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(54) Title: INHIBITION-BASED HIGH-THROUGHPUT SCREEN STRATEGY FOR CELL CLONES

(57) Abstract: A method for screening cells with high level expression of a target protein is disclosed. The method includes introducing into a plurality of host cells a DNA construct that encodes both a target protein and an inhibitor to an endogenous selectable marker in the host cells, screening host cells harboring the DNA construct for the expression of the endogenous selectable marker, and isolating cells with reduced expression of the selectable marker. Also disclosed is a DNA construct configured to express both the target protein and the inhibitor inside the host cell.

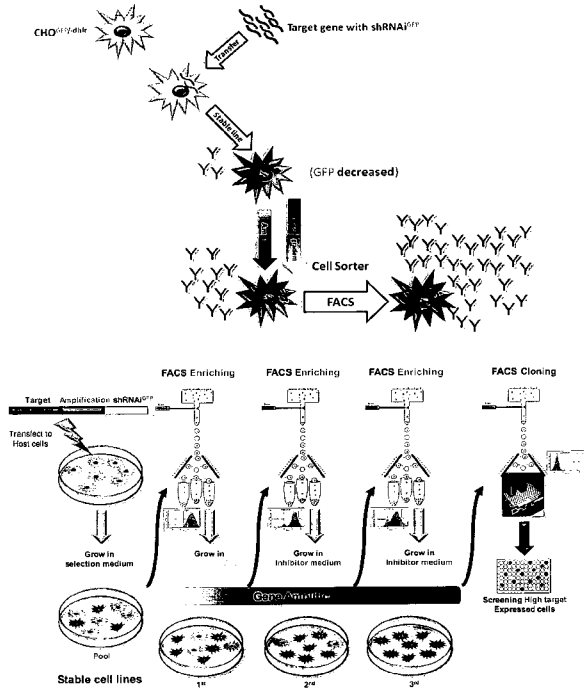

FIG. 1

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INHIBITION-BASED HIGH-THROUGHPUT SCREEN STRATEGY FOR CELL CLONES

RELATED APPLICATIONS

This application claims the priority of U.S. Provisional Application No. 61/213,459, filed on June 11, 2009, which is incorporated herein by reference in its entirety.

TECHNICAL FIELD

The disclosed technology generally relates to biotechnology and molecular biology and, in particular, to high-throughput screening of cell clones.

BACKGROUND

A key step in the generation of cell lines producing recombinant proteins is the selection of viable clones following the incorporation of the gene of interest into the host cell. For industrial-scale bio-production, a clone in which the product gene is stably integrated and yields a high level of the protein product is highly desired.

In a heterogeneous population, the integration of a recombinant gene into the host genome is a largely random event. The proportion of cells containing multiple copies of stably integrated genes would be small compared to those with low copy numbers. The high-producing subclones are rare and tend to be diluted out by the faster growing non- or low-producing cells. Thus, to isolate a subclone with an increased production rate, many wells would need to be screened and tested. In general, limited dilution methods are tedious and time consuming.

The advent of selection methods that use flow cytometry and cell sorting considerably increased the number of cells that can be screened. Several million cells can be screened in a short time, and subpopulations and single cells can be isolated from within mixed-cell populations even when they are present at frequencies as low as 10^{-6} within the population.

Flow cytometry was partnered with a non-fluorescent reporter protein for rapid, early stage identification of clones producing high levels of a target protein. This has

been facilitated by the availability of antibody and ligand-conjugated fluorochromes enabling isolation of cells based on cell-surface protein expression. For example, a cell surface protein, not normally expressed on host cells, may be co-expressed, with the target protein as a reporter.

In the absence of a correlation between surface expression and productivity, cells can be isolated based on levels of intracellular proteins using reporter molecules such as green fluorescent protein (GFP). The GFP has become an important reporter for gene expression and the selection of cells based on inducible gene products. In mammalian cell lines, GFP has been used for the selection of high-producing clones by co-expression with recombinant proteins and selection based on fluorescence intensity. A correlation between GFP fluorescence intensity and recombinant protein production has been seen for several cell lines expressing various recombinant proteins. However, expression of these selection markers increases the load on the protein expression machinery in the cells and reduces the production of the target protein.

SUMMARY

One aspect of the present invention relates to a method for screening cells with high level expression of a target protein. The method includes introducing into a plurality of host cells a DNA construct that encodes both a target protein and an inhibitor to an endogenous selectable marker in the host cells, screening host cells harboring the DNA construct for the expression of the endogenous selectable marker, and isolating cells with reduced expression of the selectable marker. The DNA construct is configured to express both the target protein and the inhibitor inside the host cell.

In an embodiment, the inhibitor is selected from the group consisting of small interfering RNA (siRNA), small hairpin RNA (shRNA), microRNA (miRNA), hybrid of miRNA and shRNA, and antisense RNA.

In another embodiment, the inhibitor is a shRNA.

In another embodiment, the endogenous selectable marker is a fluorescent marker and the isolating step comprises sorting cells with a fluorescence activated cell sorter (FACS).

In another embodiment, the DNA construct further encodes a dihydroforate reductase (DHFR) and the host cells are DHFR-deficient cells.

Another aspect of the present invention relates to a high throughput screening method for selecting transgene expressing cells. The method includes transfecting host cells that express a fluorescent protein with a vector carrying at least one transgene and an interfering RNA that inhibits the expression of the fluorescent protein; measuring fluorescence intensity in the transfected cells; and isolating cells having a fluorescence intensity that is lower than the fluorescence intensity of untransfected cells.

In one embodiment, the fluorescent protein is green fluorescent protein (GFP).

In another embodiment, the interfering RNA is a mir-30-based shRNA.

In another embodiment, the isolating step comprises sorting cells with a FACS.

In another embodiment, the cells that express a fluorescent protein are DHFR-deficient CHO cells.

In another embodiment, the at least one transgene is linked to a gene encoding DHFR by an internal ribosome entry site (IRES).

Another aspect of the present invention relates to an expression vector for high-throughput screening of cells harboring the expression vector. The expression vector comprises a first nucleotide sequence encoding a target protein, a second nucleotide sequence encoding an exogenous selection marker for a host cell, a third nucleotide sequence encoding an inhibitor to an endogenous selection marker in the host cell, and one or more regulatory elements that control the expression of the first, second and third nucleotide sequences in the host cell. The first nucleotide sequence is linked to the second nucleotide sequence by an internal ribosome entry site (IRES).

In an embodiment, the expression vector further comprises one or more anti-repressor elements.

In a related embodiment, the one or more anti-repressor elements includes a partial mouse anti-repressor element 40.

In another embodiment, the inhibitor is an interfering RNA.

In a related embodiment, the interfering RNA is a miR-30-based shRNA.

In another embodiment, the endogenous selection marker is a fluorescent protein.

In a related embodiment, the fluorescent protein is green fluorescent protein.

In another embodiment, the exogenous selectable marker is dihydroforate reductase.

In another embodiment, the one or more regulatory elements include a CMV IE enhancer.

BRIEF DESCRIPTION OF DRAWINGS

Figure 1 is a diagram showing a GFP-based screening strategy for high transgene expression cell clones.

Figure 2 is a map of expression vector pScinoDP-DHFR.

Figure 3 is a map of expression vector pScinoDP3-DHFR.

Figure 4 is a map of expression vector pScinoDP3mir-DHFR.

Figure 5 is a map of expression vector pScinoDP8mir-DHFR.

Figure 6 is a map of expression vector pScinoDP9mir-DHFR.

Figures 7A and 7B are diagrams showing the construction of expression vector pScinoDP9mir-Herceptin-DHFR.

Figure 8 is a composite of diagrams showing GFP expression and antibody expression in herceptin-CHO$^{+GFP/-dhfr}$ cells. Panel A: FACS histogram profile of herceptin-CHO$^{+GFP/-dhfr}$ cells. Panels B-C: FACS histogram profiles of herceptin-CHO$^{+GFP/-dhfr}$ cell sorted at different fluorescence intensity levels. Panel E: ELISA analysis of antibody production of cells sorted from different fluorescent populations.

Figure 9 is a composite of diagrams showing GFP expression in herceptin-CHO$^{+GFP/-dhfr}$ cells after multiple rounds of selection. Panel A: FACS histogram profile of CHO$^{/-dhfr}$ cells after GFP expression. Panel B: FACS histogram profile of herceptin-CHO$^{+GFP/-dhfr}$ cells after G418 selection. Panel C: FACS histogram profile of herceptin-CHO$^{+GFP/-dhfr}$ cells after first round of MTX (100 nM) amplification. Panel D: FACS histogram profile of herceptin-CHO$^{+GFP/-dhfr}$ cells after second round of MTX (200 nM) amplification.

Figure 10 is a diagram showing FACS analysis of herceptin-CHO$^{+GFP/-dhfr}$ cells stained with a PE-conjugated anti-human IgG(Fc) antibody. Cells secreting antibodies were PE positive and GFP negative according to experimental design and shown in upper-left panel. Fluorescence signal was determined by FACS and calibrated with standard compensation protocol.

Figure 11 shows the key features of the FACS sorting procedure for herceptin-CHO$^{+GFP/-dhfr}$ cells and ELISA results. Panel A: FACS histogram profile for Herceptin-CHO$^{+GFP/-dhfr}$ cells. Horizontal bars and gray regions indicate the low-fluorescence and high-fluorescence cell populations used for the single cell analysis. Panel B: Antibody expression levels detected by ELISA of each individual clones selected from low-fluorescence or high-fluorescence populations.

Figure 12 is a composite of diagrams showing FACS analysis of GFP expression in cells transfected with DNA constructs encoding shRNA^{GFP} and Herceptin.

DETAILED DESCRIPTION

A method for screening cells with high level expression of an exogenous protein is disclosed. In one embodiment, the method includes the steps of introducing into a plurality of host cells a DNA construct that encodes both a target protein and an inhibitor to an endogenous selectable marker in the host cells, wherein the construct is configured to express both the target protein and the inhibitor inside the host cell; screening host cells harboring the DNA construct for the expression of the endogenous selectable marker, and isolating cells with reduced expression of the endogenous selectable marker.

As used hereinafter, the terms "cell"/"host cell" and "cell line"/"host cell line" are respectively typically defined as a eukaryotic cell and homogeneous populations thereof that are maintained in cell culture by methods known in the art, and that have the ability to express heterologous proteins. In one embodiment, the cells are CHO cells. In another embodiment, the cells are CHO cells that express GFP as an endogenous selection marker. In another embodiment, the cells are CHO cells that are deficient in DHFR gene and express GFP as an endogenous selection marker.

As used hereinafter, the term "expression" is typically used to refer to the production of a specific RNA product or products, or a specific protein or proteins, in a cell. In the case of RNA products, it refers to the process of transcription. In the case of protein products, it refers to the processes of transcription, translation and optionally post-translational modifications. In the case of secreted proteins, it refers to the processes of transcription, translation, and optionally post-translational modification (e.g., glycosylation, disfulfide bond formation, etc.), followed by secretion. In the case of multimeric proteins, it includes assembly of the multimeric structure from the polypeptide monomers. The corresponding verbs of the noun "expression" have an analogous meaning as the noun.

As used hereinafter, the term "selectable marker" is typically used to refer to a gene and/or protein whose presence can be detected directly or indirectly in a cell, for example, a gene and/or a protein that inactivates a selection agent and protects the host cell from the agent's lethal or growth-inhibitory effects (*e.g.*, an antibiotic resistance gene and/or protein). Another possibility is that the selection marker induces fluorescence or a color deposit (*e.g.*, green fluorescent protein and derivatives, luciferase, or alkaline phosphatase). The term "endogenous selectable marker" refers to a selectable marker that is encoded by a polynucleotide that is present in the host cell prior to the introduction of the DNA construct into the host cell. The coding sequence for the "endogenous selectable marker" may exist in either integrated form (*i.e.*, integrated into the cell genome) or in episomal form.

