ABSTRACT
The invention provides a novel cell line development method useful to screen for recombinant protein production. The method utilizes a membrane-anchored reporter or an intracellular reporter residing in the expression vector for a gene of interest to facilitate initial cell selection by FACS or MACS. A switching mechanism can be used to delete the reporter from the chromosome by providing an appropriate DNA recombinase, which turns the selected cells into production cells that secrete the protein of interest without co-expression of the reporter.
Figure 1A

Promoter → MAR → GOI

Expression of Site Specific DNA Recombinase

Promoter → GOI

Figure 1B

Promoter → GOI → IRES/P → MAR → GOI

Expression of Site Specific DNA Recombinase

Promoter → GOI

Figure 1C

Promoter → GOI Exon 1 → MAR → GOI Exon 2

Expression of Site Specific DNA Recombinase

Promoter → GOI Exon 1 → GOI Exon 2
Figure 2

a)  

b)  

c)  

d)  

e)  

f)
Figure 3A

Splicing Donor → Splicing Acceptor 1 → Splicing Acceptor 2

GOI Exon 1 → GOI Exon 2 → MAD → GOI Exon 2

1. Alternative Splicing

GOI Exon 1 → GOI Exon 2 → MAD → Secreted POI

Membrane-anchored POI

Figure 3B

Splicing Donor → Splicing Acceptor 1 → Splicing Acceptor 2

GOI Exon 1 → MAD → GOI Exon 2

1. Alternative Splicing

GOI Exon 1 → MAD → Membrane-anchored POI (Exon 2 deleted)

Secreted POI
Figure 3C

Splicing Donor  Splicing Acceptor 1  Splicing Acceptor 2

GOI Exon 1  GOI Exon 2: Intron  GOI Exon 2  DRRS

1. Alternative Splicing

GOI Exon 1  GOI Exon 2  GOI Exon 1  GOI Exon 2  MAD

Secreted POI  Membrane-anchored POI

Figure 3D

Splicing Donor  Splicing Acceptor 1  Splicing Acceptor 2

GOI Exon 1  Intron  GOI Exon 2  Intron  MAD  DRRS

1. Alternative Splicing

GOI Exon 1  GOI Exon 2  GOI Exon 1  MAD

Secreted POI  Membrane-anchored POI (Exon 2 deleted)
Figure 4A

GOI Exon 1 → GOI Exon 2 → MAD → GOI Exon 2

Intron

DRRS

Expression of Site Specific DNA Recombinase

Intron 3

GOI Exon 1 → GOI Exon 2

DRRS

Secreted POI

Figure 4B

GOI Exon 1 → MAD → GOI Exon 2

Intron

DRRS

Expression of Site Specific DNA Recombinase

Intron

GOI Exon 1 → GOI Exon 2

DRRS

Secreted POI
Figure 4C

- GOI Exon 1 → GOI Exon 2 → GOI Exon 2 → MAD → DRRS
- Intron
- Expression of Site Specific DNA Recombinase
- GOI Exon 1 → GOI Exon 2 → DRRS
- Intron
- Secreted POI

Figure 4D

- GOI Exon 1 → GOI Exon 2 → MAD → DRRS → DRRS
- Intron
- Expression of Site Specific DNA Recombinase
- GOI Exon 1 → GOI Exon 2 → DRRS
- Intron
- Secreted POI
Figure 5

a) CH1 - Intron 1 - Hinge - Intron 2 - CH2 - Intron 3 - CH3

b) CH1 - Intron 1 - Hinge - Intron 2 - CH2 - Intron 3 - CH3 - MAD

c) CH2 - Intron 3 - CH3 - MAD - Intron 3 - CH3

LoxP

d) CH2 - Intron 3 - MAD - Intron 3 - CH3

LoxP

e) CH2 - Intron 3 - CH3 - Intron 3 - CH3 - MAD - Intron 3 - CH3

LoxP

f) CH2 - Intron 3 - CH3 - Intron 3 - MAD - Intron 3 - CH3
Figure 7A

Intron 3
CH₂  →  CH₃  MAD  →  CH₃
LoxP  LoxP

Expression of Cre Recombinase

Intron 3
CH₂  →  CH₃
LoxP

Secreted Antibody

Figure 7B

Intron 3
CH₂  →  MAD  →  CH₃
LoxP  LoxP

Expression of Cre Recombinase

Intron 3
CH₂  →  CH₃
LoxP

Secreted Antibody
**Figure 8A**

- POI expression vector with MAR cassette
  - Transfection in desired cells
    - Stable selection (optional)
      - FACS or MACS
        - Cells with high expression of MAR
          - Transient Expression of DNA recombinase
            - Cells with high expression of POI

**Figure 8B**

- Antibody library expression vectors with modified CH intron 3
  - Transfection in CHO
    - Stable selection (optional)
      - FACS or MACS
        - Cells with high expression / affinity of surface antibody
          - Transient Expression of DNA recombinase
            - Cells with high expression / affinity of secreted antibody
Figure 10B

Figure 11A

Figure 11B
Figure 14
NOVEL CELL LINE SCREENING METHOD

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the priority benefit of U.S. provisional application No. 61/732,156, filed Nov. 30, 2012, which is expressly incorporated by reference herein in its entirety.

SEQUENCE LISTING

[0002] The instant application contains a Sequence Listing which has been submitted electronically in ASCII format and is hereby incorporated by reference in its entirety. Said ASCII copy, created on Nov. 27, 2013, is named 34166-00003_SL.txt and is 32,799 bytes in size.

[0003] The present technology pertains to cell line development and protein production, and more specifically to a switch mechanism that converts cells that express a membrane-anchored receptor (MAR) into production cells that secrete a protein of interest (POI) into culture media.

BACKGROUND

[0004] Recombinant therapeutic proteins are widely used to treat numerous human diseases from cancer to infertility. They include various blood-clotting factors, insulin, growth hormones, enzymes, Fc fusion proteins, monoclonal antibodies and other proteins (Scott C., Bioprocess Int. 10(6): S72-S78, 2012). Many recombinant therapeutic proteins are manufactured using mammalian host cells because of the need for correct folding and post-translational modification including glycosylation. Among them, therapeutic antibodies represent one of the largest sectors of protein therapeutics with a global market of approximately $50 billion in 2011 for approximately 30 approved antibody therapeutics.

[0005] The predominant therapeutic antibodies come from antibody discovery programs that belong to four categories: chimeric antibodies, humanized antibodies, fully human antibodies from synthetic human antibody libraries selected with various display systems, and fully human antibodies from transgenic animals bearing human immunoglobulin genes (Chames P. et al, Br J Pharmacol. 157(2): 220-233, 2009). Chimeric antibodies containing human constant region and non-human variable region pose an immunogenicity risk in the human body and as a result have lost favor to humanized or fully human antibodies in terms of therapeutic applications. Humanized antibodies contain 90-95% human residues and 5-10% non-human residues that are essential for antigen interaction, whereas fully human antibodies contain 100% human residues. Both humanized and fully human antibodies have enjoyed great success in therapeutic applications to treat various diseases.

