U.S. Patent 8,586,713 and the art disclosed therein.

[00117] The bispecific antigen-binding proteins can have a variety of dual antigen specificities and associated useful applications.

[00118] In some examples, bispecific antigen-binding proteins that comprise binding specificity toward a tumor antigen and a T-cell antigen can be made that target an antigen on a cell, e.g., CD20, and also target an antigen on a T-cell, e.g., a T cell receptor such as CD3. In this way, the bispecific antigen-binding protein targets both a cell of interest in a patient (e.g., B cell in a lymphoma patient, via CD20 binding) as well as a T-cell of the patient. The bispecific antigen-binding protein, in various embodiments, is designed so as to activate the T-cell upon binding CD3, thus coupling T-cell activation to a specific, selected tumor cell.

[00119] In the context of bispecific antigen-binding proteins wherein one moiety binds to a T cell receptor such as binding to CD3 and the other moiety binds a target antigen, the target antigen can be a tumor-associated antigen. Non-limiting examples of specific tumor-associated antigens include, *e.g.*, AFP, ALK, BAGE proteins, BIRC5 (survivin), BIRC7, β-catenin, brc-abl, BRCA1, BCMA, BORIS, CA9, carbonic anhydrase IX, caspase-8, CALR,

CCR5, CD19, CD20(MS4A1), CD22, CD30, CD40, CDK4, CEA, CLEC-12, CTLA4, cyclin-B1, CYP1B1, EGFR, EGFRvIII, ErbB2/Her2, ErbB3, ErbB4, ETV6-AML, EpCAM, EphA2, Fra-1, FOLR1, GAGE proteins (*e.g.*, GAGE-1, -2), GD2, GD3, GloboH, glypican-3, GM3, gp100, Her2, HLA/B-raf, HLA/k-ras, HLA/MAGE-A3, hTERT, LMP2, MAGE proteins (*e.g.*, MAGE-1, -2, -3, -4, -6, and -12), MART-1, mesothelin, ML-IAP, Muc1, Muc2, Muc3, Muc4, Muc5, Muc16 (CA-125), MUM1, NA17, NY-BR1, NY-BR62, NY-BR85, NY-ESO1, OX40, p15, p53, PAP, PAX3, PAX5, PCTA-1, PLAC1, PRLR, PRAME, PSMA (FOLH1), RAGE proteins, Ras, RGS5, Rho, SART-1, SART-3, Steap-1, Steap-2, TAG-72, TGF-β, TMPRSS2, Thompson-nouvelle antigen (Tn), TRP-1, TRP-2, tyrosinase, and uroplakin-3. In some embodiments, the bispecific antigen-binding protein comprises one moiety that binds CD3. Exemplified anti-CD3 antibody moieties are described in U.S. Pat. Appln. Pub. Nos. US2014/0088295A1 and US20150266966A1, and in International Publication No. WO 2017/053856 published on March 30, 2017, all of which are incorporated herein by reference). In other embodiments, the bispecific antigen-binding protein comprises one moiety that binds to CD3 and one moiety that binds to BCMA, CD19, CD20, CD28, CLEC-12, Her2, HLA protein, MAGE protein, Muc16, PSMA, or Steap-2. In still other embodiments, the bispecific antigen-binding protein is selected from the group consisting of an anti-CD3 x anti-CD20 bispecific antibody (as described in U.S. Pat. Appln. Pub. Nos. US2014/0088295A1 and US20150266966A1, herein incorporated by reference), an anti-CD3 x anti-Mucin 16 bispecific antibody (e.g., an anti-CD3 x anti-Muc16 bispecific antibody), and an anti-CD3 x anti- Prostate-specific membrane antigen bispecific antibody (e.g., an anti-CD3 x anti-PSMA bispecific antibody).

[00120] In the context of bispecific antigen-binding proteins wherein one moiety binds to a T cell receptor such as binds to CD3 and the other moiety binds a target antigen, the target antigen can be an infectious disease-associated antigen. Non-limiting examples of infectious disease-associated antigens include, *e.g.*, an antigen that is expressed on the surface of a virus particle, or preferentially expressed on a cell that is infected with a virus, wherein the virus is selected from the group consisting of HIV, hepatitis (A, B or C), herpes virus (*e.g.*, HSV-1, HSV-2, CMV, HAV-6, VZV, Epstein Barr virus), adenovirus, influenza virus, flavivirus, echovirus, rhinovirus, coxsackie virus, coronavirus, respiratory syncytial virus, mumps virus,

rotavirus, measles virus, rubella virus, parvovirus, vaccinia virus, HTLV, dengue virus, papillomavirus, molluscum virus, poliovirus, rabies virus, JC virus, and arboviral encephalitis virus. Alternatively, the target antigen can be an antigen that is expressed on the surface of a bacterium, or preferentially expressed on a cell that is infected with a bacterium, wherein the bacterium is selected from the group consisting of chlamydia, rickettsia, mycobacteria, staphylococci, streptococci, pneumonococci, meningococci, gonococci, klebsiella, proteus, serratia, pseudomonas, legionella, diphtheria, salmonella, bacilli, cholera, tetanus, botulism, anthrax, plague, leptospira, and Lyme disease bacteria. In certain embodiments, the target antigen is an antigen that is expressed on the surface of a fungus, or preferentially expressed on a cell that is infected with a fungus, wherein the fungus is selected from the group consisting of Candida (albicans, krusei, glabrata, tropicalis, etc.), Crytococcus neoformans, Aspergillus (fumigatus, niger, etc.), Mucorales (mucor, absidia, rhizopus, etc.), Sporothrix schenkii, Blastomyces dermatitidis, Paracoccidioides brasiliensis, Coccidioides immitis, and Histoplasma capsulatum. In certain embodiments, the target antigen is an antigen that is expressed on the surface of a parasite, or preferentially expressed on a cell that is infected with a parasite, wherein the parasite is selected from the group consisting of Entamoeba histolytica, Balantidium coli, Naegleriafowleri, Acanthamoeba sp., Giardia lambia, Cryptosporidium sp., Pneumocystis carinii, Plasmodium vivax, Babesia microti, Trypanosoma brucei, Trypanosoma cruzi, Leishmania donovani, Toxoplasma gondii, Nippostrongylus brasiliensis, Taenia crassiceps, and Brugia malayi. Non-limiting examples of specific pathogen-associated antigens include, *e.g.*, HIV gp120, HIV CD4, hepatitis B glucoprotein L, hepatitis B glucoprotein M, hepatitis B glucoprotein S, hepatitis C E1, hepatitis C E2, hepatocyte-specific protein, herpes simplex virus gB, cytomegalovirus gB, and HTLV envelope protein.

[00121] Bispecific binding proteins that comprise two binding moieties that are each directed to a binding partner (i.e., each directed to a different target) on the surface of the same cell can also be made. This design is particularly suited to targeting specific cells or cell types that express both targets on the surface of the same cell. Although targets might appear individually on other cells, the binding moieties of these binding proteins are selected such that each binding moiety binds its target with a relatively low affinity (e.g., low micromolar,

or high nanomolar - e.g., over a hundred nanomolar KD, e.g., 500, 600, 700, 800 nanomolar). In this way, prolonged target binding is favored only in situations where the two targets are in proximity on the same cell.

[00122] Bispecific binding proteins that comprise two binding moieties that bind the same target, each at a different epitope of the same target, can be made. This design is particularly suited for maximizing the probability of successfully blocking a target with binding protein. Multiple extracellular loops, e.g., of a transmembrane channel or a cell surface receptor, can be targeted by the same bispecific binding molecule.

[00123] Bispecific binding proteins that comprise two binding moieties that cluster and activate negative regulators of immune signaling to result in immune suppression can be made. Repression in cis can be achieved where the targets are on the same cell; repression in trans can be achieved where the targets are on different cells. Repression in cis, e.g., can be achieved with a bispecific binding protein having an anti-IgGRIIb binding moiety and an anti-FelD1 binding moiety, such that the IgGRIIb is clustered only in the presence of FelD1, in order to down-regulate an immune response to FelD1. Repression in trans, e.g., can be achieved with a bispecific binding protein having an anti-BTLA binding moiety and a binding moiety that specifically binds a tissue-specific antigen of interest, such that clustering of the inhibitory BTLA molecule occurs only in the selected target tissue, which potentially addresses auto-immune diseases.

[00124] Bispecific binding proteins that activate multi-component receptors can be made. In this design, two binding moieties directed to two components of a receptor bind, cross-link the receptor, and activate signaling from the receptor. This can be done, e.g., using a bispecific binding protein with a binding moiety that binds IFNAR1 and a binding moiety that binds IFNAR2, where binding cross-links the receptor. Such a bispecific binding protein can provide an alternative to interferon treatment.

[00125] Bispecific binding proteins that transport binding moieties across a semi-permeable barrier, e.g., the blood-brain barrier, can be made. In this design, one binding moiety binds a target that can transit a particular selective barrier; the other binding moiety targets a molecule with a therapeutic activity, wherein the target molecule with therapeutic activity cannot normally traverse the barrier. This kind of bispecific binding protein is useful for bringing

therapeutics to tissues that the therapeutic would not otherwise reach. Some examples include targeting the pIGR receptor to transport a therapeutic into the gut or lung, or targeting the transferrin receptor to transport a therapeutic across the blood-brain barrier.

[00126] Bispecific binding proteins that transport binding moieties into specific cells or cell types can be made. In this design, one binding moiety targets a cell surface protein (e.g., a receptor) that is readily internalized into the cell. The other binding moiety targets an intracellular protein, where binding of the intracellular protein results in a therapeutic effect.

[00127] Bispecific binding proteins that bind a surface receptor of a phagocytic immune cell and a surface molecule of an infectious pathogen (e.g., a yeast or bacterium), to bring the infectious pathogen in the vicinity of a phagocytic immune cell to facilitate phagocytosis of the pathogen. An example of such a design would be a bispecific antibody that targets a CD64 or CD89 molecule and also a pathogen.

[00128] Bispecific binding proteins that have an antibody variable region as one binding moiety and a non-Ig moiety as a second binding moiety. The antibody variable region achieves targeting, whereas the non-Ig moiety is an effector or a toxin linked to an Fc. In this way, the ligand (e.g., an effector or toxin) is delivered to the target bound by the antibody variable region.

[00129] Bispecific binding proteins that have two moieties each bound to an Ig region (e.g., an Ig sequence containing a CH2 and CH3 region) such that any two protein moieties can be brought in each other's vicinity in the context of the Fc. Examples of this design include traps, e.g., homo- or heterodimeric trap molecules.

Expression-Enhancing Loci

[00130] Expression-enhancing loci suitable for use in this invention include for example, a locus that comprises a nucleotide sequence having substantial homology to SEQ ID NO: 1 as described in U.S. Patent 8,389,239 (also referred to herein as the "EESYR® locus" or "Locus 1"), a locus that comprises a nucleotide sequence having substantial homology to SEQ ID NO: 2 or SEQ ID NO: 3 as described in U.S. Application Serial No. 14/919,300 (also referred to herein as "the YARS locus" or "Locus 2"), and other expression-enhancing loci and sequences documented in the art (e.g., US 20150167020A1, and U.S. Patent 6,800,457).

[00131] In some embodiments, the two expression-enhancing loci used in this invention are selected from the group consisting of a locus that comprises a nucleotide sequence having substantial homology to SEQ ID NO: 1, a locus that comprises a nucleotide sequence having substantial homology to SEQ ID NO: 2, and a locus that comprises a nucleotide sequence having substantial homology to SEQ ID NO: 3. These loci contain sequences that not only provide for enhanced expression of genes integrated in operable linkage to the sequences (i.e., within the sequences or within close proximity to the sequences), but also exhibit greater recombination efficiency and improved integration stability, as compared to other sequences in the genome.

[00132] SEQ ID NO: 1, SEQ ID NO: 2 and SEQ ID NO: 3 have been identified from CHO cells. Other mammalian species (such as, for example, humans or mice), were found to have limited homology to the identified expression-enhancing region; however, homologous sequences may be found in cell lines derived from other tissue types of *Cricetulus griseus*, or other homologous species, and can be isolated by techniques that are well-known in the art. For example, one may identify other homologous sequences by cross-species hybridization or PCR-based techniques. In addition, changes can be made in the nucleotide sequence set forth in SEQ ID NO: 1, SEQ ID NO: 2, or SEQ ID NO: 3, by site-directed or random mutagenesis techniques that are well known in the art. The resulting sequence variants can then be tested for expression-enhancing activity. DNAs that are at least about 90% identical in nucleic acid identity to SEQ ID NO: 1, SEQ ID NO: 2, or SEQ ID NO: 3, having expression-enhancing activity are isolatable by routine experimentation, and are expected to exhibit expression-enhancing activity.

[00133] The integration site, the site or nucleotide position of insertion of one or more exogenous nucleic acids, can be at any position that is within or adjacent to any of the expression enhancing sequences (such as SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3). Whether a specific chromosomal location within or adjacent to the locus of interest supports stable integration and efficient transcription of an integrated exogenous gene can be determined in accordance with standard procedures well known in the art, e.g., as described U.S. Patent 8,389,239 and U.S. Application Serial No. 14,919,300.

[00134] The integration sites considered herein are located within the expression enhancing sequences, or within close proximity to the sequences, *e.g.,* less than about 1 kb, 500 base pairs (bp), 250 bp, 100 bp, 50 bp, 25 bp, 10 bp, or less than about 5 bp upstream (5') or downstream (3') with respect to the location of an expression enhancing sequence on the chromosomal DNA. In still some other embodiments, the employed integration site is located at about 1000, 2500, 5000 or more base pairs upstream (5') or downstream (3') with respect to the location of an expression enhancing sequence on the chromosomal DNA.