As used hereinafter, the term "DNA construct" refers to an expression or transformation construct. The DNA construct comprises at least one expression unit or expression cassette. The term "expression unit or expression cassette" is herein defined as a unit capable of expressing a coding sequence or an open reading frame. An "expression unit or expression cassette" typically comprises one or more regulatory elements operably linked to a transgene that encodes a molecule of interest (*i.e.*, a polypeptide or a polynucleotide).

A "regulatory element" is a nucleic acid sequence that regulates the expression of a transgene by being operably linked to the coding sequence. Examples of

regulatory sequences include, but are not limited to, appropriate transcription initiation, termination, promoter and enhancer sequences; efficient RNA processing signals such as splicing and polyadenylation signals; sequences that stabilize cytoplasmic mRNA; sequences that enhance translation efficiency (i.e., Kozak consensus sequence); sequences that enhance protein stability; and when desired, sequences that enhance protein secretion. A regulatory sequence may act in cis or trans configuration, or at a distance to control a gene of interest.

A "transgene" is a nucleic acid sequence that is to be delivered or transferred to a mammalian cell. A transgene may encode a protein, peptide or polypeptide that is useful as a marker, reporter or therapeutic molecule. A transgene may also encode a protein, polypeptide or peptide that is useful for protein production, diagnostic assays or for any transient or stable gene transfer *in vitro* or *in vivo*. Alternatively, a transgene may encode a functional polynucleotide, such as miRNA, RNAi, shRNA, antisense RNAs, ribozyme or other regulatory nucleic acids. Transgenes also include DNA sequences that are used to induce DNA recombination and gene repair.

A nucleic acid sequence is "operably linked" to another nucleic acid sequence when the former is placed into a functional relationship with the latter. For example, a DNA for a presequence or secretory leader peptide is operably linked to DNA for a polypeptide if it is expressed as a preprotein that participates in the secretion of the hepolypeptide; a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the sequence; or a ribosome binding site is operably linked to a coding sequence if it is positioned so as to facilitate translation. Generally, "operably linked" means that the DNA sequences being linked are contiguous and, in the case of a secretory leader, contiguous and in reading phase. However, enhancers do not have to be contiguous. Linking is accomplished by ligation at convenient restriction sites. If such sites do not exist, synthetic oligonucleotide adaptors or linkers are used in accordance with conventional practice.

The "inhibitor to a selectable marker" can be a polypeptide or a polynucleotide that inhibits, directly or indirectly, the expression or activity of the selectable marker. In one embodiment, the inhibitor is a polynucleotide, such as a small interfering RNA

(siRNA), a small hairpin RNA (shRNA), , a microRNA (miRNA), a hybrid of miRNA and shRNA, or an antisense RNA molecule that inhibits the expression of the selectable marker in the host cell. In another embodiment, the inhibitor is a polypeptide, such as transcription regulator, that inhibits the expression of the selectable marker in the host cell. In yet another embodiment, the inhibitor is a polypeptide, such as an antibody, that inhibits a biological activity of the selectable marker.

As used herein, the term "siRNA" refers to an RNA agent, preferably a double-stranded agent, of about 10-50 nucleotides in length (the term "nucleotides" including nucleotide analogs), preferably between about 15-25 nucleotides in length, more preferably about 17, 18, 19, 20, 21, 22, 23, 24, or 25 nucleotides in length, the strands optionally having overhanging ends comprising, for example, 1, 2 or 3 overhanging nucleotides (or nucleotide analogs), which is capable of directing or mediating RNA interference. Naturally-occurring siRNAs are generated from longer dsRNA molecules (e.g., >25 nucleotides in length) by a cell's RNA interference (RNAi) machinery.

The term "RNA interference" or "RNAi", as used herein, refers generally to a sequence-specific or selective process by which a target molecule (*e.g.*, a target gene, protein or RNA) is downregulated. In specific embodiments, the process of "RNA interference" or "RNAi" features degradation of RNA molecules, e.g., RNA molecules within a cell, said degradation being triggered by an RNA agent. Degradation is catalyzed by an enzymatic, RNA-induced silencing complex (RISC). RNAi occurs in cells naturally to remove foreign RNAs (*e.g.*, viral RNAs). Natural RNAi proceeds via fragments cleaved from free dsRNA which direct the degradative mechanism to other similar RNA sequences. Alternatively, RNAi can be initiated by introducing small interfering RNA molecules into a cell to silence the expression of target genes.

As used hereinafter, the term "shRNA", refers to an RNA agent having a stem-loop structure, comprising a first and second region of complementary sequence, the degree of complementarity and orientation of the regions being sufficient such that base pairing occurs between the regions, the first and second regions being joined by a loop region, the loop resulting from a lack of base pairing between nucleotides (or

nucleotide analogs) within the loop region. The shRNA hairpin structure is cleaved by the cellular machinery into siRNA, which is then bound to the RNA-induced silencing complex (RISC). This complex binds to and cleaves mRNAs which match the siRNA that is bound to it.

The DNA construct may further contain plasmid elements for replication and selection in bacteria during the construction of the DNA construct. The DNA construct may also contain other selection markers to facilitate the amplification of the DNA construct in the host cell. Selection marker(s) encoded by the DNA construct are considered exogenous selection markers. Examples of exogenous selection markers include, but are not limited to, resistance to antibiotics (*e.g.* neomycin gene encoding resistance to G418) or enzyme analogs (*e.g.* dihydrofolate reductase gene encoding methotrexate resistance).

In one embodiment, the DNA construct contains a bicistronic expression cassette in which the open reading frame for the protein of interest is linked to the coding sequence of the exogenous selection marker or the inhibitor of the endogenous marker by an internal ribosome entry site (IRES), so that they are transcribed in the same mRNA but are translated independently. Since they each arise from a common mRNA, the exogenous selection marker's or the inhibitor's expression level accurately predicts the relative expression level of the protein of interest for each clone. Preferably, the open reading frame for the protein of interest in the bicistronic expression cassette is located upstream of the coding sequence of the exogenous selection marker or the inhibitor of the endogenous selectable marker.

For example, to improve the accuracy and throughput of multiple rounds of methotrexate (MTX) amplification, the gene encoding a therapeutic protein (such as the light chain of an antibody) is linked to an exogenous selectable marker (such as DHFR) at the 3'-end by an IRES, so that they are transcribed in the same mRNA but are translated independently. The lower efficiency of IRES-mediated translation relative to 5' cap-mediated translation ensures that cellular resources are utilized mainly for production of the therapeutic protein rather than the DHFR protein. However, since

they arise from the same mRNA, the DHFR amplification level accurately predicts, for each clone, the relative expression level of the therapeutic protein.

n another embodiment, the DNA construct further contains one or more anti-repressor element (ARE) that counteract chromatin associated repression. As used hereinafter, an ARE (or anti-repressor sequence, which is used interchangeably herein) is a naturally occurring DNA element isolated from eukaryotic genomes on the basis of its ability to block transgene repression. An ARE comprises the capacity to influence transcription of genes in cis and/or provide a stabilizing and/or an enhancing effect. It has been demonstrated that when AREs flank transgenes, the transgene expression level of randomly selected recombinant cell lines can be increased to levels approaching the maximum potential expression of the transgene's promoter. Moreover, the expression level of the transgene is stable over many cell generations, and does not manifest stochastic silencing. Therefore, ARE confers a degree of position-independent expression on transgenes that is not possible with conventional transgenic systems. The position independence means that transgenes that are integrated in genomic locations that would result in transgene silencing are, with the protection of ARE, maintained in a transcriptionally active state. In one embodiment, the ARE is a partial mouse ARE40 fragment. In another embodiment, the DNA construct contains multiple partial mouse ARE40 fragments.

Methods for introducing the DNA construct into the cell are well know in the art. Examples of such methods include, but are not limited to, electroporation, lipofection, calcium phosphate or calcium chloride co-precipitation and DEAE-dextran-mediated transfection. The DNA construct may also be introduced into the cells by a virus vector. Commonly used virus vectors include, but are not limited to, adenovirus vectors, adeno-associated virus (AAV) vectors, herpes virus vectors and retrovirus vectors.

Cells transfected with the DNA construct are screened for both the expression of the exogenous and endogenous selectable markers. High level expression of the selectable marker inhibitor from the DNA construct would result in reduced expression and/or activity of the endogenous selectable marker. Cells with reduced expression of

the selectable marker are then isolated and subcloned to determine the level and stability of transgene expression.

In certain embodiments, the endogenous selectable marker is a protein that can be induced to emit fluorescence. In these embodiments, the screening step and the isolating step can be performed simultaneously using a fluorescence activated cell sorter (FACS).

In other embodiments, cells transfected with the DNA construct are first subjected to one or more rounds of selection with the exogenous marker (*e.g.*, selection of G418 resistant and/or methotrexate resistant clones). The selected cells are then screened for reduced expression and/or activity of the endogenous selectable marker.

Also disclosed is a DNA construct configured to allow high-throughput screening of cells harboring the DNA construct. In one embodiment, the DNA construct contains coding sequences for a target protein and coding sequences for an inhibitor to an endogenous selectable marker in a host cell, wherein the DNA construct is configured to express both the target protein and the inhibitor inside the host cell.

In one embodiment, the inhibitor is a small interfering RNA (siRNA), a small hairpin RNA (shRNA), , a microRNA (miRNA), a hybrid of miRNA and shRNA, or an antisense RNA molecule that inhibits the expression of the endogenous selectable marker in the host cell.

In another embodiment, the inhibitor is a polypeptide, such as transcription regulator, that inhibits the expression of the endogenous selectable marker in the host cell.

In another embodiment, the inhibitor is a polypeptide, such as an antibody, that inhibits a biological activity of the endogenous selectable marker.

In another embodiment, the endogenous selectable marker is a protein that induces fluorescence.

In another embodiment, the DNA construct further comprises one or more anti-repressor elements.

The technology described in the present invention enables rapid identification and isolation of high production clones from a heterogeneous population of transfected

cells, decreasing the labor and time associated with standard limiting dilution cloning methods. In addition, because this technology can identify desired cells that are rare events in the population, it can shorten the development timeline by reducing the number of rounds of pool amplification prior to isolating the high producing clones or by first isolating the high producing clones for drug amplification and subcloning.

EXAMPLES

The ease and efficiency of green fluorescent protein (GFP) / fluorescence activated cell sorter (FACS)-based screening methods had been demonstrated in previous studies. In these previous reports, GFP was incorporated as part of a fusion protein or part of a bicistronic construct using either an internal ribosomal entry site (IRES) or a two promoter system. Cells with a high-level of GFP correlated with a high level of the protein product of interest. This can be attributed to the stable integration of a high number of copies of the recombinant gene or that the gene had been integrated into sites of very high transcriptional activity. Although these methods have been shown to be effective, there may be some concern regarding the use of GFP-containing cell lines for the production of human therapeutics. Furthermore, it appears unnecessary to burden the cell's metabolic machinery with GFP production after the subpopulation has been isolated. This cellular resource could potentially be diverted to increasing cell growth or recombinant protein production. The following examples describe a novel technique for selecting high-producing cell clones that reversely uses GFP as a selection marker, coupled with sorting through FACS. **Figure 1** is a schematic showing the GFP-based selection process. Briefly, plasmid vectors containing the cDNA encoding for GFP are transfected into DHFR-deficient CHO cells. Cells that have successfully acquired the desired vector are fluorescent. The GFP-expression cell is set as the parental host cell for recombinant-protein expression.

For desired recombinant-protein production, the parental host cells are transfected with the shRNAmir^{eGFP}-containing vector, and then FACS sorted to screen for cells with low fluorescent intensity. Cell cultures after several rounds of increasing MTX challenge were subjected to repeated rounds of sorting and expansion. Cell clones with the lowest GFP fluorescence intensity would correspond to the clones

with the highest transgene expression. Finally, the selected clones are expanded and tested for production and stability. As FACS enables a large number of cells to be easily screened, the chance of obtaining high producing clones would be greatly increased compared to limited dilution methods. Moreover, the procedure is less labor-intensive and may significantly reduce the time required to generate clones for bio-production.