[0006] Development of a therapeutic antibody often takes 10-15 years including antibody discovery, engineering, production cell line development, manufacturing process development, and clinical studies. Among these tasks, antibody discovery may take 6-18 months and production cell line development may require an additional 6-10 months. One of the biggest problems with current antibody discovery methodologies is that they do not utilize the format of the final antibody product which is commonly a full-length human IgG. The selected antibodies are typically murine antibodies or fragments of human antibodies such as scFv or Fab, that require reformulating into the final IgG format before production cell line development. Reformating sometimes leads to unexpected problems in downstream process development, including loss of activity, low expression level, aggregation, insolubility, and/or instability. Therefore further antibody engineering and optimization may be required, resulting in loss of valuable time and increased cost.

[0007] Cell line development is a critical part of the process to obtain production cell lines for any therapeutic protein including antibodies. Production cell lines should be highly productive, stable, and have correct product quality attributes including biological activity, protein sequence homogeneity, glycosylation profile, charge variants, oxidation, deamidation and low levels of aggregation. CHO cells are the most popular mammalian cells for production of therapeutic proteins. Other mammalian cells like NS0 or SP2/0 cells have also been used to produce biological therapeutics (Jayapal K R, et al., Chemical Engineering Progress, 103: 40-47, 2007; Li F, et al., MAbs. 2(5): 466-477, 2010). Conventional cell line development utilizes gene amplification systems by incorporating Dihydrofolate Reductase (DHFR) or Glutamine Synthetase (GS) as selection markers. Typically up to 1000 clones are screened in a cell line development program by limiting dilution cloning in 96-well tissue culture plates. Obtaining a highly productive cell line requires gene amplification by adding selection pressure after stable transfection, for example, Methotrexate (MTX) in the DHFR system, or Methionine Sulfoximine (MSX) in the GS system. In most cases, productivity is often the only selection criteria until a very late stage in the process when only a handful of clones are assessed for the other quality attributes important for large scale manufacturing, resulting in increased project risk and complex issues regarding downstream development.

[0008] The process of selecting a cell population of interest for use as a recombinant protein production cell line may involve expression of a cell surface or intracellular reporter molecule. High level of expression of intracellular reporters such as GFP have been shown to be cytotoxic (Liu H S, et al., Biochem Biophys Res Commun, 260: 712-717, 1999; Wallace L M, et al., Molecular Therapy Nucleic Acids. 2, e86, 2013), however, the cytotoxicity of a reporter is minimized by cell surface display.

[0009] Display techniques have been developed for high-throughput screening of proteins, such as antibodies. Antibody display systems have been successfully applied to screen, select and characterize antibody fragments. These systems typically rely on phage display, E. coli display or yeast display. Each display system has its strengths and weaknesses, however, in general these systems lack post-translational modification functions or exhibit different post-translational modification functions from mammalian cells and tend to display small antibody fragments instead of full-length IgGs. Thus, characterization of the biological activities and further development of the isolated antibody fragments often requires conversion to whole immunoglobulins and expression in mammalian cells for proper folding and post-translational processing. This conversion process may produce antibodies with binding characteristics unlike those selected for in the initial screen.

[0010] There is a need for improved processes for selection of recombinant protein producing cell lines (such as antibody-producing cell lines), wherein the selection process facilitates rapid cell line selection based on quality attributes other than productivity. The present invention provides
improved compositions and methods for screening and selection of cell lines for recombinant protein production.

SUMMARY OF THE INVENTION

[0011] Additional features and advantages of the disclosure will be set forth in the description which follows, and in part will be obvious from the description, or can be learned by practice of the herein disclosed principles. The features and advantages of the disclosure can be realized and obtained by means of the disclosure and examples provided herein. These and other features of the invention will become more fully apparent from the following description and appended claims, or can be learned by the practice of the principles set forth herein.

[0012] The present invention relates to a switch mechanism that may turn off expression of a reporter (e.g., a cell surface or intracellular reporter) after selection of a subpopulation of cells providing for optimal expression of a POI. The reporter can be GFP or any other molecule detectable by FACS, MACS, or any other analytic method effective to detect the reporter. Expression of the reporter is functionally linked to expression of a POI such that the reporter is a surrogate for POI expression. The above-mentioned switch mechanism may be used to turn cells displaying a cell surface membrane anchored reporter (MAR) or intracellular reporter into production cells secreting a POI. Disclosed are a series of molecular designs which incorporate sequences of MAR flanked by site-specific DNA recombination recognition sequences (DRRS) inserted into an expression vector for a gene of interest (GOI). The reporter cassette could reside between the promoter and the GOI or downstream of the GOI following an internal ribosome entry site (IRES) or another promoter. In both cases, reporter expression will be first allowed to facilitate cell selection by FACS or MACS and then eliminated by transient expression or direct provision of an alternative site-specific DNA recombination to the cells in order to switch the cells to produce the POI without co-expression of the reporter.

[0013] Alternatively, the reporter cassette could reside in the middle of an intron sequence of the GOI to create alternative splicing leading to expression of the reporter. After transient expression or direct provision of an alternative site-specific DNA recombination protein, the reporter cassette is deleted from the intron enabling optimal expression and secretion of the POI without co-expression of the reporter.

BRIEF DESCRIPTION OF THE DRAWINGS

[0014] In order to describe the manner in which the above-recited and other advantages and features of the disclosure can be obtained, a more particular description of the principles briefly described above will be rendered by reference to specific embodiments thereof which are illustrated in the appended drawings. Understanding that these drawings depict only exemplary embodiments of the disclosure and are not therefore to be considered to be limiting of its scope, the principles herein are described and explained with additional specificity and detail through the use of the accompanying drawings.

[0015] FIGS. 1A-C are schematic drawings of exemplary MAR cassettes containing expression vectors and the switch mechanism that deletes the MAR. The MAR resides before the GOI (in A), or after the GOI following an IRES or another promoter (in B), or in the middle of an intron of the GOI (in C).

[0016] FIGS. 2A-F are schematic drawings of exemplary molecular structures of a model GOI, including A) a wild-type genomic sequence with one intron and two exons; B) the exon 2 fused with a membrane association domain (MAD); C) insertion of exon 2-MAD flanked by site specific DNA recombination recognition sequences (DRRS) in the intron; D) insertion of MAD flanked by DRRS into the intron; E) insertion of exon 2-MAD flanked by site specific DNA recombination recognition sequences (DRRS) downstream of exon 2 and F) insertion of MAD flanked by DRRS downstream of exon 2.

[0017] FIGS. 3A and B are schematic drawings of two alternative RNA splicing events of the molecular design in FIG. 2C (3A), FIG. 2D (3B), FIG. 2E (3C) and FIG. 2F (3D).

[0018] FIG. 4A-4D are schematic drawings of DNA recombination in the presence of an appropriate site specific DNA recombination of the molecular design in FIG. 2C (4A), FIG. 2D (4B), FIG. 2E (4C) and FIG. 2F (4D).

[0019] FIGS. 5A-F are schematic drawings of exemplary antibody heavy chain genomic sequences. A) Wild-type heavy chain constant region, including four exons (CH1, Hinge, CH2, and CH3) and three introns (Intron 1-3); B) Heavy chain fused with a MAD; C) Insertion of CH3-MAD flanked by LoxP sequences into the Intron 3; D) Insertion of MAD flanked by LoxP sequences into the Intron 3; E) Insertion of CH3-MAD flanked by LoxP sequences downstream of CH3; and F) Insertion of MAD flanked by LoxP sequences downstream of CH3. Regions before CH2 are present but not shown in C-F).