[00135] It is understood in the art that large genomic regions, such as scaffold/matrix attachment regions, are employed for efficient replication and transcription of chromosomal DNA. A scaffold/matrix attachment region (S/MAR), also known as called scaffold-attachment region (SAR), or matrix-associated or matrix attachment region (MAR), is a eukaryotic genomic DNA region where the nuclear matrix attaches. Without being bound by any one theory, S/MARs typically map to non-coding regions, separate a given transcriptional region (*e.g.* chromatin domain) from its neighbors, and also provide platforms for the machinery and/or binding of factors that enable transcription, such as recognition sites for DNAses or polymerases. Some S/MARs have been characterized at about 14-20 kb in length (Klar, et al. 2005, *Gene* 364:79-89). As such, integration of genes at an expression enhancing locus (e.g., within or near SEQ ID NO: 1, or SEQ ID NO: 2, or SEQ ID NO: 3) is expected to confer enhanced expression. In some embodiments, the host cells comprising an exogenous nucleic acid sequence encoding a bispecific antigen-binding protein integrated at a specific site within an enhanced expression locus exhibits high specific productivity. In other embodiments, the bispecific antigen-binding protein-encoding host cell has a specific productivity of at least 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 20, 25, or 30 picogram/cell/day (pcd).

[00136] In some embodiments, an exogenous nucleic acid is integrated at a site within a locus that comprises the nucleotide sequence of SEQ ID NO: 1. In specific embodiments, the integration site is within, or within close proximity to, the nucleotide sequence of SEQ ID NO: 1. In particular embodiments, the integration site is at a position within SEQ ID NO: 1 selected from nucleotides spanning positions numbered 10-13,515; 20-12,020; 1,020-11,020; 2,020-10,020; 3,020-9,020; 4,020-8,020; 5,020-7,020; 6,020-6,920; 6,120-6,820; 6,220-6,720;

6,320-6,620; 6,420-6,520; 6,460-6,500; 6,470-6,490; and 6,475-6,485. In other embodiments, the integration site is in a sequence that is selected from the group consisting of nucleotides 5,000-7,400, 5,000-6,500, 6,400-7,400 of SEQ ID NO: 1; and nucleotides 6,400-6,500 of SEQ ID NO: 1. In a specific embodiment, the integration site before, after, or within the "act" triplet of nucleotides 6471 to 6473 of SEQ ID NO: 1.

[00137] In some embodiments, an exogenous nucleic acid is integrated at a site within a locus that comprises the nucleotide sequence of SEQ ID NO: 2 or SEQ ID NO: 3. In specific embodiments, the integration site is within, or within close proximity to, the nucleotide sequence of SEQ ID NO: 2. In particular embodiments, the integration site is within, or within close proximity to, the nucleotide sequence of SEQ ID NO: 3. In some embodiments, the integration site is within nucleotides 1990-1991, 1991-1992, 1992-1993, 1993-1994, 1995-1996, 1996-1997, 1997-1998, 1999-2000, 2001-2002, 2002-2003, 2003-2004, 2004-2005, 2005-2006, 2006-2007, 2007-2008, 2008-2009, 2009-2010, 2010-2011, 2011-2012, 2012-2013, 2013-2014, 2014-2015, 2015-2016, 2016-2017, 2017-2018, 2018-2019, 2019-2020, 2020-2021 or 2021-2022 of SEQ ID NO: 3. In specific embodiments, the integration is at or within nucleotides 2001-2022 of SEQ ID NO: 3. In some embodiments, the exogenous nucleic acid is inserted at or within nucleotides 2001-2002 or nucleotides 2021-2022 of SEQ ID NO: 3 and nucleotides 2002-2021 of SEQ ID NO: 3 are deleted, as a result of the insertion.

Site-Specific Integration Into An Expression-Enhancing Locus

[00138] Integration of one or more exogenous nucleic acids into an expression-enhancing locus in a site-specific manner, i.e., into one specific site within an expression-enhancing locus as disclosed herein, can be achieved in several ways including, e.g., by homologous recombination, and recombinase mediated cassette exchange, as described in the art (see e.g., U.S. Patent 8,389,239 and the art disclosed therein).

[00139] In some embodiments, cells are provided that contain at least two, i.e., two or more, different recombinase recognition sequences (RRS) within an expression-enhancing locus convenient for integrating an nucleic acid sequence containing one or more exogenous nucleic acids or genes of interest. Such cells can be obtained by introducing an exogenous nucleic acid sequence containing two or more RRS into a desirable locus by various means including

homologous recombination, as described hereinbelow and in the art, e.g., U.S. Patent 8,389,239 and the art disclosed therein.

[00140] In specific embodiments, cells are provided that contain more than two different recombinase recognition sequences (RRS) within an expression-enhancing locus convenient for integrating multiple exogenous nucleic acids. In particular embodiments, cells are provided that contain three different recombinase recognition sequences (RRS) within an expression-enhancing locus which can mediate integration of two separate exogenous nucleic acids, for example, wherein the 5' RRS and the middle RRS in the genome match the 5' RRS and the 3' RRS flanking the first exogenous nucleic acid to be integrated, and the middle RRS and 3' RRS in the genome match the 5' RRS and the 3' RRS flanking the second exogenous nucleic acid to be integrated.

[00141] Suitable RRSs can be selected from the group comprising *Lox*P, *Lox*511, *Lox*5171, *Lox*2272, *Lox*2372, *Lox*m2, *Lox*-FAS, *Lox*71, *Lox*66 and the mutants thereof, where the site specific recombinase is Cre recombinase or its derivative is used to achieve recombinase-mediated cassette exchange (RMCE). In other examples, suitable RRS can be selected from the group comprising FRT, F3, F5, FRT mutant-10, FRT mutant+10 and the mutants thereof, and in this scenario, the site-specific recombinase Flp recombinase or its derivative is used to achieve RMCE. In yet another example, RRSs can be selected from the group comprising attB, attP and the mutants thereof, and in this case where the site-specific recombinase phiC31 integrase or its derivative is used to achieve RMCE.

[00142] In other embodiments, native cells are modified by a homologous recombination technique to integrate a nucleic acid sequence containing one or more exogenous nucleic acids into a specific site within an expression-enhancing locus.

[00143] For homologous recombination, homologous polynucleotide molecules (*i.e.* homologous arms) line up and exchange a stretch of their sequences. A transgene can be introduced during this exchange if the transgene is flanked by homologous genomic sequences. In one example, a recombinase recognition site can be introduced into the host cell genome at the integration sites via homologous recombination. In other examples, a nucleic acid sequence containing one or more exogenous nucleic acids of interest, e.g., one or more nucleic acids each encoding an HCF or LCF (such as a variable region), wherein the

nucleic sequence is flanked by sequences homologous to the sequences at the target locus ("homologous arms"), is inserted into the host genome.

[00144] Homologous recombination in eukaryotic cells can be facilitated by introducing a break in the chromosomal DNA at the integration site. This may be accomplished by targeting certain nucleases to the specific site of integration. DNA-binding proteins that recognize DNA sequences at the target locus are known in the art. Gene targeting vectors are also employed to facilitate homologous recombination.

[00145] Gene targeting vector construction and nuclease selection to achieve homologous recombination are within the skill of the artisan to whom this invention pertains. In some examples, zinc finger nucleases (ZFNs), which have a modular structure and contain individual zinc finger domains, recognize a particular 3-nucleotide sequence in the target sequence (*e.g.* site of targeted integration). Some embodiments can utilize ZFNs with a combination of individual zinc finger domains targeting multiple target sequences. Transcription activator-like (TAL) effector nucleases (TALENs) may also be employed for site-specific genome editing. TAL effector protein DNA-binding domain is typically utilized in combination with a non-specific cleavage domain of a restriction nuclease, such as FokI. In some embodiments, a fusion protein comprising a TAL effector protein DNA-binding domain and a restriction nuclease cleavage domain is employed to recognize and cleave DNA at a target sequence within the locus of the invention (Boch J et al., 2009 *Science* 326:1509-1512). RNA-guided endonucleases (RGENs) are programmable genome engineering tools that were developed from bacterial adaptive immune machinery. In this system—the clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated (Cas) immune response—the protein Cas9 forms a sequence-specific endonuclease when complexed with two RNAs, one of which guides target selection. RGENs consist of components (Cas9 and tracrRNA) and a target-specific CRISPR RNA (crRNA). Both the efficiency of DNA target cleavage and the location of the cleavage sites vary based on the position of a protospacer adjacent motif (PAM), an additional requirement for target recognition (Chen, H. et al, *J. Biol. Chem.* published online March 14, 2014, as Manuscript M113.539726). Sequences unique for a specific targeting locus (such as SEQ ID NO: 1, SEQ

ID NO: 2, or SEQ ID NO: 3) can be identified by aligning many of these sequences to the CHO genome which can reveal potential off-target sites with 16-17 base pair match.

[00146] In some embodiments, a targeting vector carrying a nucleic acid of interest (e.g., a nucleic acid containing one or more RRSs optionally flanking one or more selectable marker genes, or a nucleic acid containing one or more exogenous nucleic acids each encoding an HCF or LCF (such as a variable region), flanked by 5' and 3' homology arms, is introduced into a cell with one or more additional vectors or mRNA. In one embodiment, the one or more additional vectors or mRNA contain a nucleotide sequence encoding a site-specific nuclease, including but not limited to a zinc finger nuclease (ZFN), a ZFN dimer, a transcription activator-like effector nuclease (TALEN), a TAL effector domain fusion protein, and an RNA-guided DNA endonuclease. In certain embodiments, the one or more vectors or mRNA include a first vector comprising a guide RNA, a tracrRNA and a nucleotide sequence encoding a Cas enzyme, and a second vector comprising a donor (exogenous) nucleotide sequence. Such donor sequence contains a nucleotide sequence coding for the gene of interest, or the recognition sequence, or the gene cassette comprising any one of these exogenous elements intended for targeted insertion. Where mRNA is used, the mRNA can be transfected into the cell by means of common transfection methods known to the skilled person and may encode an enzyme, for example a transposase or endonuclease. Although an mRNA introduced into the cells may be transient and does not integrate into the genome, the mRNA may carry an exogenous nucleic acid necessary or beneficial for the integration to take place. In some instances, mRNA is chosen in order to eliminate any risk of long-lasting side effects of an accessory polynucleotide, where only short-term expression is required to achieve the desired integration of a nucleic acid.

Vectors For Site Specific Integration

[00147] Nucleic acid vectors are provided herein for introducing exogenous nucleic acids into two expression enhancing loci via site-specific integration. Suitable vectors include vectors designed to contain an exogenous nucleic sequence flanked by RRSs for integration via RMCE, and vectors designed to contain an exogenous nucleic sequence of interest flanked by homology arms for integration via homologous recombination.

[00148] In various embodiments, vectors are provided to achieve site-specific integration via RMCE. In some embodiments, vectors are designed to achieve simultaneous integration of multiple nucleic acids into two target loci. In contrast to sequential integration, simultaneous integration permits efficiency and rapid isolation of desirable clones that produce antigen-binding proteins, or other multimeric proteins of interest, suitable for large scale production (manufacturing).

[00149] In some embodiments, a set of vectors is provided for expressing a bispecific antigen-binding protein in a cell.

[00150] In some embodiments, a vector set can include two "HCF vectors", each containing a nucleic acid flanked by a 5' RRS and a 3' RRS, where the nucleic acid includes a nucleotide sequence encoding a HCF and wherein the two HCFs are different. The RRS on the two HCF vectors are different from each other, and are designed to integrate HCF-encoding nucleotide sequences to two expression-enhancing loci. The vector set also includes a nucleotide sequence encoding a LCF, which can be included in one of the HCF vectors, or in both HCF vectors (thereby providing two copies of the same LCF), or alternatively, provided in a separate "LCF vector" and flanked by a 5' RRS and a 3' RRS.

[00151] In some embodiments, the LCF-coding nucleotide sequence is included in one of the HCF vectors and positioned between the 5' RRS and the 3' RRS on that HCF vector. The LCF-coding sequence can be placed upstream or downstream of the HCF-coding sequence.

[00152] In some embodiments, the LCF-coding nucleotide sequence is included both of the HCF vectors and positioned between the 5' RRS and the 3' RRS on each HCF vector. Similarly, the LCF-coding sequence can be placed upstream or downstream of the HCF-coding sequence in each vector.

[00153] In some embodiments, the LCF-coding nucleotide sequence is provided in a separate vector, a "LCF" vector, and is flanked by a 5' RRS and a 3' RRS, with the two RRS being different from each other. The RRSs in the vector set can be designed such that the LCF-coding sequence can be "joined" with one of the HCF-coding sequences through a common RRS during RMCE with a target locus that also contains the common RRS. For example, the 3' RRS of the LCF vector can be the same as the 5' RRS of one the HCF vectors, giving rise to a LCF-HCF arrangement after integration at a target locus via RMCE. In another example,

the 3' RRS of a HCF vector can be the same as the 5' RRS of the LCF vector, giving rise to a HCF-LCF arrangement after integration at a target locus via RMCE. In some embodiments, the common RRS is designed in a split selectable marker format - that is, it is included at the 3' end of a 5' portion of a selectable marker gene included in one vector, and also included at the 5' end of the remaining, 3' portion of the same selectable marker gene included in another vector, such that upon "joining" and integration into a target locus, the properly integrated nucleic acid includes the entire selectable marker gene to allow for convenient identification of transfectants. In some embodiments, the common RRS is designed in a split gene format, i.e., included at the 3' end of a 5' portion of an gene or intron within such split gene as part of a 5' portion of said gene on one vector, and at the 5' end of the remaining portion of the gene or intron within such split gene as part of the remaining 3' portion of said split gene. In still other embodiments, the third or middle RRS in a first vector is designed to be between a promoter and the selectable marker gene to which it is operably linked (but it is separated from on the other vector); the third or middle RRS in the first vector is designed to be 3' of a promoter; and the third or middle RRS in the second vector is designed to be 5' of the selectable marker gene.