Example 1: Establishment of $CHO^{+GFP/-dhfr}$ cell line

The Chinesse Hamster Ovary dihydrofolate reductase deficient cell line ($CHO/^{dhfr-}$) was maintained in Iscove's modified Dulbecco's medium (IMDM, Gibco, Cat. No.12200) supplemented with HT (0.1 mM sodium hypoxanthine and 0.016 mM thymidine, Gibco, Cat No.11067), 10 % FBS (Biological Industries, Cat.04-001-1A) and 2 μM Methothrexate Hydrate (MTX, Sigma, SI-M8407).

The $CHO/^{dhfr-}$ cells were transfected with 5 ug of plasmid vector containing the cDNA encoding for GFP (pFLAg-eGFP-IRES-Puro,) with lipofectamine according to the instructions from Lipofectamine™ Invitrogen Puls ™ Reagent (Cat. No.11514-015). Transfected cells were selected using 5 μg/ml of puromycin dihydrochloride (Sigma, SI-P8833). After 10 days of selection in IMDM medium supplemented with HT, 10 % FBS, 2 μM MTX and selective antibiotics, cells were maintained in above medium. The GFP-expressing CHO^{-dhfr} cell ($CHO^{+GFP/-dhfr}$ cell) is cloned and set as the parental host for recombinant-protein expression.

Example 2: Construction of expression vectors

(1) The pScinoDP-DHFR vector

The pScinoDP-DHFR plasmid was constructed by substituting the EGFP gene in the pEGFP-N1 (Clontech) plasmid backbone with an IRES-DHFR fused gene, and inserting an addition SV40 polyA tail and CMV-IE promoter into the pEGFP-N1 (Clontech) plasmid backbone. Briefly, polyA tail and CMV-IE promoter sequence were obtained by PCR amplification using pCEP4 plasmid (Invitrogen) as the template. A fused polyA tail-promoter fused sequence SV40polA-CMV-IE (about 1.2 kb) was created through overlap extension by PCR (OL-PCR). The fused sequence was digested with XhoI/BglII and inserted into XhoI/BglII digested pEGFP-N1 vector.

The resulting construct was named pScinoDP.

IRES sequence and DHFR gene were obtained by PCR amplification using pIRES2-EGFP and pSV2-DHFR plasmids as template. A hybrid IRES sequence fragment and DHFR gene fragment (IRES2-DHFR) was produced through overlap extension by PCR (OL-PCR). The hybrid fragment (~1.1 kb) was digested with AgeI/NotI and inserted into AgeI/NotI digested pEGFP-N1 to substitute the EGFP gene in the pEGFP-N1 vector. The resulting construct was named pIRES2-DHFR. A site-directed mutagenesis was performed to eliminate the ApaLI site in the IRES sequence.

Plasmid pIRES2-DHFR, with mutated restricted enzyme ApaLI site in IRES sequence, was digested with AgeI / NotI and a 1.2 kb fragment containing IRES sequence and the entire DHFR coding region gene was ligated into AgeI/NotI-digested pScinoDP, generating pScinoDP-DHFR **(Figure 2)**. All constructs were confirmed by restriction analyses and/or by nucleotide sequencing.

(2) The pScinoDP3-DHFR vector

The pScinoDP3-DHFR vector is a pScinoDP-DHFR based vector that carries a hEF1α promoter with the CMV-IE enhancer. Briefly, the hEF1α promoter was amplified from pBudCE4.1 vector (Invitrogen). Hybrid CMV-IE enhancer sequence with hEF1α promoter was obtained through sub-cloning hEF1α promoter after the CMV-IE enhancer on pEGFP-N1 to form pCMVe-hEF1α-EGFP vector. The CMV^e-hEF1α fragment was PCR amplified from the $pCMV^e$-hEF1α-EGFP vector and used to replace both CMV promoters in the pScionDP-DHFR vector. The resulting vector is named pScinoDP3-DHFR **(Figure 3)**.

(3) The pScinoDP3mir-DHFR vector

The single strand 97nt "mir30-like" $shRNAi^{GFP}$ oligo was generated using PCR as described using the following synthesis skeleton DNA and primers:

Single strand 97nt "$shRNAi^{GFP}$ DNA oligo

5'-*TGCTGTTGACAGTGAGCG***AGCACAAGCTGGAGTACAACTA***TAGTGAAGCC ACAGATGTA***TAGTTGTACTCCAGCTTGTGCCT***GCCTACTGCCTCGGA***-3'

(SEQ ID NO:1)

The underlined, italicized sequences represent the flanking mir30 sequences and the non-underlined, italicized sequence represents the mir30 loop structure. The sample sense and antisense-selected target sequences are shown in bold and underlined bold, respectively. The mir30-like shRNAi^{GFP} is synthesized as a single stranded DNA oligonucleotide with common ends corresponding to part of the endogenous mir30 miRNA flanking sequence.

mirFWD-**AgeI** primer sequence (40 mers):
5'-CAGAAGGACCGGTAAGGTATAT*TGCTGTTGACAGTGAGCG*-3' **(SEQ ID NO:2)**

mirREV-HindIII primer sequence (37 mers):
5'-CTAAAGTAGCCCCTTAAGCTT*TCCGAGGCAGTAGGCA*-3' **(SEQ ID NO:3)**

The flanking regions, shown in underlined, italicized sequences, are used as universal flanks to prime a reaction, whereby the entire mir30-like shRNAi^{GFP} is amplified to produce a PCR product that can be cloned into the recipient vector.

PCR was performed using Platinum® Pfx DNA Polymerase and the following profile: 95℃ for 3 min, then 95℃ for 30 s, 54℃ for 30 s, and 75℃ for 30 s for a total of 35 cycles. The resulting PCR products (AgeI-shRNAi^{GFP}) were cloned into modified pEGFP-N1 vector (AgeI site destroyed, and additional AgeI and EcoRV sites behind Neomycin gene). The resulting construct was named pEGFP-N1-shRNAi^{GFP}. These AgeI-shRNAi^{GFP} sequences were also confirmed by DNA sequencing.

The pEGFP-N1-shRNAi^{GFP} vector was digested with ApaLI-NotI. The CMV-IE-GFP fragment was replaced with the ScinoDP3-DHFR fragment from pScinoDP3-DHFR. The resulting construct was named pScinoDP3mir-DHFR **(Figure 4)**.

(4) The pScinoDP8mir-DHFR vector

The pScinoDP8mir-DHFR vector contains a regulatory DNA element mARE40. Regulatory elements, such as anti-repressing element derived from housekeeping genes, were shown to positively affect specific productivities of recombinant proteins produced from cell lines. Plasmid pEGFP-N1 was used as backbone for this construct. Briefly, the partial mouse anti-repressor element 40 fragment was generated using overlapping-PCR as described using the following synthesis skeleton DNA and primers:

mARE40-L1(+) skeleton DNA:
5'-TTGCTCTGAGCCAGCCCACCAGTTTGGAATGACTCCTTTTTATGACTTGAA TTTTCAAGTATAAAGTCTAGTGCTAAATTTAATTTGAACAACTGTATAGTTTT TG-3' **(SEQ ID NO:4)**

mARE40-L1(-) skeleton DNA:
5'-TTAGAAATCCTCACACACAACAAGTTTTCATTTCACTTCTAATTCTGAAAAA AACACTGCCACCATTTTTTTTCCTTCCCCCAACCAGCAAAAACTATACAGTTG T-3' **(SEQ ID NO:5)**

mARE40-R1(+) skeleton DNA
5'-GTGTGTGAGGATTTCTAATGACATGTGGTGGTTGCATACTGAGTGAAGCC GGTGAGCATTCTGCCATGTCACCCCCTCGTGCTCAGTAATGTACTTTACAGA AATC-3' **(SEQ ID NO:6)**

mARE40-R1(-) skeleton DNA
5'-TGGCAGAAATGCAGGCTGAGTGAGACTACCCAGAGAAGAGACCGGATAT ACACAAGAAGCATGGTTTATATCAATCTTTTGAGTTTAGGATTTCTGTAAAG TACAT-3' **(SEQ ID NO:7)**

mARE40-5'primer: 5'-TTGCTCTGAGCCAGCCCACCAGTTT-3' **(SEQ ID NO:8)**

mARE40-3'AseI primer: 5'-GTTATTAATTGGCAGAAATGCAGGCTGAGT-3' **(SEQ ID NO:9)**

mARE40-3'AflII primer: 5'-CCCACATGTTGGCAGAAATGCAGGCTGAGT-3' **(SEQ ID NO:10)**

mARE40-3'SpeI primer: 5'-GGACTAGTTGGCAGAAATGCAGGCTGAGTG-3' **(SEQ ID NO:11)**

PCR was performed using Platinum® Pfx DNA Polymerase and the following profile: 95℃ for 3 min, then 95℃ for 30 s, 58℃ for 30 s, and 75℃ for 30 s for a total of 35 cycles. The resulting PCR products (mARE40-AseI and mARE40-AflII) were separately cloned into modified pEGFP-N1(AgeI site destroyed, and additional EcoRV before AseI, and ScaI site before AflII site) vector to form pmARE40-EGFP-N1, and (mARE40-SpeI) were cloned into the pScinoDP3-DHFR(additional EcoRV site before SpeI site) vector to form pScinoDP3-DHFR-F2. The pmARE40-EGFP-N1 vector was digested with AseI-BamHI and the CMV-IE promoter was replaced with the ~1.6 kb AseI-CMV^e-hEF1α-BamHI fragment from pScinoDP3-DHFR vector. The resulting construct was named pFmARE40ScinoDP3-EGFP-N1. The pFmARE40ScinoDP3-EGFP-N1 vector was digested with BamHI-NotI and the EGFP gene was replaced with the ~1.6 kb BamHI-SV40polA-mARE40-DP3-IRES-DHFR-NotI fragment from pScinoDP3-DHFR-F2. The resulting construct was named pScinoDP8-DHFR. The pEGFP-N1-shRNAiGFP vector was then digested with ApaLI-NotI and the CMV-IE-GFP fragment was replaced with the ScinoDP8-DHFR fragment from pScinoDP8-DHFR. The resulting construct was named pScinoDP8mir-DHFR **(Figure 5)**. The complete sequence of the cloned partial mouse anti-repressor element

40 fragment is shown below:

5'-TTgCTCTgAgCCAgCCCACCAgTTTggAATgACTCCTTTTTATgACTTgAATTT
TCAAgTATAAAgTCTAgTgCTAAATTTAATTTgAACAACTgTATAgTTTTTgCTg
gTTgggggAAggAAAAAAAATggTggCAgTgTTTTTTTCAgAATTAgAAgTgAAATg
AAAACTTGTTgTgTgTgAggATTTCTAATgACATgTggTggTTgCATACTgAgTgAA
gCCggTgAgCATTCTgCCATgTCACCCCCTCgTgCTCAgTAATgTACTTTACAgAA
ATCCTAAACTCAAAAgATTgATATAAACCATgCTTCTTgTgTATATCCggTCTC
TTCTCTGGGTAgTCTCACTCAgCCTgCATTTCTgCCA-3' **(SEQ ID NO:12)**.