[0020] FIGS. 6A and B are schematic drawings of two alternative RNA splicing events of the molecular designs in FIG. 5C (6A) and FIG. 5D (6B). Regions before CH2 are not shown.

[0021] FIGS. 7A and B are schematic drawings of DNA recombination in the presence of Cre recombinase of the molecular designs in FIG. 5C (7A) and FIG. 5D (7B). Regions before CH2 are not shown.

[0022] FIGS. 8A and B are flow charts of an antibody cell line development process (8A) and an antibody library screening process (8B).

[0023] FIGS. 9A-C are plasmid maps of an antibody heavy chain expression vector (9A), an antibody light chain expression vector (9B) and an antibody heavy and light chain expression vector (9C).

[0024] FIG. 10A shows Rituxan levels in culture media 2 days after transfection of 293F cells with Rituxan expression vectors.

[0025] FIG. 10B shows FACS plots indicating cell surface Rituxan expression 2 days after transfection of 293F cells with Rituxan expression vectors.

[0026] FIG. 11A shows that membrane-anchored antibody expression was switched off by transient transfection of a Cre expression vector ("After Cre").

[0027] FIG. 11B shows that membrane-anchored antibody expression was switched off by treatment of cells with recombinant Cre ("After Cre").

[0028] FIG. 12A shows that cell surface antibody of a CH50 cell line that expresses both membrane-anchored Humira and secreted Humira via alternative splicing.

[0029] FIG. 12B shows that the membrane antibody expression correlates strongly with the secreted antibody lev-
els in cells that express both membrane-anchored Humira and secreted Humira via alternative splicing.

[0030] FIG. 13 shows lack of surface antibody on a Humira expressing cell line after switching off membrane anchorage.

[0031] FIG. 14 shows that a CHOS cell line expressing membrane-anchored Humira stained positively after binding with biotinylated TNFα and streptavidin Phycoerythrin conjugates.

DETAILED DESCRIPTION OF THE INVENTION

[0032] Provided herein are compositions, methods and systems for improved selection of production cells that secrete a protein of interest (POI) into culture media.

[0033] The invention is not limited to the specific compositions, devices, methodology, systems, kits or medical conditions described herein, as such may, of course, vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to limit the scope of the present invention.

[0034] The present invention concerns a switch mechanism that can be used to turn cells expressing a membrane-anchored reporter (MAR) or an intracellular reporter into production cells secreting a protein of interest (POI) into culture media, e.g., an antibody or any other protein. The MAR can be any molecule including a membrane-anchored POI, a membrane-anchored GFP, or any other membrane associated molecule which can be detected or selected using high throughput methodologies such as fluorescence-activated cell sorting (FACS) or magnetic-activated cell sorting (MACS), or any other analytic method effective to detect expression of the reporter molecule. The method allows for initial screening or selection of desired cells using methodologies such as FACS or MACS by detecting a reporter molecule, followed by application of a molecular switch that transforms the cells such that they secrete the POI without co-expression of the reporter molecule for production purposes.

[0035] Various embodiments of the disclosure are discussed in detail below. While specific embodiments are discussed, it should be understood that this is done for illustration purposes only. A person skilled in the relevant art will recognize that other configurations may be used without departing from the spirit and scope of the disclosure.

DEFINITIONS

[0036] It must be noted that as used herein and in the appended claims, the singular forms “a”, “an”, and “the” include plural references unless the context clearly dictates otherwise.

[0037] Unless defined otherwise, all technical and scientific terms used herein generally have the same meaning as commonly understood to one of ordinary skill in the art to which this invention belongs.

[0038] The publications discussed herein are provided solely for their disclosure prior to the filing date of the present application. Nothing herein is to be construed as an admission that the invention is not entitled to antedate such a disclosure by virtue of prior invention.

[0039] As used herein, the term “membrane-anchored reporter” or “MAR” is used with reference to any membrane molecule or a non-membrane molecule fused with a membrane association domain (MAD).

[0040] As used herein, the term “membrane association domain” or “MAD” is used with reference to a protein domain associated with a membrane, which could be a GPI anchor signal sequence (GASS), a transmembrane domain, or any molecule that binds to a cell membrane or a membrane protein e.g., an Ab, GFP, and the like. In one aspect of the invention a host cell is characterized by the expression of a cell surface membrane anchored reporter fused to a POI wherein expression of the reporter is detected by FACS, MACS or any technique that can detect cell surface expression of a POI. Expression of the cell surface membrane anchored reporter fused to a POI is detected following transfection with a DNA construct such as shown in FIGS. 3A-D, 6A and B.

[0041] As used herein, the term “protein of interest” or “POI” is used with reference to a protein having desired characteristics that may be selected using the method of the invention. A “protein of interest” (POI) includes full length proteins, polypeptides, and fragments thereof; peptides, all of which can be expressed in the selected host cell. Exemplary POIs are antibodies, enzymes, cytokines, adhesion molecules, receptors, derivatives and any other polypeptides that can be expressed using the methods described herein. In another aspect of the invention, the protein of interest is recovered from the culture medium as a secreted polypeptide. In general, the protein of interest is produced in the culture media at a level of at least 100 mg/L, at least 150 mg/L, at least 200 mg/L, at least 300 mg/L, at least 500 mg/L, at least 1000 mg/L, e.g., 100-150 mg/L, 150-200 mg/L, 200-250 mg/L, 250-300 mg/L, 300-500 mg/L, 500-1000 mg/L. In some cases, the POI, e.g., an enzyme, may be biologically active at low concentration. In such cases, production at a level below 100 mg/L in the culture media will satisfy commercial production requirements. In general, methods teaching a skilled person how to purify a protein expressed by host cells are well known in the art.

[0042] As used herein, the term “gene of interest” or “GOI” is used with reference to a polynucleotide sequence of any length that encodes a “protein of interest” (POI). The selected sequence can be full length or a truncated gene, a fusion or tagged gene, and can be a cDNA, a genomic DNA, or a DNA fragment, preferably, a cDNA. It can be the native sequence, i.e., naturally occurring form(s), or can be mutated or otherwise modified as desired.

[0043] The term “host cells” or “expression host cells” as used herein refers to any cell line that will effectively produce a POI with correct folding and post-translational modification including glycosylation as required for biological activity. Exemplary host cells include Chinese Hamster Ovary (CHO) cells, e.g., CHOS (Invitrogen), NSO, Sp2/0, CHO derived mutant cell or derivatives or progenies of any of such cells. Other mammalian cells, including but not limited to human, mouse, rat, monkey, and rodent cells, and eukaryotic cells, including but not limited to yeast, insect, plant and avian cells, can also be used in the meaning of this invention, as appropriate for the production of a particular POI.

[0044] As used herein, the term “magnetic-activated cell sorting” or “MACS” is used with reference to a method for separation of various cell populations depending on their surface antigens (CD molecules). The term MACS is a registered trademark of Miltenyi Biotec and the method is marketed by the company as MACS Technology.

[0045] As used herein, the term “DNA recombinase recognition sequence” or “DRRS” is used with reference to a
sequence that facilitates the rearrangement of DNA segment by the activity of a site-specific recombinase which recognizes and binds to short DNA sequences resulting in cleavage of the DNA backbone such that two DNA sequences are exchanged, followed by rejoicing of the DNA strands.