[00154] In some embodiments, the vector set can include an additional nucleotide sequence encoding a LCF. That is, the vector set can include two HCF vectors, and two LCF-encoding nucleotide sequences. The two LCF-encoding sequences can encode the same or different LCF. In some embodiments, the two LCF-encoding sequences can be each included into a HCF vector, resulting in two vectors, each containing a HCF-coding sequence and a LCF-coding sequence. The two vectors can be designed to have RRSs suitable for targeting the two vector sequences into two loci. In other embodiments, one of the two LCF-encoding sequences is included into a HCF vector and positioned between the 5' RRS and the 3' RRS on that HCF vector, and the other LCF-encoding sequence is provided on a separate vector - that is, one vector having both LCF and HC (in LCF-HCF or HCF-LCF arrangement, or in short a "LCF/HCF vector"), one HCF vector, and one LCF vector. In some of these other embodiments, the vector RRSs can be designed to permit joining of the HCF-coding sequence on the HCF vector and the LCF-coding sequence on the LCF vector at a target locus via RMCE. For example, the 3' RRS of the LCF vector can be the same as the 5' RRS of the HCF

vector, and the common RRS can be designed in a split selectable marker or split intron format to facilitate selection and identification of transfectants. In still other embodiments, where the two LCFs are different, the two LCF-coding nucleotide sequences can each be provided on a separate vector - that is, the vector set includes two HCF vectors, and two LCF vectors. RRSs can be designed to permit proper "joining" of one LCF-coding sequence with one HCF-coding sequence at one target locus, and the other LCF-coding sequence with the other HCF-coding sequence at a second target locus. Figures 1, 3 and 4 are illustrative of different formats of vectors and RRS/loci combinations and not meant to be limiting. Each given vector system provides a means for simultaneously integrating each nucleotide sequence in the presence of a recombinase for the rapid and convenient selection of positive integrants (desired clones).

[00155] The nucleotide sequences encoding an HCF or LCF can encode amino acids or domain(s) from a constant region, or encode an entire constant region. In specific embodiments, a nucleotide sequences encoding an HCF or LCF can encode one or more constant domains, such as CL, CH1, hinge, CH2, CH3, or combinations thereof. In certain embodiments, a nucleotide sequence encoding a HCF domain can encode a CH3 domain. For example, the nucleotide sequence encoding the first HCF can encode a first CH3 domain, and the nucleotide sequence encoding the second HCF can encode a second CH3 domain. The first and second CH3 domains can be the same, or differ in at least one amino acid. The differences in the CH3 domains or in the constant regions can take any of the formats for bispecific antigen-binding proteins described herein, e.g., differences that result in different Protein A binding characteristics, or in a "knob-and-hole" format. Independent of any amino acid sequence differences, the two HCF-coding nucleotide sequences can also differ in that one of the two nucleotide sequences has been codon modified.

[00156] In some embodiments, each HCF or LCF-coding nucleotide sequence is independently and operably linked to a transcriptional regulatory sequence including e.g., a promoter. In some embodiments, the promoters directing transcription of the two -HCF-containing polypeptides are the same. In some embodiments, the promoters directing transcription of the two HCF-containing polypeptides, as well as the promoter directing transcription of the LCF-containing polypeptide, are all the same (e.g., a CMV promoter, or

any other suitable promoter described herein). In some embodiments, each HCF- or LCF-coding nucleotide sequence is independently and operably linked to an inducible or repressible promoter. Inducible or repressible promoters allow production to occur, for example, only in production phase (fed-batch culture) and not during growth phase (seed train culture). Inducible or repressible promoters also allow for differential expression of one or more genes of interest. In some embodiments, each HCF- and/or LCF-coding nucleotide sequence is independently and operably linked to a promoter upstream of at least one TetR operator (TetO) or Arc operator (ArcO). In still other embodiments, each HCF- and/or LCF-coding nucleotide sequence is independently and operably linked to a CMV/TetO or CMV/ArcO hybrid promoter. Examples of hybrid promoters (also referred to as regulatory fusion proteins) may be found in International Publication No. WO03101189A1, published December 11, 2003 (herein incorporated by reference).

[00157] In some embodiments, the vector set includes a nucleotide sequence encoding a recombinase that recognizes one or more RRSs, which can be included in one of the HCF or LCF-coding vectors, or provided in a separate vector.

[00158] In various other embodiments, vectors are provided to achieve site-specific integration via homologous recombination.

[00159] In some embodiments, a vector set is provided that includes two vectors, each containing an exogenous nucleic acid, flanked by a 5' homology arm and a 3' homology arm, for site-specific integration into two expression enhancing loci in a cell, wherein the exogenous nucleic acids from the two vectors together encode an antigen binding protein. Thus, the homology arms on one vector are designed for integration into one of the two loci, and the homology arms on the other vector are designed for integration into the other locus. In these embodiments, the antigen-binding protein can be monospecific or bispecific.

[00160] It is well within the skill of the artisan to select sequences homologous to sequences within an expression enhancing locus and include the selected sequences as homology arms in a targeting vector. In some embodiments, the vector or construct comprises a first homologous arm and a second homologous arm, wherein the first and second homologous arms combined comprise a targeted sequence which replaces an endogenous sequence within the locus. In other embodiments, the first and second homologous arms comprise a targeted

sequence which integrates or inserts within an endogenous sequence within the locus. In some embodiments, the homology arms contain a nucleotide sequence homologous to a nucleotide sequence present in SEQ ID NO: 1, SEQ ID NO: 2, or SEQ ID NO: 3. In specific embodiments, the vector contains a 5' homology arm having the nucleotide sequence corresponding to nucleotides 1001-2001 of SEQ ID NO: 3, and a 3' homology arm having the nucleotide corresponding to nucleotides 2022-3022 of SEQ ID NO: 3. Homologous arms, for example a first homologous arm (also called 5' homology arm) and a second homologous arm (also called 3' homology arm) are homologous to a targeted sequence within the locus. The homologous arms from 5' to 3' may expand a region or targeted sequence within the locus that comprises at least 1 kb, or at least about 2 kb, or at least about 3 kb, or at least about 4 kb, or at least 5 kb, or at least about 10 kb. In other embodiments, the total number of nucleotides of a targeted sequence selected for a first and second homologous arm comprises at least 1 kb, or at least about 2 kb, or at least about 3 kb, or at least about 4 kb, or at least 5 kb, or at least about 10 kb. In some instances, the distance between the 5' homology arm and the 3' homology arm (homologous to the targeted sequence) comprises at least 5 bp, 10 bp, 20 bp, 30 bp, 40 bp, 50 bp, 60 bp, 70 bp, 80 bp, 90 bp, 100 bp, 200 bp, 300 bp, 400 bp, 500 bp, 600 bp, 700 bp, 800 bp, 900 bp, or at least 1 kb, or at least about 2 kb, or at least about 3 kb, or at least about 4 kb, or at least 5 kb, or at least about 10 kb. In instances where nucleotides 1001-2001 and 2022-3022 of SEQ ID NO: 3 are chosen as 5' and 3' homology arms, the distance between the two homology arms can be 20 nucleotides (corresponding to nucleotides 2002-2021 of SEQ ID NO: 3); and such homology arms can mediate integration of an exogenous nucleic acid sequence within a locus comprising SEQ ID NO: 3, e.g., within nucleotides 1990-2021 or 2002-2021 of SEQ ID NO: 3, and a simultaneous deletion of nucleotides 2002-2021 of SEQ ID NO: 3.

[00161] The vectors disclosed herein for introducing exogenous nucleic acids for site-specific integration into an expression enhancing locus can include additional genes and sequences for directing the expression of exogenous nucleic acids of interest and encoded polypeptides and for the selection and identification of cells into which the exogenous nucleic acids of interest have successfully integrated. Such additional sequences include, for

example, transcriptional and translational regulatory sequences , selectable marker genes, and the like, also described hereinbelow.

Regulatory Sequences

[00162] The vectors disclosed herein for introducing exogenous nucleic acids into an expression enhancing locus in a site-specific manner, and the cells obtained as a result of site-specific integration, can include regulator sequences for directing the expression of exogenous nucleic acids of interest and encoded polypeptides. Regulatory sequences include transcriptional promoters, enhancers, sequences encoding suitable mRNA ribosomal binding sites, and sequences that control the termination of transcription and translation. Transcriptional and translational control sequences may be provided by viral sources. For example, commonly used promoters and enhancers are derived from viruses such as polyoma, adenovirus 2, simian virus 40 (SV40), mouse or human cytomegalovirus (CMV), CMV immediate early (CMV-IE) or CMV major IE (CMV-MIE) promoter, as well as RSV, SV40 late promoter, SL3-3, MMTV, ubiquitin (Ubi), ubiquitin C (UbC), and HIV LTR promoters. Viral genomic promoters, control and/or signal sequences may be utilized to drive expression, provided such control sequences are compatible with the host cell chosen. Non-viral cellular promoters can also be used (*e.g.*, the β-globin and the EF-1α promoters), depending on the cell type in which the proteins of interest are to be expressed. DNA sequences derived from the SV40 viral genome, for example, the SV40 origin, early and late promoter, enhancer, splice, and polyadenylation sites may be used to provide other genetic elements useful for expression of a exogenous DNA sequence. Early and late promoters are particularly useful because both are obtained easily from the SV40 virus as a fragment that also comprises the SV40 viral origin of replication (Fiers *et al., Nature* 273:113, 1978). Smaller or larger SV40 fragments may also be used. Typically, the approximately 250 bp sequence extending from the Hind III site toward the BglI site located in the SV40 origin of replication is included. Inducible (induced by a chemical compound, cofactor, regulatory protein, for example) and/or repressible (repressed by a chemical compound, cofactor, regulatory protein, for example) promoters can be used and are particularly useful for allowing the production of antigen binding proteins to occur only in production phase (fed-batch culture) and not during growth phase (seed train culture), or to differentially control expression of antibody components in

different loci with precision. Examples of inducible promoters include alcohol dehydrogenase I gene promoters, tetracycline-responsive promoter systems, glucocorticoid receptor promoters, estrogen receptor promoter, ecdysone receptor promoters, metallothionein-based promoters, and T7-polymerase based promoters. Examples of repressible promoters include hybrid promoters (also referred to as regulatory fusion proteins) comprising a CMV or other promoter operably linked to at least one TetR operator (TetO) or Arc operator (ArcO), and are described in International Publication No. WO03101189A1, published December 11, 2003 (herein incorporated by reference). Sequences suitable for the expression of multiple transcripts via a bicistronic vector have been described previously (Kim S. K. and Wold B. J., *Cell* 42:129, 1985) and can be used this invention. Examples of suitable strategies for multicistronic expression of proteins include the use of a 2A peptide (Szymczak et al., *Expert Opin Biol Ther* 5: 627–638 (2005)) and the use of an internal ribosome entry site ("IRES"), both well known in the art. Other types of expression vectors will also be useful, for example, those described in U.S. Pat. No. 4,634,665 (Axel *et al.*) and U.S. Pat. No. 4,656,134 (Ringold *et al.*).

Selectable Markers

[00163] The vectors disclosed herein for introducing exogenous nucleic acids into an expression enhancing locus in a site-specific manner, and the cells obtained as a result of site-specific integration, can include one or more selectable markers genes.

[00164] In some embodiments, a selectable marker gene confers drug resistance, such as, for example, those described in Table 1 of Kaufman, R. J. (1988) *Meth. Enzymology* 185:537, and include DHFR-MTX resistance, P-glycoprotein and multiple drug resistance (MDR)-various lipophilic cytotoxic agents (*e.g.*, adriamycin, colchicine, vincristine), and adenosine deaminase (ADA)-Xyl-A or adenosine and 2'-deoxycoformycin. Other dominant selectable markers include microbially derived antibiotic resistance genes, for example neomycin, kanamycin or hygromycin resistance. Several suitable selection systems exist for mammalian hosts (Sambrook supra, pgs 16.9-16.15). Co-transfection protocols employing two dominant selectable markers have also been described (Okayama and Berg, *Mol. Cell Biol* 5:1136, 1985).

[00165] In other embodiments, a selectable marker gene encodes a polypeptide that provides or is capable of generating a detectable signal for the recognition of gene cassettes that have or have not been successfully inserted and/or replaced, as the case may be. Suitable examples include a fluorescent marker or protein, an enzyme that catalyzes a chemical reaction that generates a detectable signal, among others. Examples of fluorescent markers are well-known in the art, including, but not limited to *Discosoma* coral (DsRed), green fluorescent protein (GFP), enhanced green fluorescent protein (eGFP), cyano fluorescent protein (CFP), enhanced cyano fluorescent protein (eCFP), yellow fluorescent protein (YFP), enhanced yellow fluorescent protein (eYFP) and far-red fluorescent protein (*e.g.* mKate, mKate2, mPlum, mRaspberry or E2-crimson. See also, e.g., Nagai, T., et al. 2002 *Nature Biotechnology* 20:87-90; Heim, R. et al. 23 February 1995 *Nature* 373:663-664; and Strack, R.L. et al. 2009 *Biochemistry* 48:8279-81.