(5) The pScinoDP9mir-DHFR vector

The pScinoDP9mir-DHFR vector (**Figure 6**) was constructed using procedures similar to those used for the construction of pScinoDP8mir-DHFR vector and substituting the two CMV enhancers in pScinoDP8mir-DHFR with CAG promoter (CMV-IE enhancer fused with chicken β-actin promoter). The complete sequence of pScinoDP9mir-DHFR is shown in **SEQ ID NO:13**.

pScinoDP9mir-DHFR contains the internal ribosome entry site (IRES) of the encephalomyocarditis virus (ECMV) between the MCSII and the dihydrofolate reductase (DHFR) coding region. This permits both the gene of interest (for example: light chain cloned into the MCSII) and the DHFR gene to be translated from a single bicistronic mRNA. Sequences flanking DHFR have been converted to a Kozak consensus translation initiation site to further increase the translation efficiency in eukaryotic cells. The MCSI in pScinoDP9mir-DHFR is between the immediate early promoter of CMV (PCMV IE) and SV40 polyadenylation signals sequences. SV40 polyadenylation signals downstream of the MCSI direct proper processing of the 3' end of the first transcription. The MCSII in pScinoDP-dhfr is between the second immediate early promoter of cytomegalovirus (PCMV IE) and the IRES sequence. SV40 polyadenylation signals downstream of the DHFR gene direct proper processing of the 3' end of the bicistronic mRNA.

Because pScinoDP9mir-DHFR is derived from pEGFP-N1 vector, it contains

an SV40 origin for replication in mammalian cells expressing the SV40 T antigen. A neomycin-resistance cassette (Neo^r), consisting of the SV40 early promoter, the neomycin/kanamycin resistance gene of Tn5, and polyadenylation signals from the Herpes simplex virus thymidine kinase (HSV TK) gene, allows stably transfected eukaryotic cells to be selected using G418. A bacterial promoter upstream of this cassette expresses kanamycin resistance in *E. coli*. The pScinoDP-DHFR backbone also contains a pUC origin of replication for propagation in E. coli and an f1 origin for single-stranded DNA production.

(6) The pGFP/Puromycin vector

IRES sequence and DHFR gene were obtained by PCR amplification used pIRES2-EGFP and pLKO-AS3w-puro plasmid as template. A hybrid IRES sequence fragment and puromycin gene fragment (IRES2-puromycin) was obtained through overlap extension by PCR (OL-PCR). The IRES2-puromycin fragment was inserted into SalI-BamHI digested pFLAG-CMV2 vector (Kodak). The resulting construct was named pIRES2-Puro. The EGFP gene was obtained from the pEGFP-N1 vector and inserted into the pIRES-Puro vector to form pGFP/Puromycin vector.

(7) The pScinoDP9mir-Herceptin-DHFR vector

The construction of the pScinoDP9mir-Herceptin-DHFR vector is shown in **Figures 7A and 7B**. Briefly, the 4-1Leader sequence and 4-2Leader sequence were PCR amplified from oligo-synthesis skeleton fragments, while heavy chain constant region sequence of human IgG_1($hIgG_1C_H$) and light chain constant region sequence of human IgG_1($hIgG_1C_L$) were obtained from recombinant plasmids containing human IgG_1 sequence. Hybrid leader sequence and $hIgG_1$ constant region sequence vectors were obtained by sub-cloning $hIgG_1$ constant region to leader sequence containing vector by the way of orientation linkage.

Next, , variant region of heavy chain (V_H) and light chain (V_L) were created through repeated overlapping PCR from oligo-synthesis skeleton fragments and sub-cloned into p4-1Leader-$hIgG_1C_H$ or p4-2Leader-$hIgG_1C_L$ vector. After correcting PCR errata and removing additional sequences introduced during cloning process, the correctness of the leader-peptide-HerceptinVC sequence was verified by sequencing

(Figure 7A).

Finally, the hybrid 4-1Leader-Herceptin heavy chain (4-1-HerceptinVC$_H$), and 4-2Leader-Herceptin light chain (4-2-HerceptinVC$_L$) were individually sub-cloned into the MCSI and MCSII sites in pScinoDP9mir-DHFR vector to form the pScinoDP9mir-Herceptin-DHFR vector **(Figure 7B).**

The amino acid and nucleotide sequences for the variant region of anti-HER2 heavy chain 1 are shown in **SEQ ID NOS: 14 and 15**, respectively. The skeleton fragments and PCR primers used for the variant region of the heavy chain (V_H) have the following sequences:

Skeleton fragments:

HerceptinV$_H$-L1(+) (108 mer)

5'-gAggTgCAgCTCgTggAgAgTggTggCgggTTggTCCAgCCAggCgggTCTCTgCgATTgAgCTgTgCTgCCTCTggATTTAACATCAAAgACACgTACATCCATTgg-3' **(SEQ ID NO:16)**

HerceptinV$_H$-L2(-) (105 mer)

5'-TTTAACgCTATCAgCgTATCTggTgTAgCCgTTAgTgggATAgATTCTAgCTACCCATTCAAggCCCTTgCCgggggCCTgTCTCACCCAATggATgTACgTgTC-3' **(SEQ ID NO:17)**

HerceptinV$_H$-R1(+) (105 mer)

5'-TACgCTgATAgCgTTAAAggAAggTTTACTATTTCTgCCgACACCTCCAAgAATACCgCATATCTACAgATgAACTCCCTgCgCgCTgAggACACCgCTgTgTAT-3' **(SEQ ID NO:18)**

HerceptinV$_H$-R2(-) (108 mer)

5'-CTTAgTAgAgCACTgCTAACTgTCACTAAggTACCCTggCCCCAgTAgTCCATTgCgTAgAATCCgTCTCCCCCCCAACgTgAgCAgTAATACACAgCggTgTCCTC-3' **(SEQ ID NO:19)**

Primers for Amplification:

Herceptin-V_H-5HindIII (30 mer) (sense)

5'-gCCAAgCTTgAggTgCAgCTCgTggAgAgT-3' **(SEQ ID NO:20)**

Herceptin-V_H-3ApaI (30 mer) (antisense)

5'-AgggggCCCTTAgTAgAggCACTgCTAACT-3' **(SEQ ID NO:21)**

The amino acid and nucleotide sequences for the variant region of anti-HER2 light chain 1 are shown in **SEQ ID NOS:22 and 23**, respectively. The skeleton fragments and PCR primers used for the variant region of the light chain (V_L) have the following sequences:

Skeleton Fragments:

HerceptinV$_L$-L1(+) (93 mer)

5'-gATATACAgATgACACAgTCTCCgTCAAgTCTgAgCgCAAgCgTgggCgACCgg GTAACAATTACCTgTAgAgCCAgCCAggACgTAAATACA-3' **(SEQ ID NO:24)**

HerceptinV$_L$-L2(-) (95 mer)

5'-CCgCTATAAAggAACgAggCAgAgTAgATCAgAAgCTTAggAGCTTTACCAggT TTTTgCTgATACCAggCCACggCTgTATTTACgTCCTggCT-3' **(SEQ ID NO:25)**

HerceptinV$_L$-R1(+) (94 mer)

5'-CTCgTTCCTTTATAgCggggTgCCAAgCCgCTTCTCCggATCTAggTCTggAACA gACTTTACTCTgACCATTTCCAgTCTCCAgCCCgAAgAC-3' **(SEQ ID NO:26)**

HerceptinV$_L$-R2(-) (93 mer)

5'-CTTgATCTCgACCTTggTgCCCTgCCCAAATgTgggTggAgTCgTgTAATgTTgCT ggCAATAgTAggTAgCAAAgTCTTCgggCTggAgACT-3' **(SEQ ID NO:27)**

Primers for Amplification

Herceptin-V_L-5HindIII (30 mer) (sense)

5'-gCCAAgCTTgATATACAgATgACACAgTCT-3' **(SEQ ID NO:28)**

Herceptin-V_L-3BamHI (30 mer) (antisense)

5'-CgCggATTCCTTgATCTCgACCTTggTgCC-3' **(SEQ ID NO:29)**

Example 3: Establishment of herceptin/CHO$^{+GFP/-dhfr}$ cell line

CHO$^{/+GFP/-dhfr}$ cells were suspended in PBS buffer. 40 μg of linearized plasmid (pScinoDP9mir-Herceptin-DHFR) DNA was added to the cells and incubated on ice for 10 min. The cells were then electroported by two pulses at a voltage setting of 750 V and a capacitance setting at 25 μF (Gene Pulser II with capacitance extender, and pulse controller from Bio-Rad). The electroporated cells were plated in T-175 flask with 25 mL of medium (IMDM supplemented with HT, 10 % FBS, 2 μM MTX and 5 μg/ml puromycin dihydrochloride) for 24 hours. The cells were then selected using 800 μg/ml of G418 sulfate (Calbiochem, Cat# 345810) in Minimum Essential medium Alpha medium (Alpha-MEM, Gibco, Cat. No.12000) supplemented with 10% D-FBS (Gibco, Cat. No. 30067-334) and 5 μg/ml of puromycin dihydrochloride. After 14 days of selection in Alpha-MEM medium supplemented with 10 % D-FBS and selective antibiotics, the cells were subjected to MTX treatment at increasing concentrations for gene amplification.

The cells were FACS sorted to screen for cells with low GFP fluorescent intensity. Since the expression of the target gene and shRNA^{GFP} would lead to reduced GFP production in the CHO$^{+GFP/-dhfr}$ cells, cells with least green fluoresence (GFP negative) would have the highest level of target gene expression. FACS was performed using a MoFlo^{TM} XDP (Beckman Coulter) equipped with Summit software, an laser emitting at 488 nm and a cell deposition unit for sorting.

Low-fluorescence and high-fluorescence cell populations were sorted with the single cell deposited using FACS into 96-well cell culture plates containing 220 μl

Alpha-MEM supplemented with 10%D-FBS, G418, puromycin dihydrochloride and MTX. Clones were incubated at 37℃ and 5% carbon dioxide in a humidified incubator for 12 days.

Example 4: Characterization of herceptin-CHO$^{+GFP/-dhfr}$ cell line

(1) Detection of surface antibodies by immunostaining

Trypsinized herceptin-CHO$^{+GFP/-dhfr}$ cells were centrifuged 5 min at 200 rpm. The cells were washed twice with PBS and resuspended in PBS to a final concentration of about 1 × 10^7 cells/ml. The cells were then incubated with phycoerythrin (PE)-conjugated mouse anti-human IgG(Fc) (Beckman Coulter, Cat. No. 736007) at dilutions according to the manufacturer's recommendations at 4℃ for 30 min in the dark, washed twice with PBS and kept on ice for FACS analysis.

(2) Detection of secreted antibodies by ELISA

Briefly, 96-well plates were coated with anti human IgG antibody (Sigma: I 1886) diluted in 0.05 M Carbonate-Bicarbonate buffer (pH 9.7), and incubated at 4℃ for 16h. Plates were blocked with blocking buffer (10 mM Tris, 0.15 M NaCl, 1% skim milk, pH 8.0) at 37℃ for 30 min. Culture supernatants were loaded on the wells and incubated at 37℃ for 2 h. Horseradish peroxidase conjugated anti-human IgG-F(c) antibody (Abcam: ab7499), diluted in dilution buffer (10 mM Tris, 0.15 M NaCl, 0.05% Tween 20, pH 8.0) according to the manufacturer's recommendations and incubated at 37℃ for 1 h. The reactions were detected using the substrate (1- stepTM ultra TMB-ELISA, Pierce, Cat. 34028) and plates were read on microplate reader (Bio-Rad).

(3) Results

Figure 8 presents a histogram profile of Herceptin-CHO$^{+GFP/-dhfr}$ cells sorted at different fluorescent intensities using FACS. Panel A shows the GFP fluorescence in a pool of Herceptin-CHO$^{+GFP/-dhfr}$ cells. The cells were divided into several subpopulations (R2, R3 and R4) based on the fluorescent intensity. Panels B-D show levels of GFP expression in each sub-population. Panel E shows the anti-herceptin antibody titles in the pool and each subpopulation. The data showed that cells with the lowest level of GFP fluorescence (Herceptin/R4/CHO$^{+GFP/-dhfr}$) had the highest level

of anti-herceptin titer.