[0046] As used herein, the term “GPI anchored signal sequence” or “GASS” is used with reference to a glycolipid that can be attached to the C-termius of a protein during posttranslational modification. It is composed of a phosphatidylinositol group linked through a carbohydrate-containing linker (glucosamine and mannose glycosidically bound to the inositol residue) to the C-terminal amino acid of a mature protein. The hydrophobic phosphatidylinositol group anchors the protein to the cell membrane.

[0047] The term “productivity” or “specific productivity” describes the quantity of a specific protein (e.g., a POI) which is produced by a defined number of cells within a defined time. One exemplary way to measure “productivity” is to seed cells into fresh culture medium at defined densities. After a defined time, e.g., after 24, 48, or 72 hours, a sample of the cell culture fluid is taken and subjected to ELISA measurement to determine the titer of the protein of interest. The productivity can be reported as mg/L of culture media. In the context of industrial manufacturing, the specific productivity is usually expressed as amount of protein in picogram produced per cell per day (“pg/cell/day”).

[0048] The term “biological activity” describes and quantifies the biological functions of the protein within the cell or in vitro assays.

DESCRIPTION

[0049] The present invention relates to a series of molecular designs incorporating sequences of a membrane-anchored reporter (MAR) or an intracellular reporter flanked by site specific DNA recombinase recognition sequences (DRRS) inserted into an expression vector for the GOI. The reporter cassette could reside between the promoter and the GOI (FIG. 1A), or after the GOI following an IRES or another promoter (FIG. 1B), or in the middle of an intron of the GOI (FIG. 1C). The reporter is first expressed allowing cell selection e.g., by FACS or MACS, and then deleted by transient expression or direct provision of an appropriate site-specific DNA recombinase to switch the cells to secretion of the POI without co-expression of the reporter. The MAR needs to be detected or selected using high throughput methodologies such as FACS or MACS, and could be any membrane protein or non-membrane protein fused with a membrane association domain (MAD), which could be a GPI anchor signal sequence (GASS), or a transmembrane domain, or any peptide that binds to a cell surface protein. For example, the MAR could be membrane-anchored GFP or membrane-anchored POI. One of the advantages of using a membrane anchored reporter protein instead of an intracellular reporter protein is to avoid any potential cellular toxicity after accumulation of high concentrations of the reporter protein. SEV ID NO: 1 shows the nucleotide sequence of GFP fused to the IL2 signal peptide at the N-terminus and DAF GASS at the C-terminus. Any GFP variants or other fluorescence proteins, functional signal peptides or membrane association domains can be used here.

[0050] After being transfected with reporter-containing expression vectors, the host cells such as CHO cells can be allowed to grow in the presence of appropriate antibiotics for selection of stable cells with the expression vector integrated into the chromosome. Alternatively, the transfected cells can be selected directly for the reporter expression by FACS or MACS for 1-2 weeks until the reporter expression is stable. The advantage of not using antibiotics is for better health of the cells and potentially less gene-silencing of the expression cassette (Kaufman W. L. et al., Nucleic Acids Res., 36(17), e11, 2008). Desired cells with high expression levels of the reporter or other properties (such as stability, protein sequence homogeneity, proper glycosylation profile, proper charge variants, and acceptable aggregate levels), can then be selected with FACS or MACS or another analytic technique. Subsequently the reporter cassette can be deleted by providing an appropriate site-specific DNA recombinase to switch the selected cells into production cells that produce the POI. The DNA recombinase can be supplied to the cells by transient transfection with an expression vector, or by direct provision of the DNA recombinase protein to the culture media, or by any other means.

[0051] The present invention also provides a series of molecular designs to modify the intron sequence of a GOI. FIG. 2A shows the genomic sequence of a model secreted GOI containing two exons and an intron. It would be anchored on cell surface if fused with a MAD, which could be a GASS, or a transmembrane domain, or any peptide that binds to a cell surface protein (FIG. 2B). In order to create an alternative splicing site, the DNA sequence of GOI Exon 2 fused with MAD flanked by DRRS can be inserted into the Intron (FIG. 2C). MAD flanked by DRRS can be inserted into the Intron (FIG. 2D), the DNA sequence of GOI Exon 2 fused with MAD flanked by DRRS can be inserted downstream of Exon 2 (FIG. 2E), or MAD flanked by DRRS can be inserted downstream of Exon 2 (FIG. 2F).

[0052] The mRNA may contain an unaltered splicing donor or any functional splicing donor for the Intron and two splicing acceptors shown in FIG. 3A. The two splicing acceptors may be identical to the splicing acceptor in the wild-type GOI mRNA or any functional splicing acceptor. This exemplary alternative splicing would lead to membrane-anchored POI using the acceptor 1 or secreted POI using the acceptor 2 (FIG. 3A). If only secreted POI is desired, appropriate site-specific DNA recombinase recognizing the DRRS can be transiently expressed in the cell and the sequence between the DRRS deleted as shown in FIG. 4A.

[0053] Another way to manipulate the Intron is to insert a MAD sequence flanked by DRRS directly (FIG. 2D). Alternative splicing shown in FIG. 3B would lead to either membrane-anchored POI with Exon 2 deleted or secreted POI. When an appropriate site-specific DNA recombinase is supplied, the sequence between DRRS is deleted and all the expressed POI is secreted (FIG. 4B).

[0054] Yet another way to manipulate the Intron is to insert Exon-2-MAD flanked by site specific DNA recombinase recognition sequences (DRRS) downstream of Exon 2 (FIG. 2E). Alternative splicing shown in FIG. 3C would lead to either membrane-anchored POI with Exon-1 and Exon-2 fused to MAD, or secreted POI with Exon1 and Exon2. When an appropriate site-specific DNA recombinase is supplied, the sequence between DRRS is deleted and all the expressed POI is secreted (FIG. 4C).

[0055] A further way to manipulate the Intron is to insert a MAD sequence flanked by DRRS downstream of Exon 2 (FIG. 2F). Alternative splicing shown in FIG. 3D would lead to either membrane-anchored POI with Exon 2 deleted, or secreted POI with Exon1 and Exon2. When an appropriate
site-specific DNA recombinase is supplied, the MAD sequence between DRRS is deleted and all the expressed POI is secreted (FIG. 4D).

[0056] FIGS. 5-7 describe how the above-mentioned intron modifying designs are applied to manipulate a human immunoglobulin gamma genomic sequence. The genomic structure of the wild-type human antibody IgG1 heavy chain constant region contains four exons and three introns as shown in FIG. 5A. The DNA sequence is shown in SEQ ID NO: 3. FIG. 5B shows a membrane-anchored antibody heavy chain prepared by fusing with a MAD. It has been reported that membrane-anchored antibodies can be constructed by fusing a GASS or a transmembrane domain (TM) at the C-terminus of the heavy chain (Zhou C, et al., Mabs, 2:508-518, 2010; Bowers P M, et al. Proc Natl Acad Sci USA, 108(51):20455-20460, 2011; Li. F, et al., Appl Microbiol Biotechnol., 96(5):1233-41 2012). The GASS of human DAF is shown in SEQ ID NO: 4 (nucleotide) and SEQ ID NO: 5 (amino acid), respectively. Any other GASS or TM or any peptide that binds to a cell surface protein can also be used for membrane anchoring. In order to create an alternative splicing site, the DNA sequence for CH3-MAD flanked by DRRS can be inserted into Intron 3 (FIG. 5C), or into the other two introns; the DNA sequence for MAD flanked by DRRS can be inserted into Intron 3 (FIG. 5D), the DNA sequence for CH3-MAD flanked by LocXp sequences may be inserted downstream of Exon 3 (FIG. 5E) or the DNA sequence for MAD flanked by LocXp sequences may be inserted downstream of Exon 3 (FIG. 5F).