Systems for Making Antigen-Binding Proteins

[00166] In a further aspect, this disclosure provides systems that include a combination of a cell (e.g., a CHO cell) with one or more vectors, and that can be utilized to make cells having integrated within two expression enhancing loci exogenous nucleic acids that together encode an antigen binding protein, either a monospecific protein or a bispecific protein. The systems can be provided in the form of a kit, for example.

[00167] In some embodiments, a system is designed to permit efficient vector construction and simultaneous integration of multiple exogenous nucleic acids via RMCE into specific sites within two enhanced expression loci. Simultaneous integration permits rapid isolation of desirable clones, and the use of two enhanced expression loci is also important for creation of a stable cell line suitable for protein production (e.g. commercially-enabled cell line).

[00168] The system provided herein includes a cell and a set of vectors. The cell contains a pair of RRSs (a 5' RRS and a 3' RRS) integrated within each of two enhanced expression loci. In some embodiments, an exogenous nucleic acid is present between the 5' RRS and the 3' RRS at each locus, and can include, e.g., one or more selectable marker genes. The vector set includes at least two vectors, with each vector containing a pair of RRSs (a 5' RRS and a 3' RRS) flanking a nucleotide sequence encoding an HCF or LCF, and with the nucleotide sequence on one of the two vectors encoding an HCF (an HCF vector), and the nucleotide

sequence on the other of the two vectors encoding an LCF (an LCF vector), and wherein the HCF and LCF are regions of an antigen-binding protein. The 5' RRS and 3' RRS within each pair of RRSs are different, and the RRSs in the system are designed such that upon introduction of the vectors into the cell, the HCF or LCF-encoding nucleotide sequences in the vectors integrate into the two enhanced expression loci through RMCE mediated by the RRSs to express the antigen-binding protein. Depending on whether the antigen-binding protein, the number of vectors, the placement of HCF or LCF- coding sequences, and the relationship among the RRSs, can be designed differently.

[00169] In some embodiments, the system is designed for integration into two expression enhancing loci and expression of monospecific antigen-binding proteins. In some embodiments, the 5' RRS and the 3' RRS on one of the two vectors (i.e., an HCF vector and an LCF vector) are identical to the 5' RRS and the 3' RRS in one of the two loci, respectively, and the 5' RRS and the 3' RRS on the other vector are identical to the 5' RRS and the 3' RRS in the other locus, respectively, essentially targeting the HCF and LCF nucleic acids separately to the two loci, one to each. In other embodiments, the HCF coding sequence and the LCF coding sequence, while on separate vectors, are designed for integration jointly into each of the two loci. According to these embodiments, the 5' RRS and the 3' RRS in the first locus are the same as the 5' RRS and the 3' RRS in the second locus, respectively; and each locus also contains an additional RRS between the 5' and 3' RRSs ("middle RRS"). In addition, the 5' RRS in a first the two vectors is the same as the 5' RRS in the first and second loci, the 3' RRS in that first vector is the same as the 5' RRS in the second vector and as the middle RRS in the first and second loci, and the 3' RRS is the second vector is the same as the 3' RRS in both loci. The vectors can be designed to have a split promoter and selection marker format (the promoter on one vector and the selection marker to which the promoter is operably linked on another vector). The vectors can be designed to have a split selectable marker format, or a split intron format, as described above, to facilitate selection of transfectants with proper integration. Further, the system can be designed to allow different relative positions of the LCF-coding sequence and the HCF-coding sequence after integration. In some embodiments, the system is designed to have the LCF-coding sequence integrated

upstream of the HCF-coding sequence. In other embodiments, the system is designed to have the LCF-coding sequence integrated upstream of the HCF-coding sequence.

[00170] In some embodiments, the system is designed for integration into two expression enhancing loci and expression of bispecific antigen-binding proteins.

[00171] In some embodiments, in addition to a HCF vector (encoding a first HCF) and a LCF vector (encoding a first LCF), the system also includes a nucleotide sequence encoding a second HCF that is different from the first HCF. The nucleotide sequence encoding the second HCF can be included, for example, in the LCF vector, or in a separate vector, i.e., a second HCF vector. In some embodiments, the second HCF-coding sequence is included in the LCF vector between the 5' RRS and 3' RRS on the LCF vector, in which case, the system includes a HCF vector and a LCF/HCF vector. The system, especially the RRSs, can be designed to integrate the HCF-coding sequence into one of the two loci, and the sequence that encodes both HCF and LCF into the other locus. In other embodiments, the nucleotide sequence encoding the second HCF is on a separate vector, flanked by a 5' RRS and a 3' RRS, in which case the system includes two HCF vectors and one LCF vector. In these other embodiments, the RRSs in the system can be designed such that the LCF-coding sequence can be "joined" via RMCE with one of the HCF-coding sequences through a common RRS which is also present in one of the two loci between the 5' RRS and the 3' RRS in that locus, and the other HCF-coding sequence will integrate into the other of the two loci. For example, the 3' RRS of the LCF vector can be the same as the 5' RRS of one the HCF vectors and also as a middle RRS on one of the two loci - this design will give rise to a LCF-HCF arrangement after integration into the locus having the middle RRS. In another example, the 3' RRS of a HCF vector can be the same as the 5' RRS of the LCF vector and as a middle RRS on one of the two loci, giving rise to a HCF-LCF arrangement after integration at the locus having the middle RRS. In some embodiments, the common RRS is designed in a split selectable marker format or a split intron format, as described hereinabove.

[00172] In some embodiments, the system also includes a nucleotide sequence encoding a second LCF, in addition to a HCF vector (encoding a first HCF) and a LCF vector (encoding a first LCF), and a nucleotide sequence encoding a second HCF that is different from the first HCF. That is, the system includes four, separate coding sequences, two encoding HCF and

two encoding LCF. The two LCFs can be the same or different. The four coding sequences can be placed in vectors in different designs. In some embodiments, the four sequences are placed in two vectors: LCF/HCF, and LCF/HCF, with LCF either upstream or downstream of HCF in either vector. The system (RRSs) can be designed such that one vector sequence integrates into one locus, and the other vector sequence integrates into the other locus. In some embodiments, the four sequences are placed in three vectors: LCF, HCF, and LCF/HCF (with LCF either upstream or downstream of HCF). The RRSs in the system can be designed such that the sequences in the LCF/HCF vector integrate into one locus, and the LCF coding sequence in the LCF vector and the HCF coding sequence in the HCF vector integrate into the other locus by utilizing a common RRS shared by the LCF vector, the HCF vector and this other locus. Similarly, the common RRS can be designed in a split marker or split intron format. In some embodiments, the four sequences are placed in four vectors: LCF, HCF, LCF, and HCF. The RRSs in the system can be designed such that the LCF coding sequence in one of the LCF vectors and the HCF coding sequence in one of the HCF vectors integrate jointly into one locus by utilizing a common RRS, and the LCF coding sequence in the other of the LCF vectors and the HCF coding sequence in the other of the HCF vectors integrate jointly into one locus by utilizing a common RRS.

[00173] In various embodiments of the system provided herein, the nucleotide sequences encoding an HCF or LCF can encode amino acids, e.g., amino acids or domain(s) from a constant region, or encode an entire constant region. In specific embodiments, a nucleotide sequences encoding an HCF or LCF can encode one or more constant domains, such as CL, CH1, CH2, CH3, or combinations thereof. In certain embodiments, a nucleotide sequence encoding a HCF domain can encode a CH3 domain. For example, the nucleotide sequence encoding the first HCF can encode a first CH3 domain, and the nucleotide sequence encoding the second HCF can encode a second CH3 domain. The first and second CH3 domains can be the same, or differ in at least one amino acid. The differences in the CH3 domains or in the constant regions can take any of the formats for bispecific antigen-binding proteins described herein, e.g., differences that result in different Protein A binding characteristics, or in a "knob-and-hole" format. Independent of any amino acid sequence differences, the two HCF-coding

nucleotide sequences can also differ in that one of the two nucleotide sequences has been codon modified.

[00174] In various embodiments of the system provided herein, each HCF or LCF-coding nucleotide sequence is independently and operably linked to a transcriptional regulatory sequence including e.g., a promoter. In some embodiments, the promoters directing transcription of the two HCF-containing polypeptides are the same. In some embodiments, the promoters directing transcription of the two HCF-containing polypeptides, as well as the promoter directing transcription of the LCF-containing polypeptide, are all the same (e.g., a CMV promoter, an inducible promoter, a repressible promoter, or any other suitable promoter described herein).

[00175] In some embodiments, the present system further includes a nucleotide sequence encoding a recombinase that recognizes one or more RRSs, which can be included in one of the variable region-coding vectors, or provided in a separate vector.

[00176] The systems disclosed herein are designed to permit efficient construction of vectors and rapid isolation of desirable clones, and the use of two enhanced expression loci is also important for creation of a stable cell line. In some embodiments, a system is designed to utilize negative selection for identifying transformants having intended site-specific integration (e.g., lack of fluoresence resulting from one or more fluroscent marker genes in the host genome being removed following RMCE). One round of negative selection may take only two weeks; however, the efficiency of isolating clones with intended recombination may be limited (about 1%). If negative selection is combined with positive selection based on a new selection marker provided by an integrated nucleic acid(s) (such as a a new fluorescence marker, or resistance to a drug or antibiotic, in a split format for example), the efficiency of isolating clones with intended recombination can be significantly improved (to about 40% up to about 80%). The systems can include additional components, reagents, or information, for examples, protocols for introducing the vector(s) in a system into the cell of the system by transfection. Non-limiting transfection methods include chemical-based transfection methods include the use of liposomes; nanoparticles; calcium phosphate (Graham et al. (1973) *Virology* 52 (2): 456–67, Bacchetti et al. (1977) *Proc Natl Acad Sci USA* 74 (4): 1590–4 and, Kriegler, M (1991) Transfer and Expression: A Laboratory Manual. New York: W. H.

Freeman and Company. pp. 96–97); dendrimers; or cationic polymers such as DEAE-dextran or polyethylenimine. Non chemical methods include electroporation; sono-poration; and optical transfection. Particle-based transfection include the use of a gene gun, magnet assisted transfection (Bertram, J. (2006) *Current Pharmaceutical Biotechnology* 7, 277–28). Viral methods can also be used for transfection. mRNA delivery includes methods using TransMessenger™ and TransIT® (Bire et al. *BMC Biotechnology* 2013, 13:75). One commonly used method of introducing heterologous DNA into a cell is calcium phosphate precipitation, for example, as described by Wigler *et al.* (Proc. Natl. Acad. Sci. USA 77:3567, 1980). Polyethylene-induced fusion of bacterial protoplasts with mammalian cells (Schaffner *et al.*, (1980) *Proc. Natl. Acad. Sci. USA* 77:2163) is another useful method of introducing heterologous DNA. Electroporation can also be used to introduce DNA directly into the cytoplasm of a host cell, for example, as described by Potter *et al.* (*Proc. Natl. Acad. Sci. USA* 81:7161, 1988) or Shigekawa *et al.* (BioTechniques 6:742, 1988). Other reagents useful for introducing heterologous DNA into a mammalian cell have been described, such as Lipofectin™ Reagent and Lipofectamine™Reagent (Gibco BRL, Gaithersburg, Md.). Both of these commercially available reagents are used to form lipid-nucleic acid complexes (or liposomes) which, when applied to cultured cells, facilitate uptake of the nucleic acid into the cells.

Methods for Making Antigen-Binding Proteins

[00177] This disclosure also provides methods of making bispecific antigen-binding proteins. By utilizing the methods disclosed herein, a desired antigen-binding protein can be produced at high titers and/or high specific productivity (pg/cell/day). In some embodiments, an antigen-binding protein is produced at a titer of at least 1 g/L, 1.5 g/L, 2.0 g/L. 2.5 g/L, 3.0 g/L, 3.5 g/L, 4.0 g/L, 4.5 g/L, 5.0 g/L, 10 g/L, or greater. In some embodiments, an antigen-binding protein is produced at a specific productivity of at least 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15 picogram/cell/day (pcd), or higher, determined based on total antigen-binding proteins (in pg) produced per cell per day. In some embodiments, a bispecific antigen-binding protein is produced at a ratio of the bispecific antigen-binding protein titer versus the total antigen-binding protein titer of at least 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 50%, 60% or higher.

[00178] In one embodiment, the method utilizes a system disclosed herein and introduces the vectors in the system into the cell of the system by transfection. Transfected cells where the exogenous nucleic acids have been properly integrated into two enhanced expression loci of the cell through RMCE can be screened and identified. In some embodiments, identification of transfected cells is achieved by negative selection against one or more selection markers present in a host cell before the transfection. In other embodiments, identication of transfected cells is achieved by negative selection against one or more selection markers present in a host cell before the transfection, in combination with positive selection based on one or more selection markers provided by the nucleic acids in the vectors that are designed to be integrated. HCF-containing polypeptides and LCF-containing polypeptides can be expressed from the integrated nucleic acids, and the antigen-binding protein of interest can be obtained from the identified transfected cell, and purified using known methods.