Figure 9 shows that the GFP expression level in $CHO^{+GFP/-dhfr}$ cells was reduced upon transfection with DNA constructs containing a target protein (Herceptin) and $shRNAi^{GFP}$. The FACS profiles indicate that the GFP expression level was further reduced after two rounds of MTX challenge (panels C and D). These results demonstrate that the level of target gene amplification correlated to GFP expression levels and the strength of gene amplification.

Figure 10 shows a representative FACS analysis of Herceptin-$CHO^{+GFP/-dhfr}$ cells stained with PE-conjugated mouse anti-human IgG(Fc). The result demonstrated that the antibody-producing cells (i.e., cells with higher PE staining) exhibited low levels of GFP expression.

Figure 11A shows a histogram profile for Herceptin-$CHO^{+GFP/-dhfr}$ cells. Horizontal bars and shaded regions indicate the low-fluorescence and high-fluorescence cell populations used for the single cell analysis. **Figure 11B** shows that 8 out of 18 low-fluorescent cell clones yielded ELISA values greater than 0.3, while only 2 out of 18 high-fluorescent cells clones yielded ELISA values grater than 0.3. These results demonstrated that the low-fluorescent cell population contains a higher frequency of high-yield antibody producing cells and confirmed the reversal GFP-based screen strategy.

Figure 12 shows that GFP expression in $CHO^{+GFP/-dhfr}$ cells is reduced by the transfection of the DNA construct encoding Herceptin and $shRNA^{GFP}$.

(4) Conclusion

The use of the $shRNAmir^{eGFP}$ coupled with flow cytometry substantially improves the accuracy and efficiency of cell line development at two crucial points. First, for early stage clone screening, the FACS method is a better predictor of clone productivity than the analysis of conditioned media for therapeutic protein titer. Second, clones with unstable transgene expression are easily identified by an observed increase in fluorescence during amplification stage. Thus, the present method provides the novel benefits of accurate 96-well clone screening to identify good candidates for further development and elimination of unstable clones at an earlier stage in the development

process than traditional methods.

These results demonstrate that the DNA constructs and screening methods of the present invention reliably yield high-expression clones homogenous protein preparations. Since the isolation of mammalian cell lines capable of high-yield expression of recombinant antibodies is not performed by screening multiple individual clones with limiting dilution techniques, the procedure is less labor-intensive and may significantly reduce the time required to generate clones for bioproduction. The procedure requires no additional reagent for clone selection and offers additional benefits to monitor genome stability. The expression level of the target gene is also more correlative to the strength of amplification gene. The bicistronic design allows synchronously expressions of two foreign genes in the same chromosome. The inhibitive approach enhance the intensity of the target gene amplification by reducing the intensity of the reporter gene amplification. The use of ARE increases the expression of the foreign recombinant protein by eliminating the difference caused by the inhibition of inserting the foreign gene into the different chromosome areas.

What is claimed is:

1. A method for screening cells with high level expression of a target protein, comprising:

introducing into a plurality of host cells a DNA construct that encodes both a target protein and an inhibitor to an endogenous selectable marker in the host cells, wherein the DNA construct is configured to express both the target protein and the inhibitor inside the host cell;

screening host cells harboring the DNA construct for the expression of the endogenous selectable marker, and

isolating cells with reduced expression of the selectable marker.

2. The method of claim 1, wherein the inhibitor is selected from the group consisting of small interfering RNA (siRNA), small hairpin RNA (shRNA), microRNA (miRNA), hybrid of miRNA and shRNA, and antisense RNA.

3. The method of claim 1, wherein the inhibitor is a shRNA.

4. The method of claim 1, wherein the endogenous selectable marker is a fluorescent marker and wherein the isolating step comprises sorting cells with a fluorescence activated cell sorter (FACS).

5. The method of claim 1, wherein the DNA construct further encodes a dihydroforate reductase (DHFR) and wherein the host cells are DHFR-deficient cells.

6. A high throughput screening method for selecting transgene expressing cells, comprising:

transfecting the cells with a vector carrying at least one transgene and an interfering RNA that inhibits the expression of the fluorescent protein;

measuring fluorescence intensity in the transfected cells; and

isolating cells having a fluorescence intensity that is lower than the fluorescence intensity of untransfected cells.

7. The method of claim 6, wherein the fluorescent protein is green fluorescent protein (GFP).

8. The method of claim 6, wherein the interfering RNA is a mir-30-based shRNA.

9. The method of claim 6, wherein the isolating step comprises sorting cells with a FACS.

10. The method of claim 6, wherein the cells that express a fluorescent protein are dihydroforate reductase (DHFR)-deficient CHO cells.

11. The method of claim 6, wherein the at least one transgene is linked to a gene encoding DHFR by an internal ribosome entry site (IRES).

12. An expression vector for high-throughput screening of cells harboring the expression vector, comprising:

a first nucleotide sequence encoding a target protein;

a second nucleotide sequence encoding an exogenous selection marker for a host cell;

a third nucleotide sequence encoding an inhibitor to an endogenous selection marker in the host cell; and

one or more regulatory elements that control the expression of the first, second and third nucleotide sequences in the host cell,

wherein the first nucleotide sequence is linked to the second nucleotide sequence by an internal ribosome entry site (IRES).

13. The expression vector of claim 12, further comprising one or more anti-repressor elements.

14. The expression vector of claim 13, wherein the one or more anti-repressor elements includes a partial mouse anti-repressor element 40.

15. The expression vector of claim 12, wherein the inhibitor is an interfering RNA.

16. The expression vector of claim 15, wherein the interfering RNA is a miR-30-based shRNA.

17. The expression vector of claim 12, wherein the endogenous selection marker is a fluorescent protein.

18. The expression vector of claim 17, wherein the fluorescent protein is green fluorescent protein.

19. The expression vector of claim 12, wherein the exogenous selectable marker is dihydroforate reductase.

20. The expression vector of claim 12, wherein the one or more regulatory elements include a CMV IE enhancer.

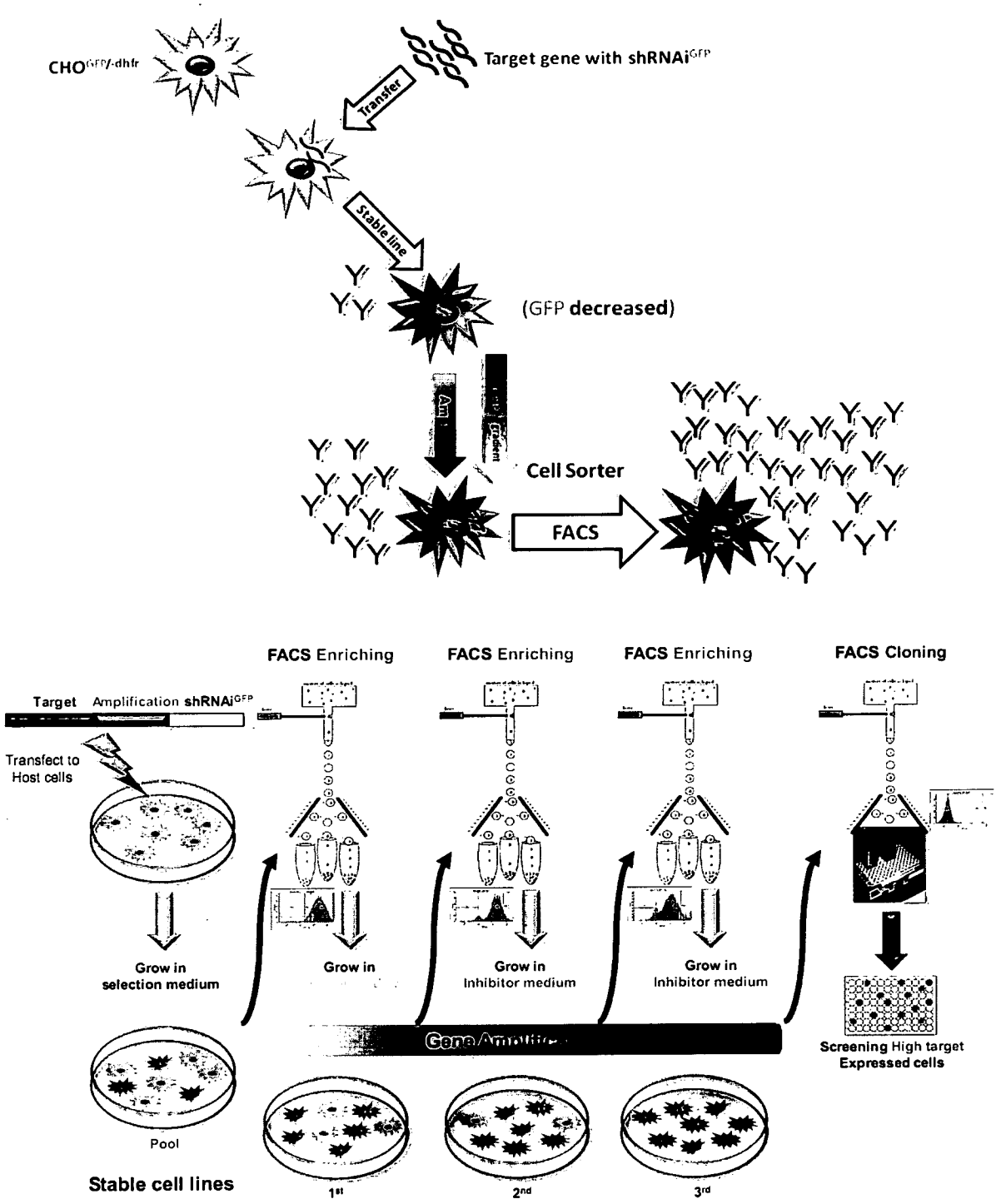

CHO GFP/-dhfr
Target gene with shRNAi GFP
Transfer
Stable line
(GFP decreased)
Am
Gradient
Cell Sorter
FACS
FACS Enriching
FACS Enriching
FACS Enriching
FACS Cloning
Target
Amplification shRNAi GFP
Transfect to Host cells
Grow in selection medium
Grow in
Grow in Inhibitor medium
Grow in Inhibitor medium
Gene Amplifi
Screening High target Expressed cells
Pool
Stable cell lines
1st
2nd
3rd

FIG. 1

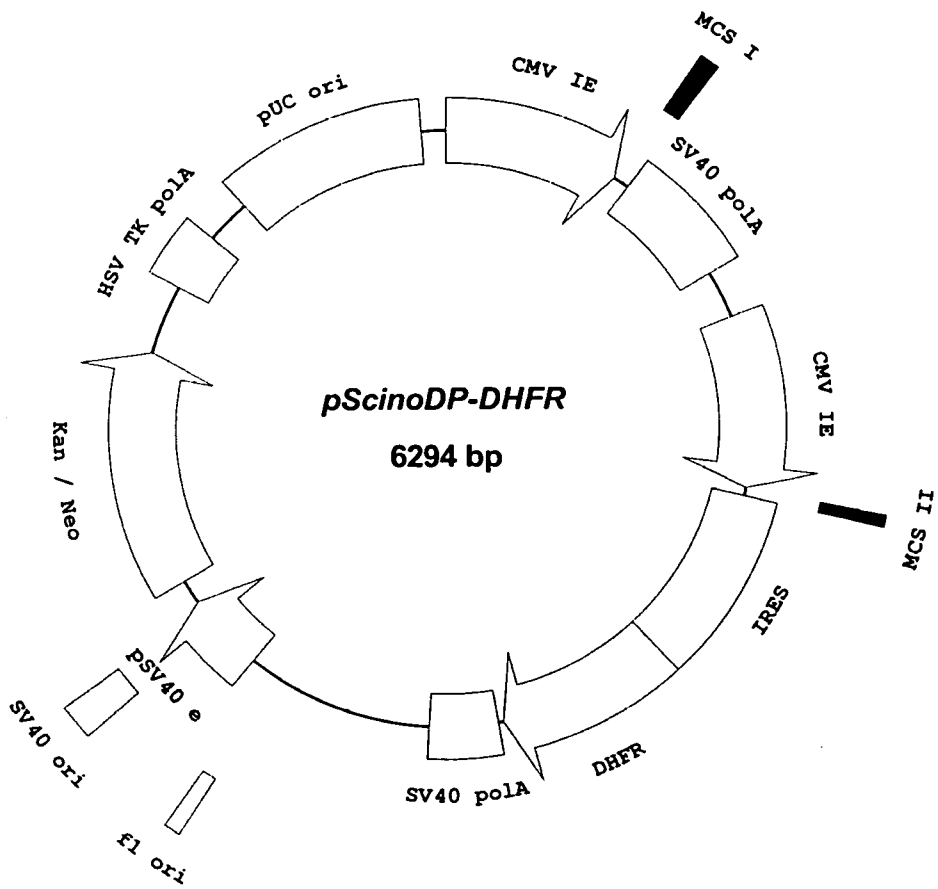

pScinoDP-DHFR
6294 bp
MCS I
CMV IE
SV40 polA
CMV IE
MCS II
IRES
DHFR
SV40 polA
PSV40 e
f1 ori
SV40 ori
Kan / Neo
HSV TK polA
PUC ori