[0057] One commonly used recognition sequence for DNA recombinase Cre is LocXp the sequence of which is shown in SEQ ID NO: 8. Similarly, any other LocXp variant sequences or recognition sequences for other site specific DNA recombinases can be used here, for example, an FRT sequence (DRRS for FLP), or an attB or attP (DRRS for 4C31 integrase (Wang Y, et al., Plant Cell Rep.; 30(3):267-85, 2011) or a specific DNA recognition sequence for any other tyrosine recombinase or serine recombinase. The mRNA may have an unaltered splicing donor for the Intron 3 and two identical splicing acceptors as shown in FIG. 6A.

[0058] The alternative splicing would lead to membrane-anchored antibody using the acceptor 1 or secreted antibody using the acceptor 2. If only secreted antibody is desired, DNA recombinase Cre can be transiently expressed in the cell or via the culture media and the sequence between the two LocXp sites will be deleted as shown in FIG. 7A.

[0059] For example, if one were to manipulate Intron 3 to insert a DNA sequence for MAD flanked by LocXp sites directly (FIG. 5D), alternative splicing shown in FIG. 6B would give membrane-anchored antibody with CH3 deleted or secreted antibody. When DNA recombinase Cre is expressed, the sequence between LocXp sites is deleted and all the expressed antibody is secreted (FIG. 7B). Similarly, the MAD flanked by LocXp sites can also be inserted into the other two introns to create alternative splicing.

[0060] FIG. 8A illustrates an exemplary cell line development process utilizing the MAR cassette and the switching mechanism. Expression vector of the GOI is modified with the MAR cassette as shown in FIG. 1. It could carry a selection marker gene. If the POI contains more than one subunit, they may be cloned into the same vector or into separate expression vectors. The expression vector or vectors are transfected into desired cells. After 1-2 weeks allowing stable integration into the chromosome, with or without antibiotic selection, the cells are analyzed and selected for high expression of the MAR by any high throughput cell selection or enrichment methodology, such as FACS or MACS. In one approach, the selected cells are transfected transiently with an expression vector for an appropriate DNA recombinase to induce site-specific DNA recombination. Deletion of the MAR cassette results in cells that produce the POI. Following single cell sorting or limiting dilution cloning into 96-well plates, clones are screened for the POI expression levels in the culture media and/or other desired product quality attributes. Selected clones are expanded and cryopreserved.

[0061] In one application of the invention, an human immunoglobulin gamma expression vector comprising a membrane association domain (MAD) flanked by site specific DNA recombinase recognition sequences (DRRS) can be inserted into the intron region between CH2 and CH3 sequences. The MAD can be a GPI anchored signal sequence (GASS) or a transmembrane domain or a peptide that binds to any cell surface protein. Alternative splicing results in a portion of expressed antibodies to be membrane-anchored and thus readily detected by fluorescence-labeled antigen or secondary antibody. After selection of cells with high expression levels of membrane-anchored antibodies by FACS, the cells may then be switched into production cells secreting the antibody into culture media by transient expression of an appropriate site-specific DNA recombinase in order to delete the sequences responsible for membrane association in the intron. The switch mechanism can be used for cell line development with greatly reduced time and cost, and can be used for production of antibody or any other recombinant protein.

[0062] Library display techniques have been developed for high-throughput screening of proteins having desired characteristics, WHO 2010/022961 discloses a method for generating or selecting a eukaryotic host cell expressing a desired level of a polypeptide of interest from a population of host cells by use of a fusion polypeptide including an immunoglobulin transmembrane anchor such that the fusion polypeptide is being displayed on the surface of the host cell.


[0064] DuBridge et al. U.S. Pat. No. 7,947,495, disclose dual display vector compositions and methods which provide for expression of secreted and membrane-bound forms of an immunoglobulin based on splice sites and recombinase recognition sites, allowing for simultaneous expression of transcripts for a membrane-bound immunoglobulin and a secreted form of the same immunoglobulin in a single host cell.

[0065] Beeri, R., et al., (PNAS, vol. 105 (38), 14336-14341, 2008) describe a technology for the rapid isolation of fully human mAbs by isolation of antigen-specific B cells from human peripheral blood mononuclear cells (PBMC) and generation of recombinant, antigen-specific single-chain Fv (scFv) libraries which are screened by mammalian cell surface display using a Sindbis virus expression system, which is followed by isolation of fully human high-affinity antibodies following a single round of selection. Another display system used to screen, select and characterize antibody fragments based on display of full-length functional antibodies on the
surface of mammalian cells relies on recombinase-mediated DNA integration coupled with high throughput FACS screening for selection of antibodies with very high antigen binding affinities is disclosed by Zhou et al. (mAbs 2:5, 508-518; 2010).

Mammalian cell based immunoglobulin libraries that rely on use of “removable-tether display vectors,” or “transmembrane display vectors,” which can be used for the expression of cell surface-bound immunoglobulins for affinity-based screening and the expression of secreted immunoglobulin are disclosed by Akamatsu et al., U.S. Pat. No. 8,163,546. In these “removable-tether display vectors”, the polynucleotide encoding the cell surface tether domain is flanked by a first and a second restriction endonuclease site.

The invention disclosed herein provides improved libraries and screening methods for selecting a POI with desired characteristics.

FIG. 8B illustrates an exemplary antibody library screening process utilizing alternative splicing and the switching mechanism. In one exemplary approach, a library of VH sequences can be cloned into an expression vector with modified Intron 3 as shown in FIG. 5C or 5D. A library of light chain sequences may be cloned into the same expression vector or into a separate vector. Expression of the antibody library vectors will result in an expression library of membrane-anchored antibodies. After transfection into CHO cells or other expression host cells, cells expressing antigen binding antibodies may be sorted or selected by FACS or MACS. Multiple rounds of sorting or selection may be performed under different stringent conditions to isolate production cells for antibodies with the best affinity for the antigen. The antibody sequences can be obtained from the selected cells by PCR or RT-PCR. The selected cells may be turned into production cells by expression of an appropriate site specific DNA recombinase to delete the MAD sequence. Similar designs may be applied to any engineered libraries of antibody or any other proteins. See, Example 8.

EXAMPLES

Example 1

Expression of Rituxan from Expression Vector Containing a LoxP Site in the 3rd Intron of the Heavy Chain Genomic Sequence

The Rituxan heavy chain variable sequence (VH) was gene synthesized and cloned into a mammalian expression vector containing the human IgG1 heavy chain constant region genomic sequence between restriction sites Xba I and Nhe I, to make vector LB0-H. The Rituxan VH sequence including signal peptide is shown in SEQ ID NO: 9. Expression of the antibody heavy chain was under the control of an EF1α promoter. The vector carries a Puromycin resistance gene for stable cell selection and an Ampicillin resistance gene for E. coli propagation. The plasmid map is shown in FIG. 9A.