[00179] In another embodiment, the method simply utilizes a cell described hereinabove, which contains exogenous nucleic acids integrated at two enhanced expression loci that together encode an antigen-binding protein, and expresses the antigen-binding protein from the cell. Each cloned expression cassette(s) is contiguous within each specific integration site.

[00180] The present description is further illustrated by the following examples, which should not be construed as limiting in any way. The contents of all cited references (including literature references, issued patents, and published patent applications as cited throughout this application) are hereby expressly incorporated by reference.

EXAMPLES

Example 1: Expression of Monospecific Antibodies (Abs) in Two Specific Expression Enhancing Loci (via Site-Specific Integration)

[00181] Ab chains (AbC1, AbC2) were cloned into vectors in which RSS sites are flanking the Ab expression cassettes and the expression cassette for the selectable marker as depicted in Figure 1. Two Ab chains could be cloned into separate vectors or combined into one vector in which 2 expression cassettes are arranged in tandem in any one of the possible

orders: AbC1, AbC2, and the selectable marker, for example AbC1 is equivalent to a conventional LC and AbC2 is equivalent to a conventional heavy chain.

[00182] Briefly, DNA encoding VH and VL domains may be isolated directly from single antigen positive B cells by PCR. Heavy chain and light chain PCR products were cloned into Sap I-linearized antibody vector containing IgG heavy chain constant region and kappa light chain constant region, respectively. The heavy chain plasmid (AbC2) has an RRS3 and an RRS2 site flanking the heavy chain expression cassettes. In addition, immediately downstream of the RRS3 in the heavy chain plasmid there is a split selection marker gene (*e.g.*US7582298). The light chain plasmid has a RRS1 and RRS3 site flanking the light chain expression cassette. In addition, the light chain plasmid has a strong promoter immediately before an ATG at the RRS3, such that upon integration into the host cell locus the RRS3-proximal promoter and initiating ATG from the light chain plasmid is brought adjacent to the selection marker gene in the heavy chain plasmid in the proper reading frame to allow transcription and translation of the selection gene. Purified recombinant plasmids having a heavy chain variable region sequence and plasmids having a light chain variable region sequence from the same B cell were then combined and transfected, together with a plasmid that expresses a recombinase, into a modified CHO host cell line having the appropriate RSSs and selection markers at the SEQ ID NO:1 (EESYR®; Locus 1) and SEQ ID NO:2 loci. The modified CHO host cell line contains 4 different selection markers at two transcriptionally active loci. Consequently, where the selection markers are different fluorescent markers, the production CHO cell can be isolated by flow cytometry for positive-negative combinations that represent desired cell recombinants. When recombinant plasmids expressing heavy chain and light chain genes are transfected together with a plasmid expressing recombinase, site-specific recombination mediated by the recombinase results in the integration of the antibody plasmids at each chromosomal locus containing the RRSs and replacement. Accordingly, recombinant cells expressing monospecific antibody were isolated and subjected to 12 day fed batch production, followed by harvest and an Octet titer assay using immobilized Protein A. The cells were observed to be isogenic and stable. Overall titer in small shaker flask was observed to increase for the expression of monospecific antibodies when utilizing the two site

integration method, with Antibody B resulting in a significant increase, nearly doubling titer (Figure 2).

Example 2: Expression of Bispecific Antibodies (BsAbs) in Two Specific Expression Enhancing Loci (via Site-Specific Integration)

[00183] For the expression of bispecific antibodies, three antibody chains and two selectable markers were cloned into plasmids analogous to Example 1, such that AbC1, AbC2, and selectable marker 1 are flanked by RRS sites compatible with the first locus, or integration site (EESYR®, SEQ ID NO:1; Locus 1), and AbC1, AbC3, selectable marker 2 are compatible with the second locus, or integration site (SEQ ID NO:2). In our observations, AbC1 as a conventional LC does not require two gene copies for adequate expression. For each site, 1 or 2 plasmids were made, where the 3 expression cassettes are either arranged in tandem or arranged into 2 plasmids where 2 expression cassettes are cloned into one vector and the remaining expression cassette is cloned into the second vector. See Figure 3.

[00184] When recombinant plasmids expressing the heavy chain and light chain genes are transfected together with a plasmid expressing recombinase, site-specific recombination mediated by the recombinase results in the integration of the antibody plasmids at each chromosomal locus containing the RRSs and replacement. Accordingly, recombinant cells expressing bispecific antibody were isolated and subjected to 12 day fed batch production, followed by harvest and an Octet titer assay using immobilized anti-Fc, and a second anti-Fc* (modified Fc detection antibody, see US 2014-0134719 A1, published May 15, 2014). The cells were observed to be isogenic and stable. Overall titer in small shaker flask was observed to increase significantly from 1.75 to more than 2 fold from utilization of the two site integration method (Figure 5).

Example 3: Large Scale Production of Bispecific and Monospecific Antibodies following Site-Specific Integration

[00185] Host cells (CHO-K1) were created as described above analogous to Example 1 (see also Figure 3 for bispecific antibodies and Figure 1 for monospecific antibodies). Host cells enabled for RMCE of gene cassettes in the EESYR® locus (Locus 1) and SEQ ID NO: 2

(Locus 2) were compared to host cells enabled for RMCE of gene cassettes into only one integration site (Locus 1/EESYR®). Vectors carrying antibody light chain and heavy chains (AbC1, AbC2, AbC3) and the requisite RRS and selection marker nucleic acids (see Figure 3) were recombined into production cell lines (RSX^{2BP}) to create host cells expressing Ab E, Ab F, Ab G, and Ab H. As such, each bispecific antibody host cell expresses one common light chain, and two heavy chains that bind different antigens where one of the heavy chains is engineered in its CH3 domain to differentially bind Protein A (as described in U.S. Patent No. 8,586,713, herein incorporated by reference).

[00186] For monospecific antbodies, antibody light chain and heavy chain (AbC1, AbC2) were cloned into vectors in which RSS sites are flanking the Ab expression cassettes (and the expression cassette also provides a selectable marker gene) as depicted in Figure 1. Recombination was perfomed to create host cells (RSX^2) expressing Ab J and Ab K.

[00187] 2L, 15L, or 50L bioreactors were inoculated from a seed culture of the antibody-producing cell line RSX^2 derived from CHO-K1. The inoculated cells were grown at 36.5°C for thirteen days and fed glucose and other supplemental nutrients as needed. Cells were grown in chemically defined (hydrolysate-free and serum-free) base media. Total antibody was harvested and subjected to purification.

[00188] Total IgG antibody (titer) was determined following Protein A/Protein G chromatography. For bispecific antibodies, total IgG antibody as well as each of the three species of antibody including bispecific (heterdimeric Fc/Fc*), homodimer with wild-type heavy chains (Fc/Fc) and homodimer with modified heavy chains (Fc*/Fc*), was measured in order to determine the ratio of the desired bispecific antibody species. Total titers were determined by an HPLC method utilizing Protein A /Protein G columns using elution techniques as described in U.S. Patent No. 8,586,713. Briefly, the three bioreactor species bind to columns during sample loading and the bispecific species (Fc/Fc*) elutes first off of the Protein A column employing a pH step gradient in the presence of an ionic modifier. The bispecific species is collected during the first elution step, followed by elution of the two homodimeric species.

[00189] Table 1 shows that the overall (total) IgG titer and bispecific antibody titer (Figure 6A) in pilot large scale production culture were highly improved by utilizing host cells expressing antibody via two integration sites.

[00190] Table 1: Total IgG and Bispecifc IgG Titer Measurement

	Bispecific titer (g/L)		Total titer (g/L)	
Antibody	Two sites (Locus 1/Locus 2)	One site (Locus 1)	Two sites (Locus 1/Locus 2)	One site (Locus 1)
Ab E	2.25	0.87	4.47	2.7
Ab F	1.45	0.7	2.9	2.4
Ab G	1.6	0.5	2.7	1.3
Ab H	2	1	3.2	2.5

[00191] Bispecific titers were determined as described above. As seen in Table 2, the titers of bispecific antibody as a ratio of the total IgG titer produced by the cell is significantly higher in production cultures of host cells having the two integration site construction. See Figure 6B. In fact, it was unexpected that 50% or greater Bispecific ratios were consistently achieved.

[00192] Table 2: Ratio of Bispecific Antibodies per Total IgG Production

	Bispecific Ratio (% total IgG)	
	Two sites (Locus 1/Locus 2)	One site (Locus 1)
Ab E	50%	32%
Ab F	50%	29%
Ab G	59%	38%
Ab H	63%	40%

[00193] Monospecific antibodies were expressed using the two integration site method to determine improvements in overall IgG titers. Table 3 illustrates that overall titer in large

bioreactor scale was observed to increase significantly from 0.6 to 1.3 fold. See also Figure 7. For production bioreactors used in manufacturing, especially those at 500 L up to 10,000 L in culture volume, observed increases in titer by use of these improved cell lines equates to a significant amount of increased product yield per batch.

[00194] Table 3: Total IgG (Monospecific) Titer Measurement

	Total Titer (g/L)	
	Two sites (Locus 1/Locus 2)	One site (Locus 1)
Ab J	3.3	2.2
Ab K	3.2	2

[00195] Although the foregoing invention has been described in some detail by way of illustration and example, it will be readily apparent to those of ordinary skill in the art that certain changes and modifications may be made to the teachings of the invention without departing from the spirit or scope of the appended claims.

SEQUENCES

SEQ ID NO: 1
13515 bases
DNA
Cricetulus griseus

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WHAT IS CLAIMED IS:

1. A cell comprising,

a first exogenous nucleic acid integrated within a first enhanced expression locus; and a second exogenous nucleic acid integrated within a second enhanced expression locus; wherein the first and second exogenous nucleic acids together encoding an antigen-binding protein.

2. The cell of claim 1, wherein the first exogenous nucleic acid comprises a nucleotide sequence encoding a first HCF, and the second exogenous nucleic acid comprises a nucleotide sequence encoding a LCF.

3. The cell of claim 2, wherein the second exogenous nucleic acid further comprises a nucleotide sequence encoding a second HCF.

4. The cell of claim 3, wherein the first and second HCFs are different.

5. The cell of claim 3, wherein the nucleotide sequence encoding the first HCF encodes a first CH3 domain, and wherein the nucleotide sequence encoding the second HCF rencodes a second CH3 domain.

6. The cell of claim 5, wherein the first and second CH3 domains differ in at least one amino acid position.

7. The cell of claim 5, wherein the nucleotide sequences encoding the first and second CH domains differ from each other in that one of the nucleotide sequences has been codon modified.

8. The cell of claim 3, wherein the first exogenous nucleic acid further comprises a nucleotide sequence encoding a second LCF.

9. The cell of claim 8, wherein the first and second LCFs are the same.

10. The cell of any of claims 2, 3, 8, wherein each of the nucleotide sequences encoding a HCF or LCF is operably linked to a promoter.

11. The cell of claim 2, wherein a first RRS and a second RRS are positioned 5' and 3', respectively, relative to the first exogenous nucleic acid, and a third RRS and a fourth RRS are positioned 5' and 3', respectively, relative to the second exogenous nucleic acid, wherein the first and second RRSs are different, and the third and fourth RRSs are different.

12. The cell of claim 11, wherein the first, second, third and fourth RRS are different from each other.

13. The cell of claim 3, wherein a first additional RRS is present between the nucleotide sequence encoding the first LCF and the nucleotide sequence encoding the second HCF, and wherein said additional RRS is different from each of the first, second, third and fourth RRSs.

14. The cell of claim 13, wherein the first additional RRS is included within an intron of a selectable marker gene that is present between the nucleotide sequence encoding the first LCF and the adjacent nucleotide sequence encoding the second HCF.

15. The cell of claim 8, wherein a first RRS and a second RRS are positioned 5' and 3', respectively, relative to the first exogenous nucleic acid, and a third RRS and a fourth RRS are positioned 5' and 3', respectively, relative to the second exogenous nucleic acid, wherein the first and second RRSs are different, and the third and fourth RRSs are different.

16. The cell of claim 15, wherein the first and second HCFs are the same, and the first and second LCFs are the same, and wherein the first and third RRS are the same, and the second and fourth RRS are the same.

17. The cell of claim 15, wherein the first and second HCFs are different, and the first and second LCFs are the same, and wherein the first, second, third and fourth RRSs are different from each other.

18. The cell of claim 16 or 17, wherein a first additional RRS is present between the nucleotide sequence encoding the first LCF and the nucleotide sequence encoding the second HCF in the second exogenous nucleic acid, wherein the first additional RRS is different from the first, second, third and fourth RRSs.

19. The cell of claim 18, wherein the first additional RRS is included within an intron of a first selectable marker gene that is present between the nucleotide sequence encoding the first

LCF and the nucleotide sequence encoding the second HCF in the second exogenous nucleic acid.

20. The cell of claim 18, wherein a second additional RRS is present between the nucleotide sequence encoding the second LCF and the nucleotide sequence encoding the first HCF, wherein the first and second RRS are the same or different, and are each different from the first, second, third and fourth RRSs.

21. The cell of claim 20, wherein the first additional RRS is included within an intron of a first selectable marker gene that is present between the nucleotide sequence encoding the first LCF and the nucleotide sequence encoding the second HCF in the second exogenous nucleic acid, and the second additional RRS is included within an intron of a second selectable marker gene that is present between the nucleotide sequence encoding the second LCF and the nucleotide sequence encoding the first HCF, wherein the first and second selectable marker genes are different.