FIG. 2

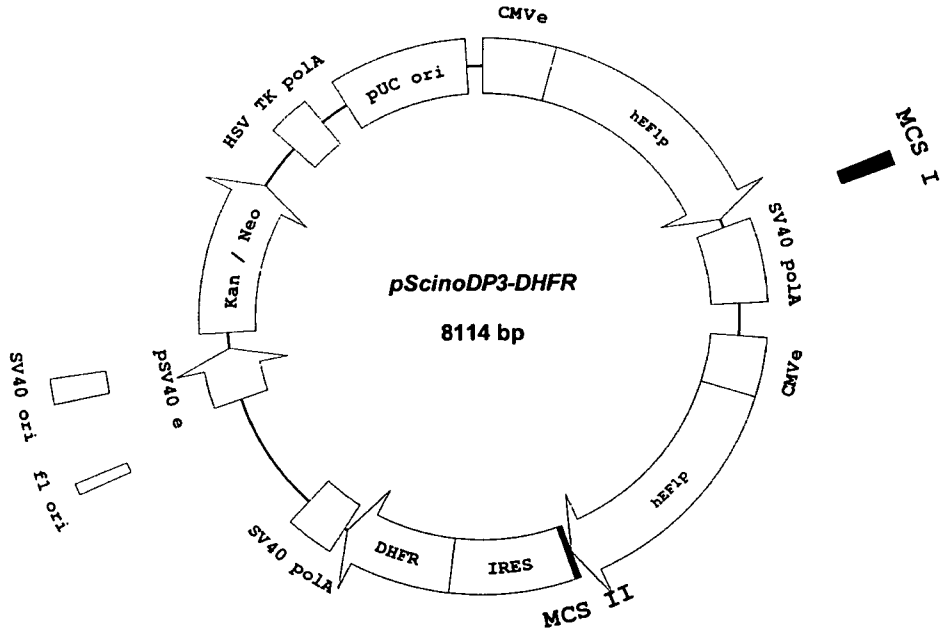
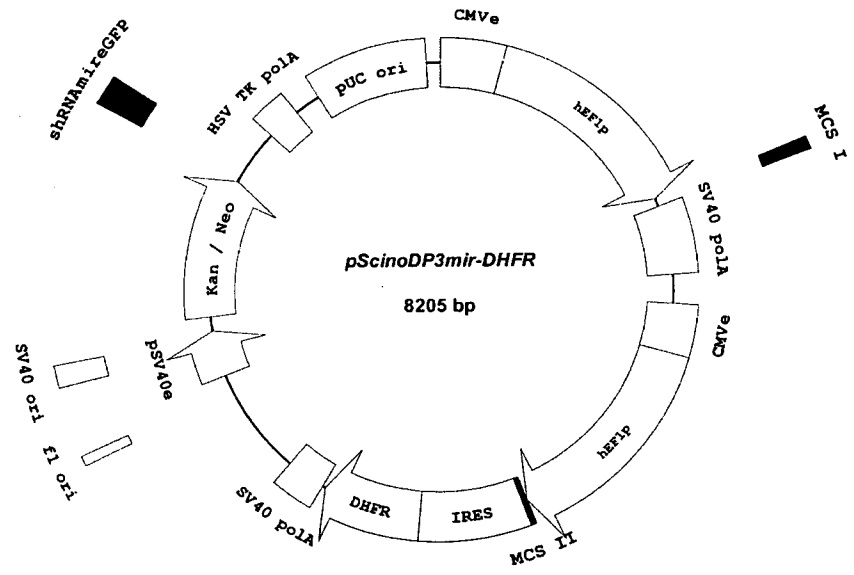

CMVe
HEF1P
SV40 polA
eMVe
HEF1P
IRES
DHFR
SV40 polA
MCS II
pSV40 e
Kan / Neo
HSV TK polA
pUC ori
MCS I
SV40 ori
f1 ori
pScinoDP3-DHFR
8114 bp

FIG. 3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hEF1p
MCS I
SV40 polA
CMVe
hEF1p
MCS II
IRES
DHFR
SV40 polA
pSV40e
Kan / Neo
HSV TK polA
shRNAmireGFP
pUC ori
SV40 ori
f1 ori
pScinoDP3mir-DHFR
8205 bp

FIG. 4

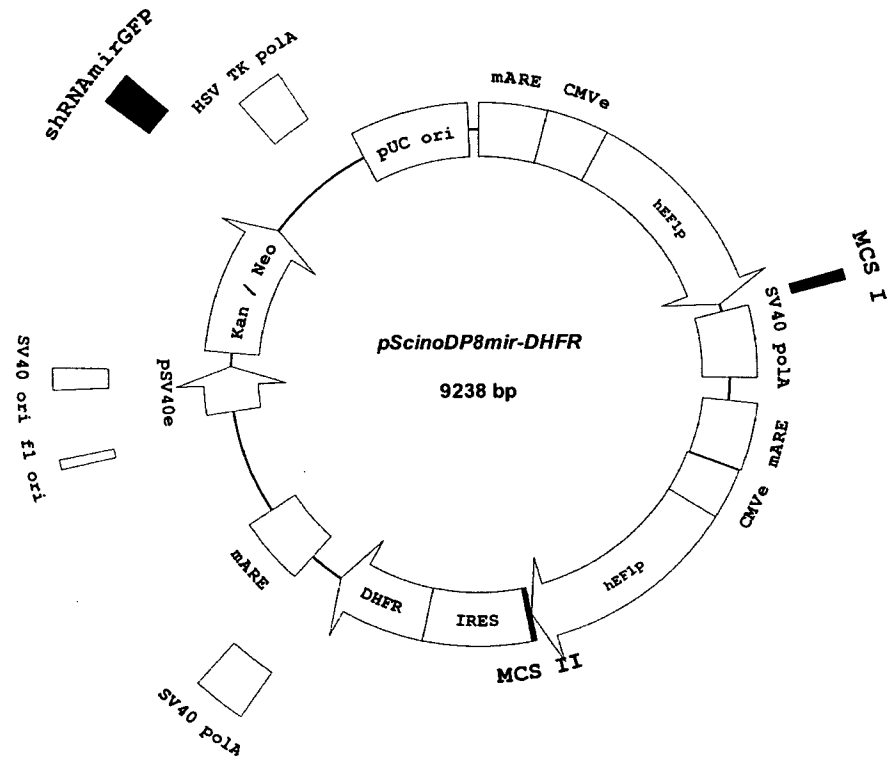

mARE
CMVe
hEF1p
MCS I
SV40 polA
mARE
CMVe
hEF1p
MCS II
IRES
DHFR
mARE
SV40 polA
pSV40e
Kan / Neo
HSV TK polA
shRNAmirGFP
pUC ori
SV40 ori
f1 ori
pScinoDP8mir-DHFR
9238 bp

FIG. 5

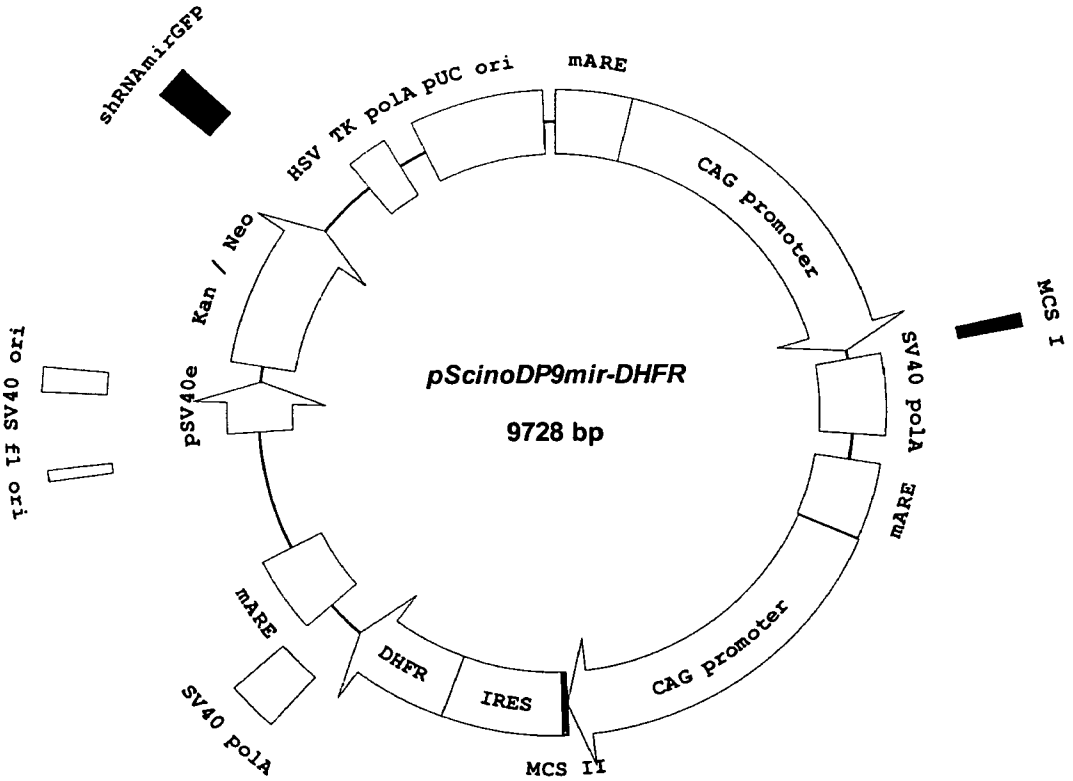

shRNAmirGFP
HSV TK polA
pUC ori
mARE
CAG promoter
MCS I
SV40 polA
mARE
CAG promoter
MCS II
IRES
DHFR
mARE
SV40 polA
pSV40e
Kan / Neo
f1 ori
SV40 ori
pScinoDP9mir-DHFR
9728 bp