The Rituxan light chain cDNA was gene synthesized and cloned into a separate mammalian expression vector between restriction sites Xba I and BamHI to make vector LB0-K. The sequence of the light chain is shown in SEQ ID NO: 11. Expression of the antibody light chain was under the control of an EF1α promoter. It carries a Neomycin resistance gene for stable cell selection and an Ampicillin resistance gene for E. coli propagation. The plasmid map is shown in FIG. 9B.

A LoxP site was inserted into the middle of the 3rd intron of Rituxan genomic sequence in LB0-H by Bridge PCR to make vector LB1. The sequence of the heavy chain constant region is shown in SEQ ID NO: 13.

To express Rituxan, 293F’ cells (Invitrogen Inc.) were co-transfected with LB0-H or LB1, together with LB0-K. Transfection conditions were optimized with Freestyle Max transfection reagent (Invitrogen) and a GFP expression vector. 50 μg of DNA and 37.5 μl of Freestyle Max were used to transfect 30 ml of cells (1×10⁸ cells/ml). The cells were typically diluted 3 times the next day and subjected to flow cytometry analysis for GFP expression after one more day of culturing. Transfection efficiencies were determined to be ~80% for 293F’ cells under these conditions. To assay Rituxan expression, antibody levels in media were determined by dilution ELISA in which Rituxan was captured with goat anti-human IgG Fc (100 ng/ml, Bethyl) and detected with the goat anti-human Kappa antibody HRP conjugates (1:10,000 dilution, Bethyl).

Human IgG antibody (2 μg/ml of IgG, Sigma) was used as the standard for IgG quantitation. The expression levels are shown in FIG. 10A. LB1-transfected cells produced a similar amount of antibody as the wild-type control LB0-H, suggesting that the LoxP sequence inserted in the middle of the intron between CH2 and CH3 did not affect antibody expression levels. The heavy chain constant regions in both transfected cells were amplified by RT-PCR and sequenced. The RNA splicing was found to be identical with or without a LoxP site inside of the gamma chain intron.

Example 2

Expression of Rituxan Anchored on Cell Surface

The human IgG1 CH3 sequence fused with the DAF GPI anchor signal sequence (SEQ ID NO: 4) or the PDGFR TM domain sequence (SEQ ID NO: 6) followed by LoxP and intron 3 sequences were synthesized and inserted into the 3rd intron in LB1 to make vector LB3 or LB4, respectively, as shown in FIG. 5C. The sequences of the heavy chain constant region of LB3 and LB4 are shown in SEQ ID NO: 14 and SEQ ID NO: 15, respectively. The light chain expression cassette in LB0-K was digested with restriction enzymes EcoRV and Asc I. The DNA fragment of 2625 bp was then cloned into LB4 between EcoRV and Asc I to make Rituxan expression vector LB37. The plasmid map is shown in FIG. 9C. To assess Rituxan expression, 293F’ cells were co-transfected with heavy chain vectors LB3, or LB4, together with the light chain vector LB0-K using Freestyle Max transfection reagent.

The antibody titers in media were determined by dilution ELISA in which Rituxan was captured with goat anti-human IgG Fc (100 ng/ml, Bethyl) and detected with the goat anti-human Kappa antibody HRP conjugates (1:10,000 dilution, Bethyl). Human IgG antibody (2 μg/ml of IgG, Sigma) was used as the standard for IgG quantitation. The expression levels are shown in FIG. 10A. Vectors employing the modified intron demonstrate robust expression of Rituxan ranging from 4-10 μg/ml in culture media after 2 days when cell densities are typically about 1×10⁶ cells/ml. LB1-transfected cells produced a similar amount of antibody as the wild-type control LB0-H, suggesting that the LoxP sequence
inserted in the middle of the intron between CH2 and CH3 did not affect antibody expression levels. LB3 secreted 2-3 times more antibody into the media than LB4, consistent with the fact that GPI-linked membrane anchorage is not 100%. This result has been reproduced in 3 independent experiments.

[0076] The transfected 293F cells were also labeled with goat anti-human Fe antibody FITC conjugate (1:1,000 dilution, Bethyl) and subjected to flow cytometric analysis. 293F cells transfected with the wild-type CH3 exon vector (LB0-H, FIG. 10B.a) or LoxP modified CH3 exon vector (LB1, FIG. 10B.b) did not show cell surface antibody expression, whereas 293F cells transfected with alternatively spliced CH3-GASS vector (LB3, FIG. 10B.c) or CH3-TM vector (LB4, FIG. 10B.d) exhibited cell surface antibodies in 20-30% of the cells (Table 1).

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<th>Transfection</th>
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<td>LB1 + LB0-K</td>
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<td>LB3 + LB0-K</td>
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Example 3
Expression of CH3 Deleted Rituxan Anchored on Cell Surface

[0077] The DAF GPI anchor signal sequence or the PDGFR TM domain sequence followed by LoxP and intron3 sequences were synthesized and inserted into the 3′′ intron in LB1 to make vector LB9 or LB10, respectively, as shown in Figure 4 The sequences of the heavy chain constant region of LB9 and LB10 are shown in SEQ ID NO: 16 and SEQ ID NO: 17, respectively.

[0078] 293F cells were co-transfected with LB9 or LB10, together with LB0-K using Freestyle Max transfection reagent. After 2 days the antibody levels in the media were assayed by ELISA similarly as described in Example 1. LB9-transfected cells secreted more antibodies into media than LB10-transfected cells (FIG. 10A), consistent with the result described in Example 2. The transfected 293F cells were also labeled with goat anti-human Fe antibody FITC conjugate (1:1,000 dilution, Bethyl) and subjected to flow cytometric analysis. 293F cells transfected with alternatively spliced vectors LB3 (FIG. 10B.c) or LB4 (FIG. 10B.d) exhibited cell surface expression of antibody in 8-20% of the cells (Table 1).

Example 4
Expression of Membrane-Anchored GFP Upstream of the Rituxan Heavy Chain

[0079] The membrane-anchored GFP (SEQ ID NO: 2) carrying a Kosaak consensus sequence was flanked by two LoxP sites, and inserted between the EF1α promoter and the Rituxan gamma sequence in the vector LB0-H to make vector LB11, as described in FIG. 1A. The sequence between the 2 LoxP sites was deleted in LB11 and only one LoxP site remained to make vector LB11-LoxP. 293F cells were co-transfected with LB0-H, LB11, or LB11-LoxP, together with LB0-K using Freestyle Max transfection reagent. After 2 days, antibody levels were assayed by ELISA as described in Example 1. LB11-transfected cells produced less than 10% of relative to LB0-H-transfected cells (FIG. 10A), suggesting that the presence of the GFP cassette upstream of the Rituxan heavy chain gene greatly diminished expression of Rituxan. After removal of the GFP cassette, LB11-LoxP produced Rituxan at a similar level to LB0-H (FIG. 10A). Expression of GFP in LB11-transfected cells was confirmed by flow cytometric analysis.