22. The cell of claim 2, wherein the antigen-binding protein is monospecific.

23. The cell of claim 4, wherein the antigen-binding protein is bispecific.

24. A cell comprising,

integrated within a first enhanced expression locus, from 5' to 3': a first RRS, a first exogenous nucleic acid, and a second RRS;

integrated within a second enhanced expression locus, from 5' to 3': a third RRS, a second exogenous nucleic acid, and a fourth RRS;

wherein the first and second RRS are different, and the third and fourth RRSs are different.

25. The cell of claim 24, wherein the first exogenous nucleic acid comprises a first selectable marker gene, and the second exogenous nucleic acid comprises a second selectable marker gene, wherein the first and the second selectable marker genes are different.

26. The cell of claim 25, wherein the first exogenous nucleic acid further comprises a first additional RRS, wherein the first additional RRS is different from first and second RRSs.

27. The cell of claim 26, wherein the second exogenous nucleic acid further comprises a second additional RRS, and the second additional RRS is different from the third and fourth RRSs.

28. The cell of claim 24, where the first exogenous nucleic acid comprises a first selectable marker gene, a first additional RRS, and a first additional selectable marker gene, wherein the first and first additional selectable marker genes are different, and the first additional RRS is different from the first and second RRSs.

29. The cell of claim 27, wherein the second exogenous nucleic acid comprises a second selectable marker gene, a second additional RRS, and a second additional selectable marker gene, wherein the second and second additional selectable marker genes are different from each other and also from the first and first additional selectable marker genes, and wherein the second additional RRS is different from the third and fourth RRSs.

30. The cell according to any of the preceding claims, wherein the cell is a CHO cell.

31. The cell of claim 30, wherein one of two enhanced expression loci is selected from the group consisting of a nucleotide sequence at least 90% identical to SEQ ID NO: 1, a nucleotide sequence at least 90% identical to SEQ ID NO: 2, and a nucleotide sequence at least 90% identical to SEQ ID NO:3.

32. A set of vectors for expressing a bispecific antigen-binding protein in a cell, comprising

a first vector comprising from 5' to 3', a first RRS, a first nucleic acid comprising a nucleotide sequence encoding a first HCF, and a second RRS;

a second vector comprising from 5' to 3', a third RRS, a second nucleic acid comprising a nucleotide sequence encoding a second HC, a fourth RRS;

and a nucleotide sequence encoding a first LC that is either within the first nucleic acid in the first vector, or is in a third vector different from the first and second vectors;

wherein the first, second, third, and fourth RRSs are different;

and wherein the bispecific antigen-binding protein comprises the first HCF, the second HCF and the first LCF, and wherein the first and second HCFs are different.

33. The set of vectors of claim 32, wherein the nucleotide sequence encoding the first LCF is within the first nucleic acid in the first vector.

34. The set of vectors of claim 34, wherein the first nucleic acid further comprises a first selectable marker gene.

35. The set of vectors of claim 32, wherein the nucleotide sequence encoding the first LCF is in the third vector and is flanked by a 5' RRS and 3' RRS, wherein (i) the 3' RRS is the same as the first RRS, and the 5' RRS is different from the first and second RRSs, or (ii) the 5' RRS is the same as the second RRS, and the 3' RRS is different from the first and second RRSs.

36. The set of vectors of claim 35, wherein the common RRS between the first and third vectors is placed at the 3' end of a 5' portion of a selectable marker gene on one of the first and third vectors, and is placed at the 5' end of the remaining 3' portion of the selectable marker gene on the other vector.

37. The set of vectors of claim 32, further comprising a nucleotide sequence encoding a second LCF that is either within the second nucleic acid in the second vector, or is in a fourth vector separate from the first, second and third vectors.

38. The set of vectors of claim 37, wherein the first and second LCFs are the same.

39. The set of vectors of claim 38, wherein the nucleotide sequence encoding the first LCF is within the first nucleic acid in the first vector, and the nucleotide sequence encoding the second VL is on the fourth vector.

40. The set of vectors of claim 39, wherein the nucleotide sequence encoding the second LCF on the fourth vector is flanked by a 5' RRS and 3' RRS, wherein (i) the 3' RRS is the same as the third RRS, and the 5' RRS is different from the third and fourth RRSs, or (ii) the 5' RRS is the same as the fourth RRS, and the 3' RRS is different from the third and fourth RRSs.

41. The set of vectors of claim 40, wherein the common RRS between the second and fourth vectors is placed at the 3' end of a 5' portion of a selectable marker gene on one of the second and fourth vectors, and is placed at the 5' end of the remaining 3' portion of the selectable marker gene on the other vector.

42. The set of vectors of claim 38, wherein the nucleotide sequence encoding the first LCF is within the first nucleic acid in the first vector, and the nucleotide sequence encoding the second VL is within the second nucleic acid on the second vector.

43. The set of vectors of claim 38, wherein the nucleotide sequence encoding the first LCF is on the third vector, and the nucleotide sequence encoding the second VL is on the fourth vector.

44. The set of vectors of claim 43, wherein the nucleotide sequence encoding the first LCF on the third vector is flanked by a 5' RRS and 3' RRS, wherein (i) the 3' RRS on the third vector is the same as the first RRS, and the 5' RRS on the third vector is different from the first and second RRSs, or (ii) the 5' RRS on the third vector is the same as the second RRS, and the 3' RRS on the third vector is different from the first and second RRSs; and wherein the nucleotide sequence encoding the second LCF on the fourth vector is flanked by a 5' RRS and 3' RRS, wherein (i) the 3' RRS on the fourth vector is the same as the third RRS, and the 5' RRS on the fourth vector is different from the third and fourth RRSs, or (ii) the 5' RRS on the fourth vector is the same as the fourth RRS, and the 3' RRS on the fourth vector is different from the third and fourth RRSs.

45. The set of vectors of claim 32, wherein the nucleotide sequence encoding the first HCF encodes a first CH3 domain, and the nucleotide sequence encoding the second HCF encodes a second CH3 domain.

46. The set of vectors of claim 45, wherein the first and second CH3 domains differ in at least one amino acid.

47. The set of vectors of claim 45, wherein the nucleotide sequences encoding the first and second CH3 domains differ in that one of the nucleotide sequences has been codon modified.

48. The set of vectors of claim 32, wherein each of the nucleotide sequences encoding a variable region is independently linked a promoter.

49. The set of vectors of claim 32, further comprising a nucleotide sequence encoding a recombinase that recognizes the first and second RRSs, and/or the third and fourth RRSs.

50. A set of vectors, comprising

a first vector comprising a first nucleic acid, flanked by a 5' homology arm and a 3' homology arm for integration into a first expression enhancing locus of a cell; and

a second vector comprising a second nucleic acid, flanked by a 5' homology arm and a 3' homology arm for integration into a second expression enhancing locus of the cell;

wherein the first and second nucleic acids together encode an antigen-binding protein.

51. A system comprising a cell and a set of vectors,

wherein the cell comprises,

integrated within a first enhanced expression locus: from 5' to 3', a first RRS, a first exogenous nucleic acid, and a second RRS;

integrated within a second enhanced expression locus: from 5' to 3', a third RRS, a second exogenous nucleic acid, and a fourth RRS;

wherein the first and second RRSs are different, and the third and fourth RRSs are different; and wherein the first and second enhanced expression loci are different;

wherein the set of vectors comprises,

a first vector comprising from 5' to 3', a first vector 5' RRS, a first nucleic acid, and a first vector 3' RRS, wherein the first vector 5' and 3' RRSs are different;

a second vector comprising from 5' to 3', a second vector 5' RRS, a second nucleic acid, and a second vector 3' RRS, wherein the second vector 5' and 3' RRSs are different;

a nucleotide sequence encoding a first HCF and a nucleotide sequence encoding a first LCF, wherein one of the nucleotide sequences is in the first nucleic acid and the other

nucleotide sequences is in the second nucleic acid; wherein the first HCF and the first LCF are regions of an antigen-binding protein; and

wherein upon introduction of the vectors into the cell, the first and second nucleic acids in the vectors integrate into the first enhanced expression locus and the second enhanced expression locus, respectively, through recombination mediated by the RRSs.

52. The system of claim 51, wherein the antigen-binding protein is a monospecific antigen-binding protein.

53. The system of claim 52, wherein the first and third RRSs are the same, and the second and fourth RRSs are the same.

54. The system of claim 53, wherein a first additional RRS is present between the first and second RRS in the first locus.

55. The system of claim 54, wherein the first vector 5' RRS is the same as the first and third RRS; the first vector 3' RRS, the second vector 5' RRS, and the first additional RRS are the same; and the second vector 3' RRS is the same as the second and fourth RRS.

56. The system of claim 55, wherein the VL-encoding nucleotide sequence is in the first vector, and the HCF-encoding nucleotide sequence is in the second vector.

57. The system of claim 55, wherein the first vector 3' RRS is placed at the 3' end of a 5' portion of a selectable marker gene, and the second vector 5' RRS is placed at the 5' end of the remaining 3' portion of the selectable marker gene.

58. The system of claim 52, wherein the first vector 5' RRS is the same as the first RRS, and the first vector 3' RRS is the same as the second RRS; and wherein the second vector 5' RRS is the same as the third RRS, and the second vector 3' RRS is the same as the fourth RRS.

59. The system of claim 52, wherein the antigen-binding protein is a bispecific antigen-binding protein.

60. The system of claim 59, further comprising a nucleotide sequence encoding a second HCF that is different from the first HCF.

61. The system of claim 60, wherein the nucleotide sequence encoding the first LCF and the nucleotide sequence encoding the second HCF are both included in the first nucleic acid in the first vector, and the nucleotide sequence encoding the first HCF is in the second vector.

62. The system of claim 61, wherein the first vector 5' RRS is the same as the first RRS, the first vector 3' RRS is the same as the second RRS, the second vector 5' RRS is the same as the third RRS, and the second vector 3' RRS is the same as the fourth RRS.

63. The system of claim 60, wherein the nucleotide sequence encoding the second HCF is on a third, separate vector, flanked by a third vector 5' RRS and a third vector 3' RRS.

64. The system of claim 63, wherein the nucleotide sequence encoding the first LCF is in the first vector, the nucleotide sequence encoding the first HCF is in the second vector, the first vector 5' RRS is the same as the first RRS, the first vector 3' RRS is the same as the second vector 5' RRS and as a first additional RRS, and the second vector 3' RRS is the same as the second RRS, the third vector 5' RRS is the same as the third RRS, and the third vector 3' RRS is the same as the fourth RRS, wherein the first additional RRS is included in the first locus between the first and second RRSs.

65. The system of claim 64, wherein the first vector 3' RRS is placed at the 3' end of a 5' portion of a selectable marker gene included in the first vector, and the second vector 5' RRS is placed at the 5' end of the remaining selectable marker gene included in the second vector.

66. The system of claim 61, further comprising a nucleotide sequence encoding a second LCF.

67. The system of claim 66, wherein the first and second LCFs are the same.

68. The system of claim 67, wherein the nucleotide sequence encoding the second LCF is in the second nucleic acid of the second vector, wherein the first vector 5' RRS is the same as the first RRS, the first vector 3' RRS is the same as the second RRS, the second vector 5' RRS is the same as the third RRS, and the second vector 3' RRS is the same as the fourth RRS.

69. The system of claim 67, wherein the nucleotide sequence encoding the second LCF is in a third, separate vector, flanked by a third vector 5' RRS and a third vector 3' RRS.

70. The system of claim 69, wherein the first vector 5' and 3' RRS are identical to the first and second RRS in the first locus, respectively; the third vector 5' RRS is the same as the third RRS, the third vector 3' RRS is the same as the second vector '5 RRS and as an additional RRS present between the third and fourth RRSs in the second locus, the second vector 3' RRS is the same as the fourth RRS.

71. The system of claim 70, wherein the third vector 3' RRS is placed at the 3' end of a 5' portion of a selectable marker gene included in the third vector, and the second vector '5 RRS is placed at the 5' end of the remaining 3' portion of the selectable marker gene included in the second vector.

72. The system of claim 60, wherein the nucleotide sequence encoding the first HCF encodes a first CH3 domain, and the nucleotide sequence encoding the second HCF encodes a second CH3 domain.

73. The system of claim 72, wherein the first and second CH3 domains differ in at least one amino acid.

74. The system of claim 72, wherein the nucleotide sequences encoding the first and second CH3 domains differ in that one of the nucleotide sequences has been codon modified.

75. The system of claim 51, wherein the nucleotide sequence encoding the first HCF and the nucleotide sequence encoding the first LCF are each operably linked to a promoter.

76. The system of claim 51, wherein the cell is a CHO cell.

77. The system of claim 76, wherein the two enhanced expression loci include a locus comprising the nucleotide sequence of SEQ ID NO: 1 and a locus comprising the nucleotide sequence of SEQ ID NO: 2.