FIG. 6

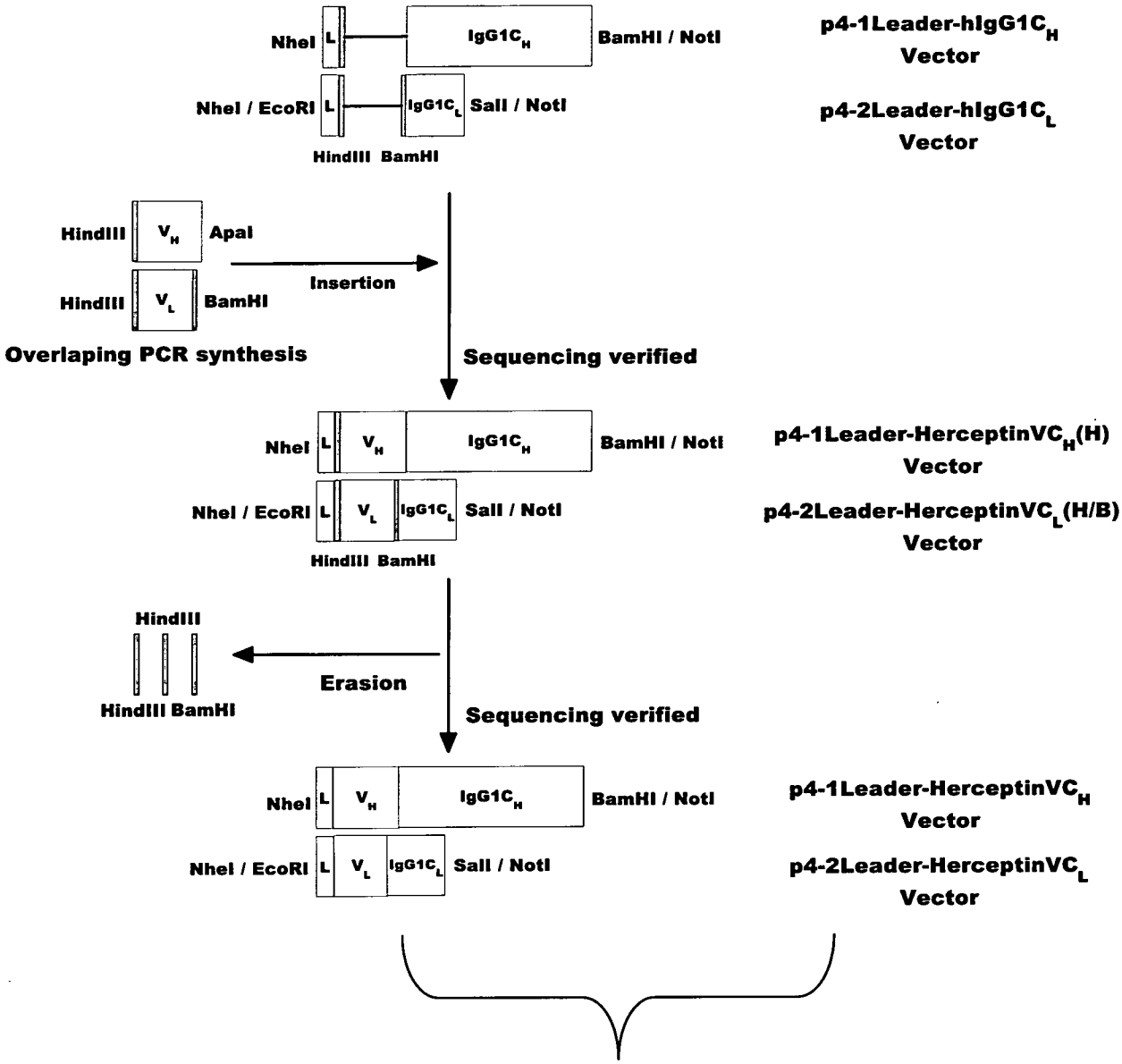

NheI L IgG1C_H BamHI / NotI
NheI / EcoRI L IgG1C_L SalI / NotI
HindIII BamHI
p4-1Leader-hIgG1C_H Vector
p4-2Leader-hIgG1C_L Vector
HindIII V_H ApaI
HindIII V_L BamHI
Overlaping PCR synthesis
Insertion
Sequencing verified
NheI L V_H IgG1C_H BamHI / NotI
NheI / EcoRI L V_L IgG1C_L SalI / NotI
HindIII BamHI
p4-1Leader-HerceptinVC_H(H) Vector
p4-2Leader-HerceptinVC_L(H/B) Vector
HindIII
HindIII BamHI
Erasion
Sequencing verified
NheI L V_H IgG1C_H BamHI / NotI
NheI / EcoRI L V_L IgG1C_L SalI / NotI
p4-1Leader-HerceptinVC_H Vector
p4-2Leader-HerceptinVC_L Vector

FIG. 7A

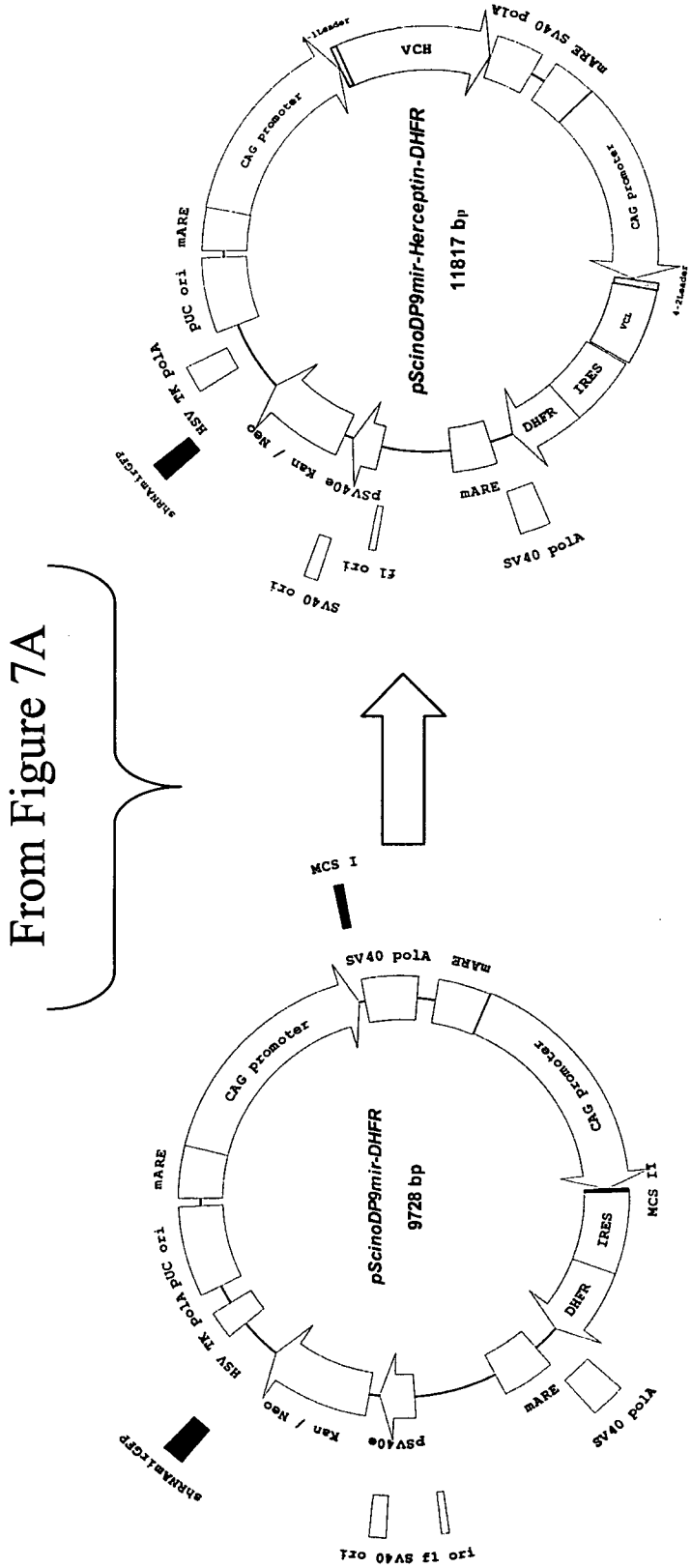

From Figure 7A
pScinoDP9mir-Herceptin-DHFR
11817 bp
VCH
MARE SV40 polA
CAG promoter
VCL
IRES
DHFR
mARE
SV40 polA
f1 ori
SV40 ori
PSV40e Kan / Neo
HSV TK polA
shRNAmirGFP
pUC ori
mARE
CAG promoter
MCS I
SV40 polA
MARE
CAG promoter
MCS II
IRES
DHFR
mARE
SV40 polA
pScinoDP9mir-DHFR
9728 bp
PSV40e Kan / Neo
HSV TK polA pUC ori
mARE
shRNAmirGFP
ori f1 SV40 ori

FIG. 7B

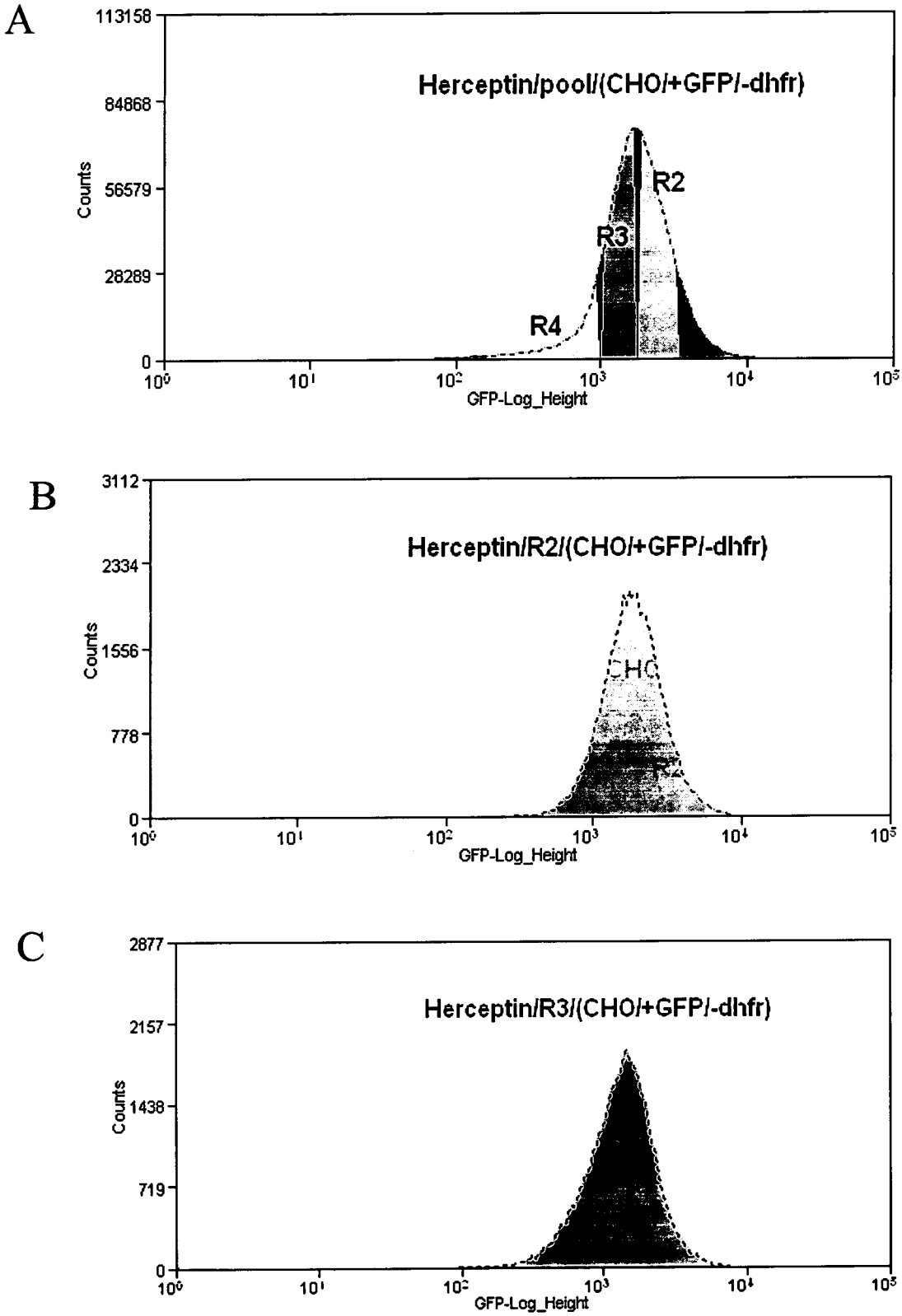
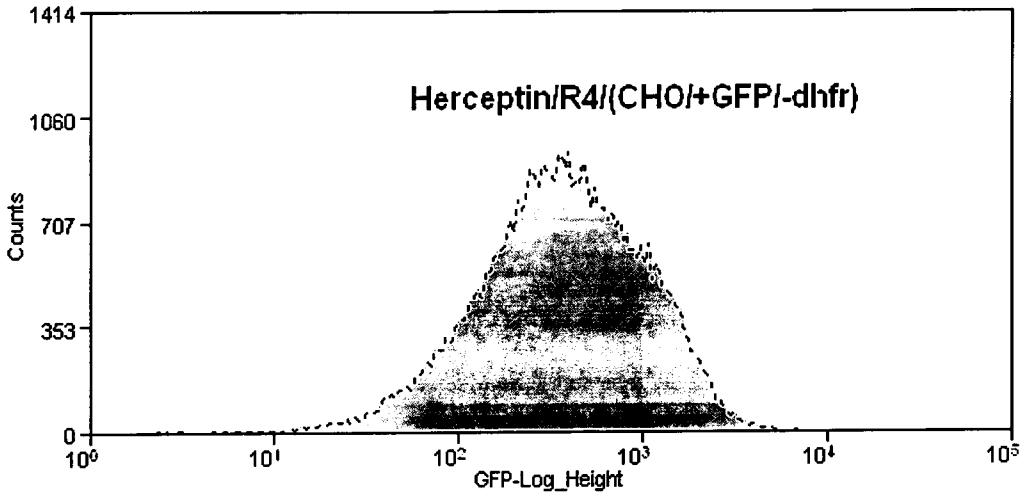