Example 5
Expression of Membrane-Anchored GFP Downstream of the Rituxan Heavy Chain

[0080] An IRES sequence (SEQ ID NO: 18) was fused with the membrane-anchored GFP (SEQ ID NO: 1) carrying a Kosaak consensus sequence. A LoxP site was then added at both N- and C-terminals. The whole sequence was inserted downstream of the Rituxan gamma stop codon and before the poly A signal in the vector LB0-H to make vector LB14, as described in FIG. 1B. 293F cells were co-transfected with LB4 and LB0-K using Freestyle Max transfection reagent. After 2 days, the antibody level in the media was assayed by ELISA as described in Example 1, and was shown in FIG. 10A. Expression of GFP in LB11-transfected cells was confirmed by flow cytometric analysis.

Example 6
Switching Off the Membrane-Anchored Antibody by Providing Cre

[0081] A Cre Expression Vector LB30 Was Constructed. The Cre cDNA Was Human Codon optimized and fused with a peptide of MPKKKKRK (SEQ ID NO: 19) at the N-terminus for nuclear localization. Expression of Cre was driven by a human EF1α promoter.

[0082] The 293F cells were transfected with LB37 linearized with restriction enzyme AscI using Freestyle Max transfection reagent, and cultured in the presence of 1 μg/ml of Puromycin and 400 μg/ml of G418. After selection for approximately 2 weeks, the stable pool was transiently transfected with the Cre expression vector LB30. After one more week of culture, the cells were labeled with goat anti-human Fe antibody FITC conjugate (1:1,000 dilution, Bethyl) and subjected to flow cytometric analysis to assess cell surface Rituxan expression. Most of the cells lost membrane-anchored antibody after Cre transfection as shown in FIG. 11A ("After Cre").

[0083] Switching off the membrane-anchored antibody was also achieved by providing recombinant Cre in the cell culture. A cell line expressing membrane-anchored antibody cloned from the stable pool described above was treated with 1 μM of recombinant Cre fused with TAT-NLS for nuclear localization (Excellgen, Inc.) for 2 hours. After one additional week of culture, the cells were assayed for cell surface antibody expression, as described above. Most of the cells lost membrane-anchored antibody as shown in FIG. 11B ("After Cre").
Example 7
Screening of Highly Productive Humira Production Cell Lines

[0084] The variable sequence of Humira light chain (SEQ ID NO: 20) was gene synthesized and cloned into LB0-K between restriction sites Xba1 and BsiWI to make vector LB42. The variable sequence of Humira heavy chain (SEQ ID NO: 22) was gene synthesized and cloned into LB4 between restriction sites Xba1 and HaeI to make vector LB25. The light chain expression cassette in LB42 was digested with restriction enzymes EcoRV and AscI. The DNA fragment of 2641 bp was then cloned into LB25 between EcoRV and AscI to make Humira expression vector LB29.

[0085] CHO cells (Invitrogen, Inc.) were cultured in Freestyle CHO media (Invitrogen, Inc.). 1 x 10^5 CHO cells were transfected with LB29 linearized with restriction enzyme AscI using Freestyle Max transfection reagent, and then selected with 10 ug/ml of Puromycin for 2 weeks. 1 x 10^7 stable cells were labeled with goat anti-human Fc antibody FITC conjugate (1:1,000 dilution, Bethyl) and subjected to FACS sorting. The 0.01% of the cells with the highest expression of cell surface antibodies were sorted into five 96-well plates. Approximately 100 colonies grew out after 2-3 weeks. The culture media was screened for expression of Humira by ELISA as described in Example 1. The 24 highest expressing clones were picked, expanded, and cryopreserved. Six clones with different levels of antibody expression were picked for cell surface antibody assessment. They were labeled with goat anti-human Fc antibody FITC conjugate (1:1,000 dilution, Bethyl) and subjected to flow cytometric analysis to confirm membrane-anchored antibody expression (FIG. 12A). They were also subjected to 30 ml of shaking culture. The antibody production in the culture media was assessed by ELISA as described in Example 1 after 7-day non-fed batch culture. Higher membrane antibody expression was found to be strongly correlated with increased, secreted antibody production (FIG. 12B). The 0.01% of stable cells transfected with linearized LB29 having the highest expression of cell surface antibodies was also sorted into a pool. After culturing for a week, the selected pool was transiently transfected with the Cre expression vector described in Example 6 with Neon Transfection System (Invitrogen, Inc.). After one more week of culturing, the cells were cloned into 96-well plates by limiting dilution. Approximately 200 colonies grew out after 2-3 weeks. The culture media was screened for expression of Humira by ELISA as described in Example 1. The 24 clones having the highest level of antibody in the media were picked and expanded to a 24-well plate. After 3 days of culturing, the culture media was screened again for expression of Humira. The 12 clones having the highest level of antibody in the media were picked, expanded, and cryopreserved. The cells were confirmed to lack of membrane-anchored antibody (FIG. 13A). The antibody production in culture media was assessed after 7-day non-fed batch culture in a 30 ml of shaking culture. Antibody yields of the 5 clones having the highest level of antibody in the media are shown in Table 2.

<table>
<thead>
<tr>
<th>Clone</th>
<th>Peak cell density (cells/ml)</th>
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</table>

Example 8
A Model Screening of Antibody Library

[0086] One cell line selected in Example 7 and designated #27 expresses membrane-anchored Humira. Cells from cell line #27 were treated with 1 ug/ml of biotinylated human TNFα (ACRO Biosystems, Inc.) for 30 min. After washing once with PBS, the cells were labeled with streptavidin Phycoerythrin conjugate (Vector.Labs, Inc.) for 30 min. After washing twice with PBS, the cells were subjected to flow cytometric analysis and exhibited positive binding of TNFα on cell surface Humira (FIG. 14). The cell line #27 was spiked at a ratio of 1:1000 into a stable pool of CHO cells transfected with Rituxan expression vector LB37. After binding with biotinylated TNFα and then streptavidin Phycoerythrin conjugates, the cells were subjected to FACS sorting. 1000 positive cells were sorted into a pool. After culturing for 2 weeks, the antibody sequence in the FACS positive cells was amplified by RT-PCR and confirmed to be the Humira antibody sequence.

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Val Ser Gly Glu Gly Gly Asp Ala Thr Tyr Gly Lys Leu Thr Leu 50 55 60
Lys Phe Ile Cys Thr Thr Thr Gly Lys Leu Pro Val Pro Trp Pro Thr Leu 65 70 75 80
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35 40
Glu Trp Ile Gly Ala Ile Tyr Pro Gly Asn Gly Asp Thr Ser Tyr Asn
45 50
Gln Lys Phe Lys Gly Lys Ala Thr Leu Thr Ala Asp Lys Ser Ser Ser
55 60
Thr Ala Tyr Met Gln Leu Ser Ser Leu Thr Ser Glu Asp Ser Ala Val
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50 55 60
Trp Ile Tyr Ala Thr Ser Asn Leu Ala Ser Gly Val Pro Val Arg Phe
65 70 75 80
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<210> SEQ ID NO: 15
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<220> FEATURE: OTHER INFORMATION: Description of Artificial Sequence: Synthetic polynucleotide

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<210> SEQ ID NO: 16
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE: OTHER INFORMATION: Description of Artificial Sequence: Synthetic polynucleotide
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20 25 30
Leu Ser Ala Ser Val Gly Asp Arg Val Thr Ile Thr Cys Arg Ala Ser
35 40 45
Gln Gly Ile Arg Asn Tyr Leu Ala Trp Tyr Gln Gln Lys Pro Gly Lys
50 55 60
Ala Pro Lys Leu Leu Ile Tyr Ala Ser Thr Leu Gln Ser Gly Val
65 70 75 80
Pro Ser Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr
85 90 95
Ile Ser Ser Leu Gln Pro Glu Asp Val Ala Thr Tyr Tyr Cys Gln Arg
100 105 110
Tyr Asn Arg Ala Pro Tyr Thr Phe Gly Gln Gly Thr Lys Val Glu Ile
115 120 125

Lys

<210> SEQ ID NO 22
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<400> SEQUENCE: 22

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420
gct
423
What is claimed is:

1. A DNA construct comprising (a) a promoter; (b) a gene of interest (GOI); and (c) a reporter gene flanked by a DNA recombinase recognition sequence (DRRS).