78. A method, comprising:

(i) providing a system according to any one of claims 51-77;

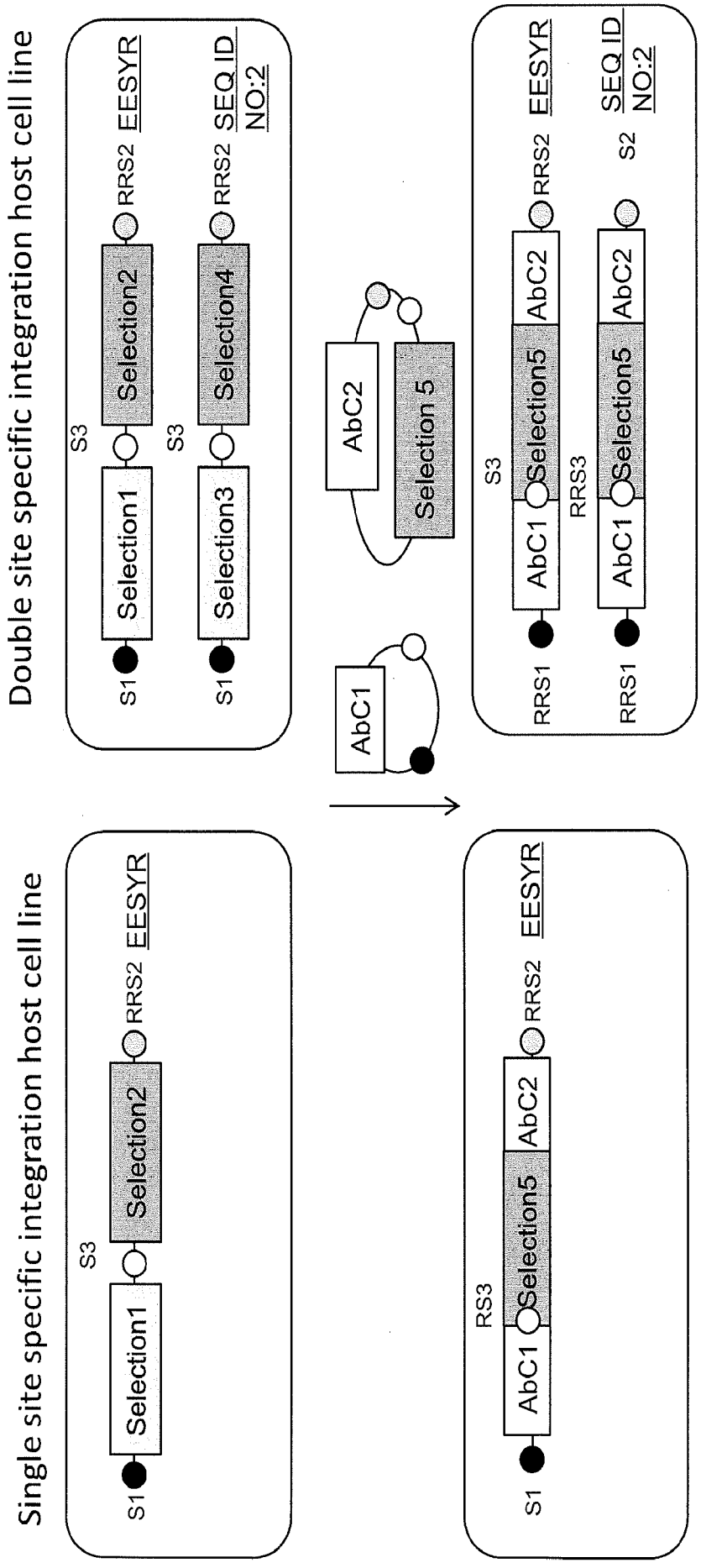
(ii) introducing the vectors into the cell by transfection; and

(iii) selecting a transfected cell where the nucleic acids in the vectors have integrated into the first and second enhanced expression loci through recombination mediated by the RRSs.

79. The method of claim 78, further comprising ;

(iv) expressing and obtaining the antigen-binding protein from the selected transfected cell.

80. A method of making an antigen-binding protein, comprising: providing a cell according to any one of claims 1-23, and expressing and obtaining the antigen-binding protein from the cell.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Selection2
RRS2
EESYR
S3
S1
Selection3
Selection4
RRS2
SEQ ID NO:2
AbC2
Selection 5
AbC1
Single site specific integration host cell line
S3
S1
Selection1
Selection2
RRS2
EESYR
RSX production cell line
RS3
S1
AbC1
Selection5
AbC2
RRS2
EESYR
RSX2 production cell line
S3
RRS1
AbC1
Selection5
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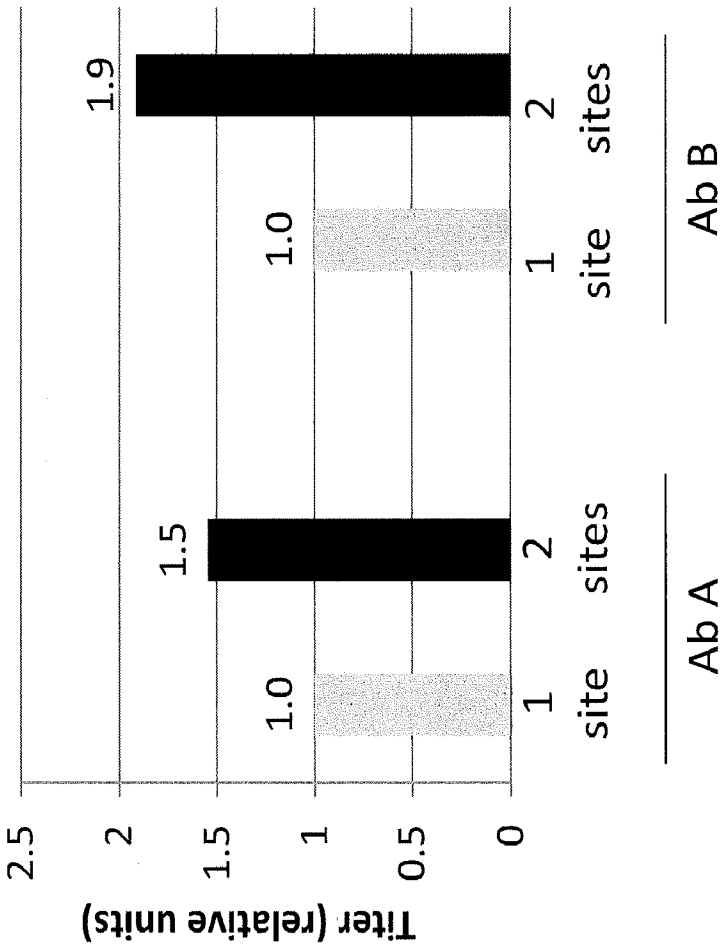
S2
SEQ ID NO:2

FIGURE 1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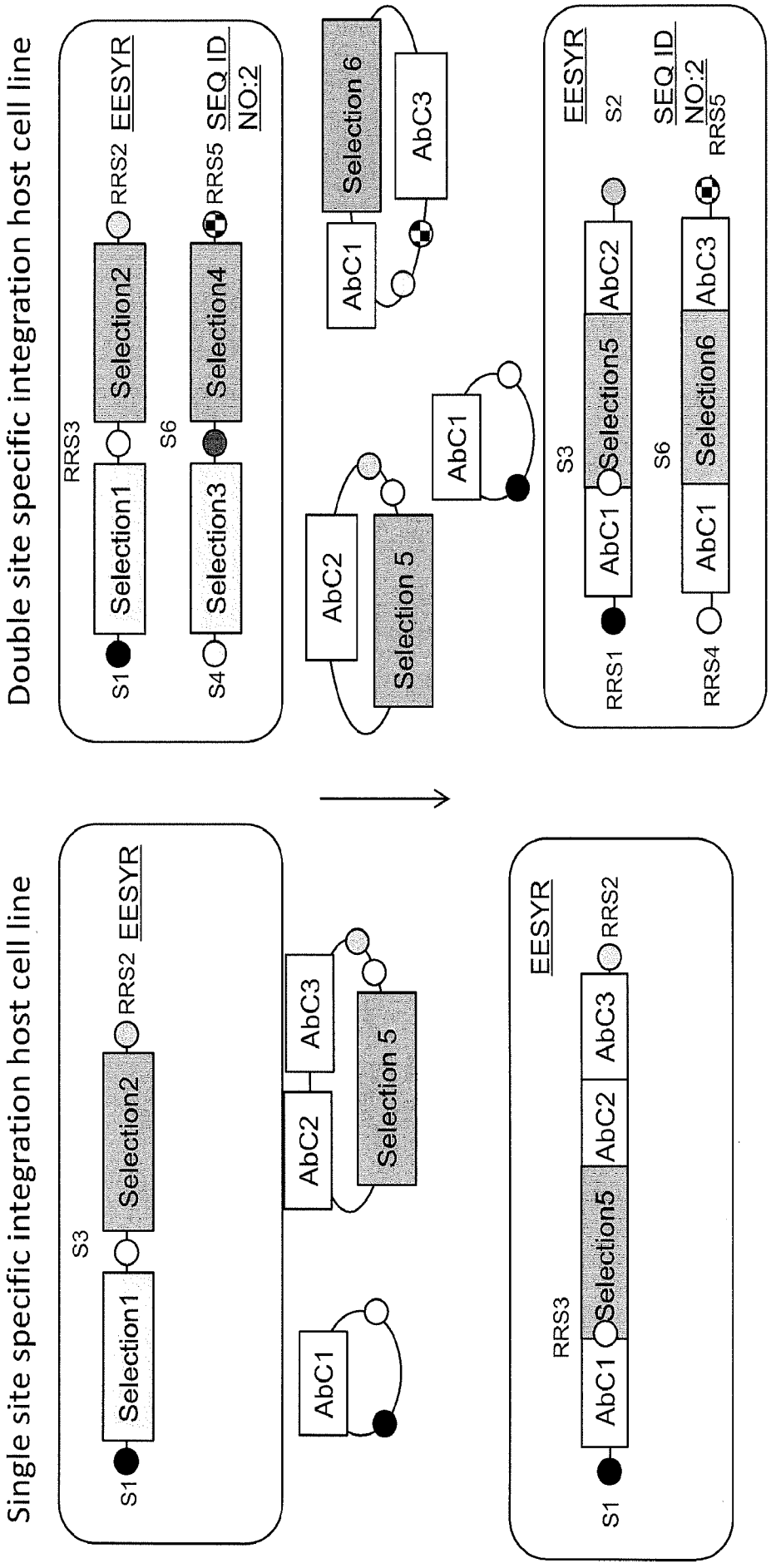
2 sites
1 site
2 sites
Ab A
Ab B

FIGURE 2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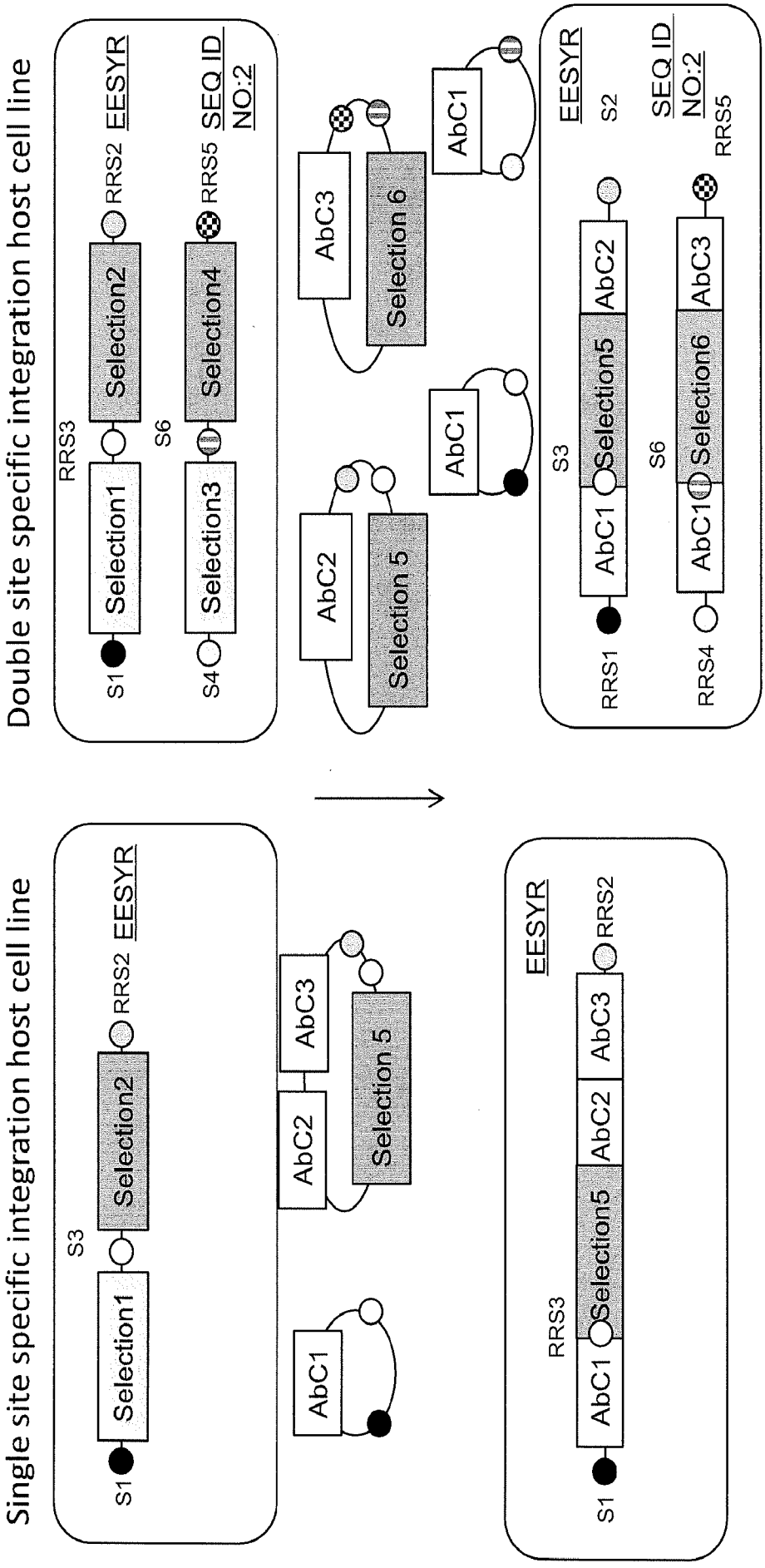
S3
Selection2
RRS2
EESYR
AbC1
AbC2
AbC3
Selection 5
RSX^BP production cell line
EESYR
RRS3
AbC1
Selection5
AbC2
AbC3
RRS2
S1
Double site specific integration host cell line
RRS3
S1
Selection1
Selection2
RRS2
EESYR
S6
S4
Selection3
Selection4
RRS5
SEQ ID NO:2
AbC2
Selection 5
AbC1
AbC1
Selection 6
AbC3
RSX^2BP production cell line
S3
AbC1
Selection5
AbC2
EESYR
S2
RRS1
S6
AbC1
Selection6
AbC3
SEQ ID NO:2
RRS4
RRS5