2. The DNA construct of claim 1, wherein the DRRS is selected from the group consisting of an FRT sequence (DRRS for flippase or Flp), an LoxP (DRRS for Cre), and an attB or attP (DRRS for φC31 integrase).

3. The DNA construct of claim 1, further comprising an IRES upstream of the reporter gene.

4. The DNA construct of claim 1, wherein the reporter gene encodes a membrane anchored reporter (MAR) molecule, wherein the MAR is any molecule that can be detected on the cell surface.

5. The DNA construct of claim 4, wherein the MAR is selected from the group consisting of membrane anchored GFP, and a membrane anchored protein of interest.

6. The DNA construct of claim 1, wherein the reporter gene encodes an intracellular reporter molecule.

7. The DNA construct of claim 1, wherein the gene of interest has two or more exons and one or more introns.

8. The DNA construct of claim 7, wherein an intron of the gene of the interest comprises a splice acceptor sequence followed by a membrane association sequence.

9. The DNA construct of claim 8, wherein a splice acceptor sequence provides a means for alternative splicing wherein the expressed protein of interest can be membrane associated or secreted.

10. The DNA construct of claim 1, wherein the gene of interest encodes a recombinant protein.

11. The DNA construct of claim 10, wherein the gene of interest encodes an antibody.

12. The DNA construct of claim 11, wherein the gene of interest encoding said antibody comprises a splice acceptor sequence in the third intron of the antibody gamma constant region coding sequence.

13. An expression vector for delivering a gene of interest into a host cell, comprising the DNA construct of claim 1.

14. A cell comprising the DNA construct of claim 1.

15. A cell culture comprising the cell of claim 14.

16. The cell culture of claim 15, wherein a site specific DNA recombinase is added to the culture media.

17. The cell culture of claim 16, wherein the site specific DNA recombinase is provided by transfection of an expression vector encoding the DNA recombinase into the cells of the cell culture.

18. The cell culture of claim 16, wherein the sequence encoding said reporter is deleted in the cells of the cell culture.

19. The cell culture of claim 18, wherein the gene of interest is expressed and the corresponding protein of interest (POI) is secreted into the cell culture media.

20. A DNA construct comprising (a) a promoter; (b) a library containing a plurality of genes of interest; and (c) a reporter gene flanked by a DNA recombinase recognition sequence (DRRS).

21. The DNA construct of claim 20, wherein the genes of interest in the library comprise an intron with a splice acceptor sequence followed by a membrane association sequence.

22. The DNA construct of claim 21, wherein the splice acceptor sequence provides a means for alternative splicing wherein the expressed genes of interest can encode membrane associated or secreted proteins of interest.

23. The DNA construct of claim 20, wherein the library is an antibody library.

24. The DNA construct of claim 23, wherein the antibody library is an affinity maturation library.
25. A cell library comprising the DNA construct of claim 24.
26. A cell culture comprising the cell library of claim 25.
27. A cell line screening method comprising:
(a) providing host cells transfected with a DNA construct according to claim 1;
(b) culturing the transfected cells wherein the reporter protein is expressed and a protein of interest may also be secreted into the cell culture media;
(c) screening the transfected host cells and selecting host cells expressing a reporter molecule;
(d) exposing the selected cells to a DNA recombinase, wherein following such exposure the cells no longer express the reporter protein; and
(e) screening for cells that secrete a protein of interest into the cell culture media.
28. The cell line screening method of claim 27, wherein the reporter molecule is a membrane anchored reporter (MAR).
29. The cell line screening method of claim 27, wherein the reporter molecule is an intracellular reporter.
30. The cell line screening method of claim 27, wherein the DNA construct comprises a DRSS selected from the group consisting of an FRT sequence (DRRS for flippase or FLP), a LoxP (DRRS for Cre), and an attB or attP (DRRS for φC31 integrase).
31. The cell line screening method of claim 28, wherein the MAR is any protein that can be detected on the cell surface.
32. The cell line screening method of claim 31, wherein the MAR is detected on the cell surface by FACS or MACS.
33. The cell line screening method of claim 27, wherein the gene of interest encoded by the DNA construct has two or more exons and one or more introns.
34. The cell line screening method of claim 33, wherein an intron of the gene of the interest comprises a splice acceptor sequence followed by a membrane association sequence.
35. The cell line screening method of claim 27, wherein the transfected host cell is exposed to a site specific DNA recombinase.
36. The cell line screening method of claim 27, wherein the protein of interest is an antibody.
37. A cell line screening method comprising the steps of:
(a) providing host cells in culture media;
(b) transfecting the host cells with a DNA construct according to claim 20 to generate a cell library expressing a library of membrane-anchored protein of interest;
(c) screening the cell library and selecting host cells expressing a membrane-anchored protein of interest with a desired property;
(d) exposing the selected host cells to a DNA recombinase.
(e) screening the cell culture media for a protein of interest with a desired property.
38. The cell line screening method of claim 37, wherein the reporter molecule is a membrane anchored reporter (MAR).
39. The cell line screening method of claim 37, wherein the reporter molecule is an intracellular reporter.
40. The cell line screening method of claim 37, wherein the DNA construct comprises a DRSS selected from the group consisting of an FRT sequence (DRRS for flippase or FLP), an LoxP (DRRS for Cre), an attB or attP (DRRS for φC31 integrase).
41. The cell line screening method of claim 38, wherein the MAR is any protein that can be detected on the cell surface.
42. The cell line screening method of claim 41, wherein the MAR is detected on the cell surface by FACS or MACS.
43. The cell line screening method of claim 37, wherein the gene of interest encoded by the DNA construct has two or more exons and one or more introns.
44. The cell line screening method of claim 43, wherein an intron of the gene of the interest comprises a splice acceptor sequence followed by a membrane association sequence.
45. The cell line screening method of claim 37, wherein the transfected host cell is exposed to a site specific DNA recombinase.
46. The cell line screening method of claim 37, wherein the protein of interest is an antibody.
47. A method of screening a cell library screening for a protein of interest comprising switching a cell having a membrane anchored reporter protein into a cell that secretes a protein of interest into culture media, comprising:
(a) providing host cells in culture media;
(b) exposing the selected host cells to a DNA recombinase.
(c) screening the cell culture media for the protein of interest for the protein of interest with desired property; and
48. The screening method of claim 47, wherein the desired property is binding of an antigen.
49. The screening method of claim 47, wherein the desired property is bioactivity.