FIGURE 3


Single site specific integration host cell line
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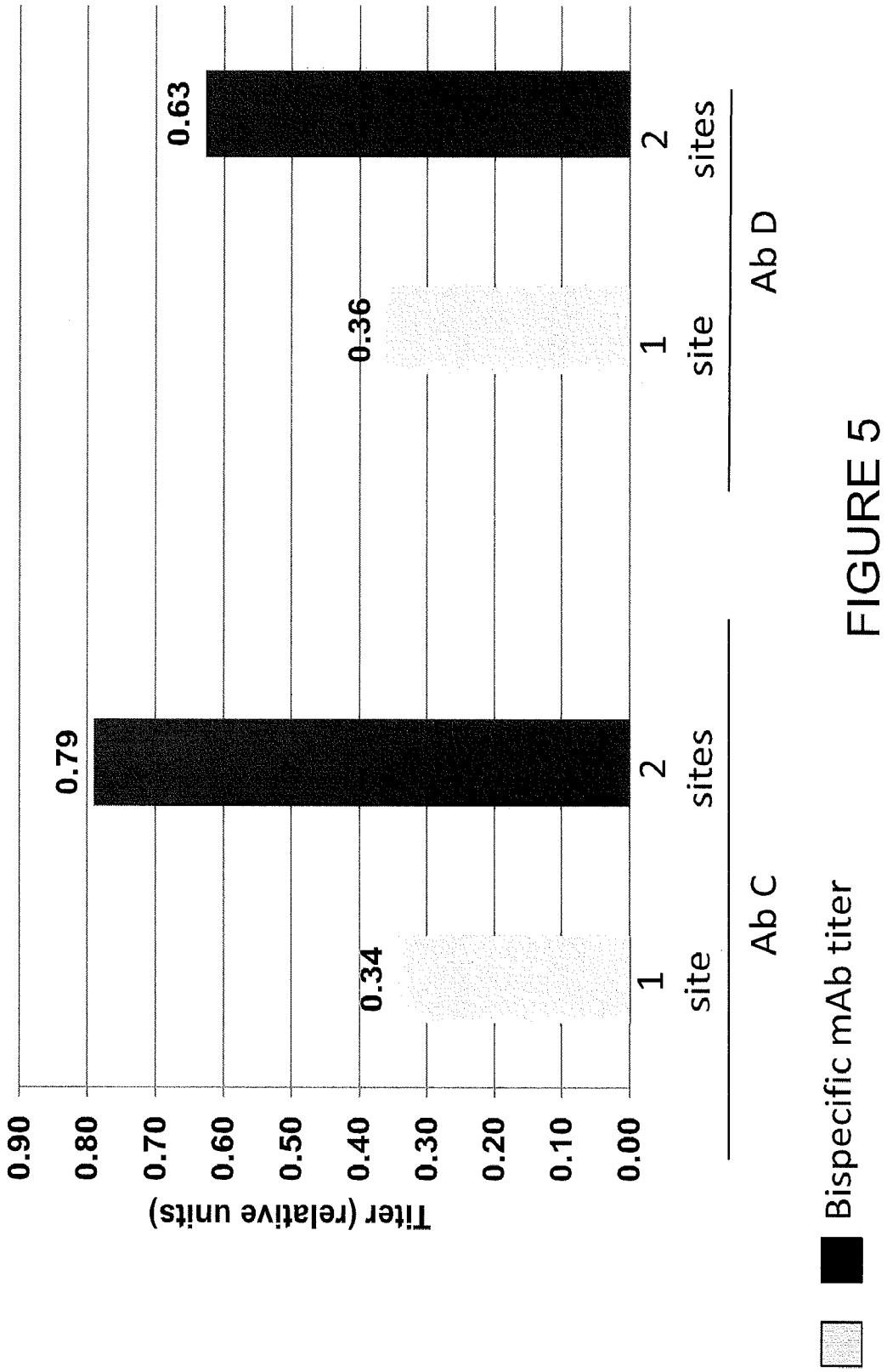
Selection1
S3
Selection2
RRS2
EESYR
AbC1
AbC2
AbC3
Selection 5
RSX^BP production cell line
EESYR
RRS3
AbC1
Selection5
AbC2
AbC3
RRS2
S1
Double site specific integration host cell line
RRS3
S1
Selection1
Selection2
RRS2
EESYR
S6
S4
Selection3
Selection4
RRS5
SEQ ID NO:2
AbC2
Selection 5
AbC3
Selection 6
AbC1
AbC1
S3
EESYR
S2
RRS1
AbC1
Selection5
AbC2
S6
SEQ ID NO:2
RRS5
RRS4
AbC1
Selection6
AbC3
RSX^2BP production cell line

FIGURE 4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0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
0.79
0.34
0.63
0.36
1 site
2 sites
1 site
2 sites
Ab C
Ab D
Bispecific mAb titer

FIGURE 5

FIGURE 6A

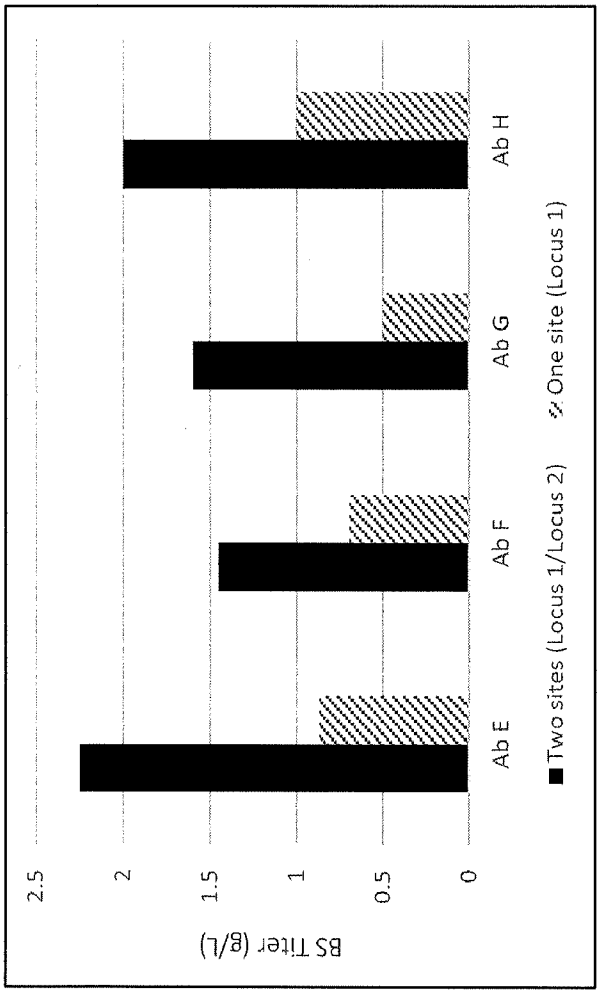
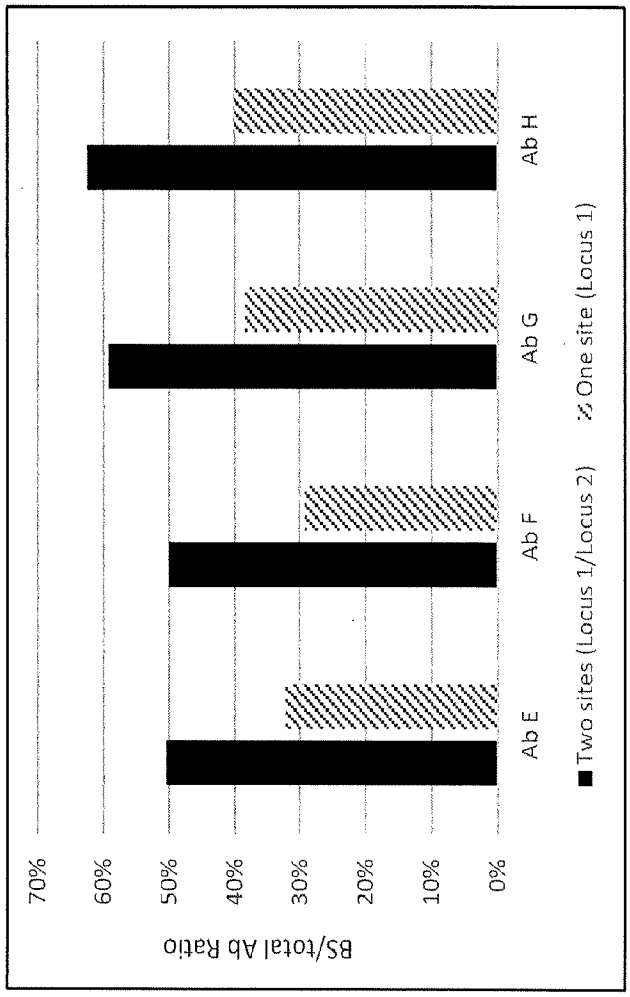


FIGURE 6B



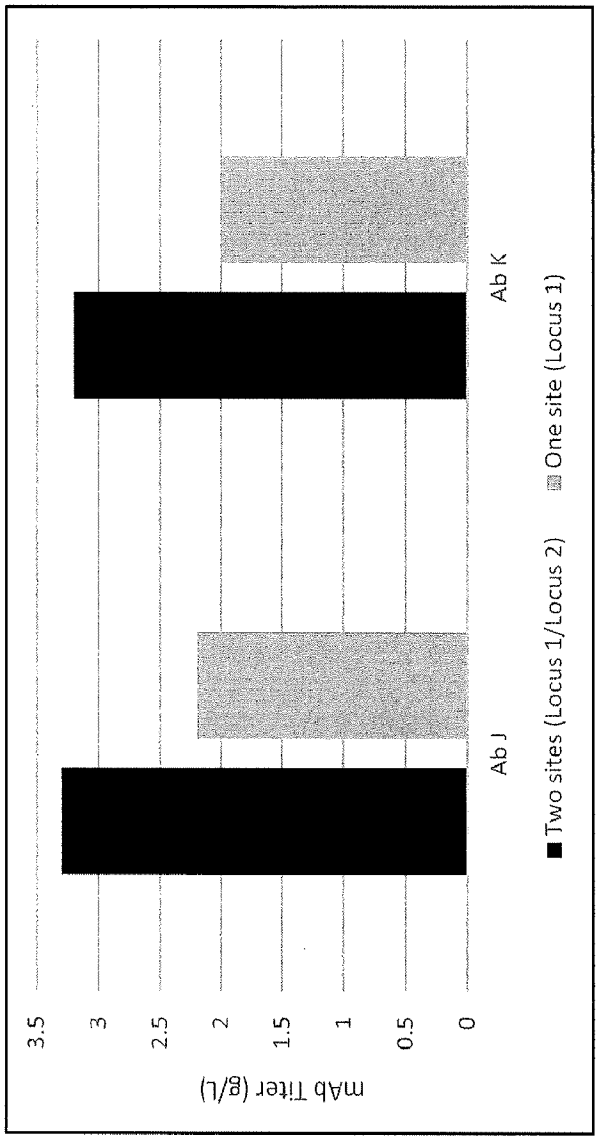

mAb Titer (g/L)
3.5
3
2.5
2
1.5
1
0.5
0
Ab J
Ab K
Two sites (Locus 1/Locus 2)
One site (Locus 1)

FIGURE 7

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2017/028555

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07K16/00 C07K16/46 C12N15/90
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C07K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, BIOSIS, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 2008/151219 A1 (REGENERON PHARMA [US]; CHEN GANG [US]; BABB ROBERT [US]; FANDL JAMES P) 11 December 2008 (2008-12-11) cited in the application page 1 - page 8; claims 1-24; examples 1-7; sequence SEQ ID No. 5 -----	1-30, 32-76, 78-80
Y	WO 2014/121087 A1 (REGENERON PHARMA [US]) 7 August 2014 (2014-08-07) paragraphs [0006], [0036], [0043], [0046], [0066], [0107] - [0109], [0160] - [0166], [0192] paragraphs [0195] - [0198] example 2 ----- -/--	1-30, 32-76, 78-80

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
22 June 2017	05/07/2017

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Marinoni J-C

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Form PCT/ISA/210 (second sheet) (April 2005)

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2017/028555

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 2013/190032 A1 (LONZA BIOLOGICS PLC [GB]; PFIZER [US]) 27 December 2013 (2013-12-27)	1-80
A	WO 2010/141478 A1 (REGENERON PHARMA [US]; CHEN GANG [US]; BURAKOV DARYA [US]; FANDL JAMES) 9 December 2010 (2010-12-09)	1-80
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