A
113158
84868
56579
28289
0
Counts
Herceptin/pool/(CHO/+GFP/-dhfr)
R2
R3
R4
10⁰
10¹
10²
10³
10⁴
10⁵
GFP-Log_Height
B
3112
2334
1556
778
0
Counts
Herceptin/R2/(CHO/+GFP/-dhfr)
CHO
10⁰
10¹
10²
10³
10⁴
10⁵
GFP-Log_Height
C
2877
2157
1438
719
0
Counts
Herceptin/R3/(CHO/+GFP/-dhfr)
10⁰
10¹
10²
10³
10⁴
10⁵
GFP-Log_Height

FIG. 8

D


Herceptin/R4/(CHO/+GFP/-dhfr)
Counts
1414
1060
707
353
0
10^0
10^1
10^2
10^3
10^4
10^5
GFP-Log_Height

E

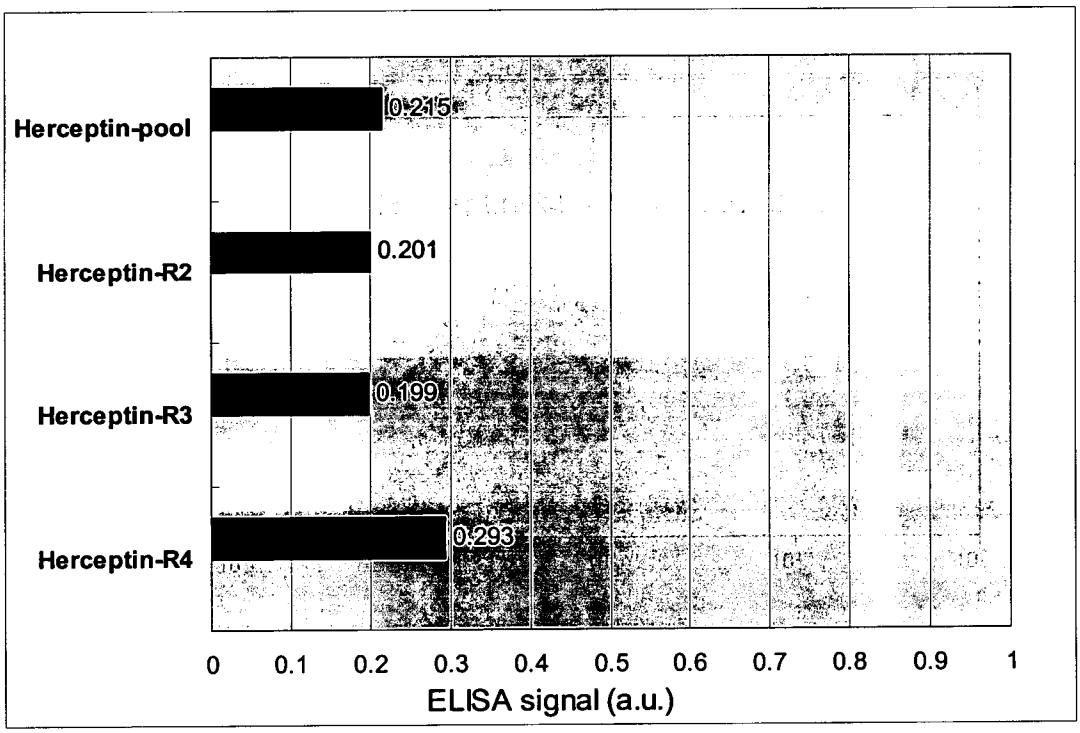
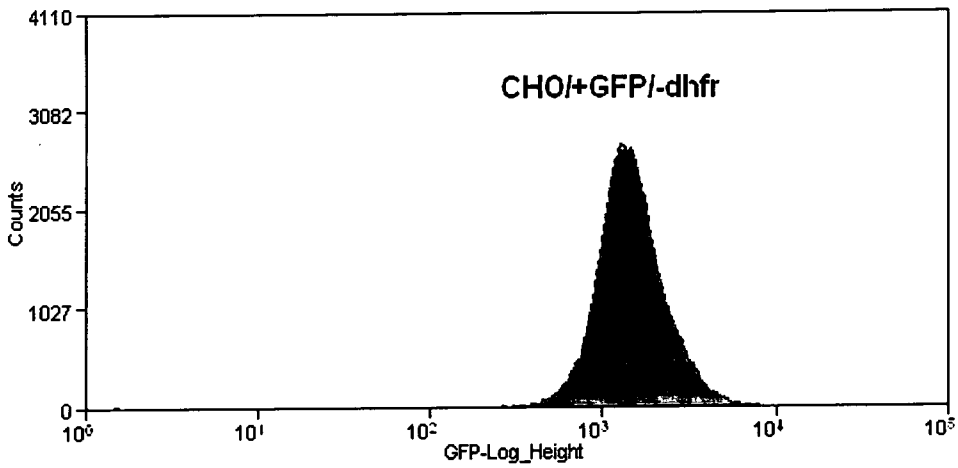

Herceptin-pool
0.215
Herceptin-R2
0.201
Herceptin-R3
0.199
Herceptin-R4
0.293
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1
ELISA signal (a.u.)

FIG. 8

A


4110
3082
2055
1027
0
Counts
CHO/+GFP/-dhfr
GFP-Log_Height

B

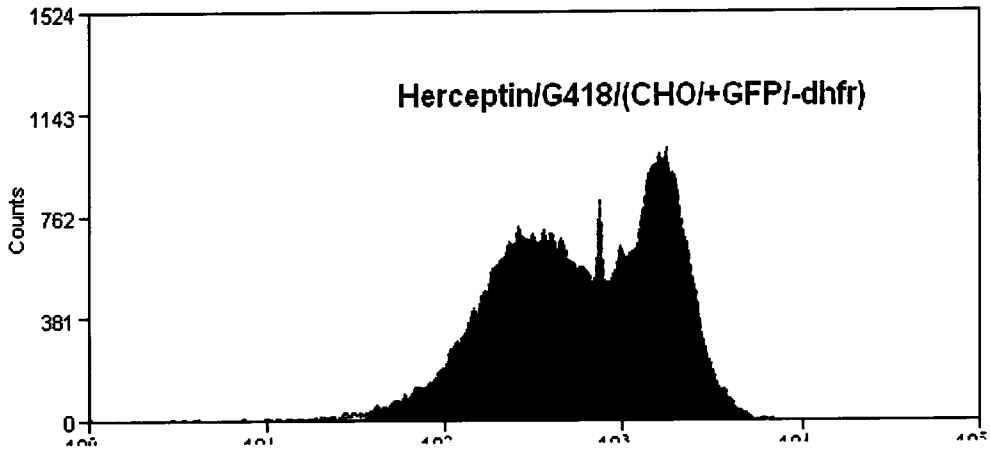
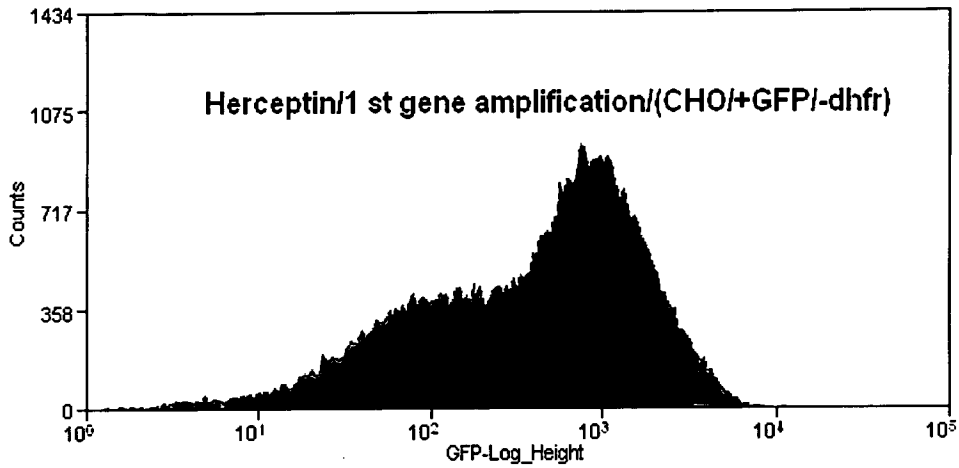

1524
1143
762
381
0
Counts
Herceptin/G418/(CHO/+GFP/-dhfr)

FIG. 9

C


Herceptin/1 st gene amplification/(CHO/+GFP/-dhfr)
Counts
1434
1075
717
358
0
10⁰
10¹
10²
10³
10⁴
10⁵
GFP-Log_Height

D

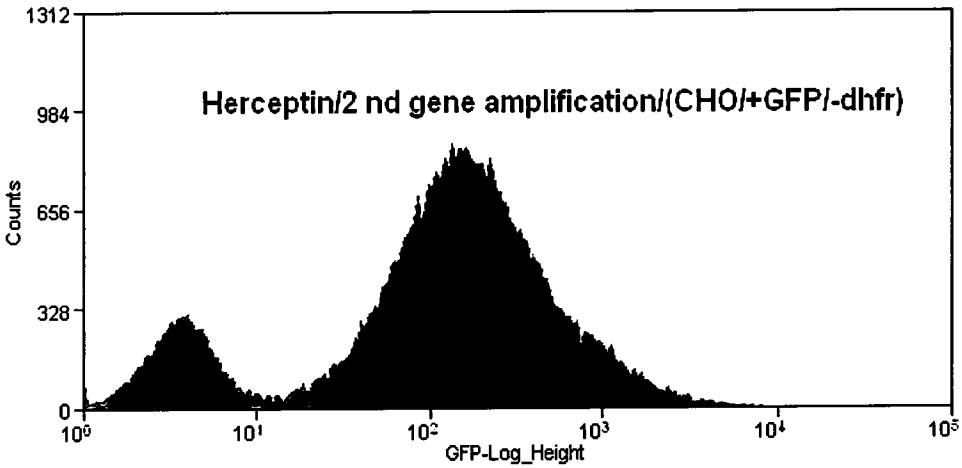

Herceptin/2 nd gene amplification/(CHO/+GFP/-dhfr)
Counts
1312
984
656
328
0
10⁰
10¹
10²
10³
10⁴
10⁵
GFP-Log_Height

FIG. 9

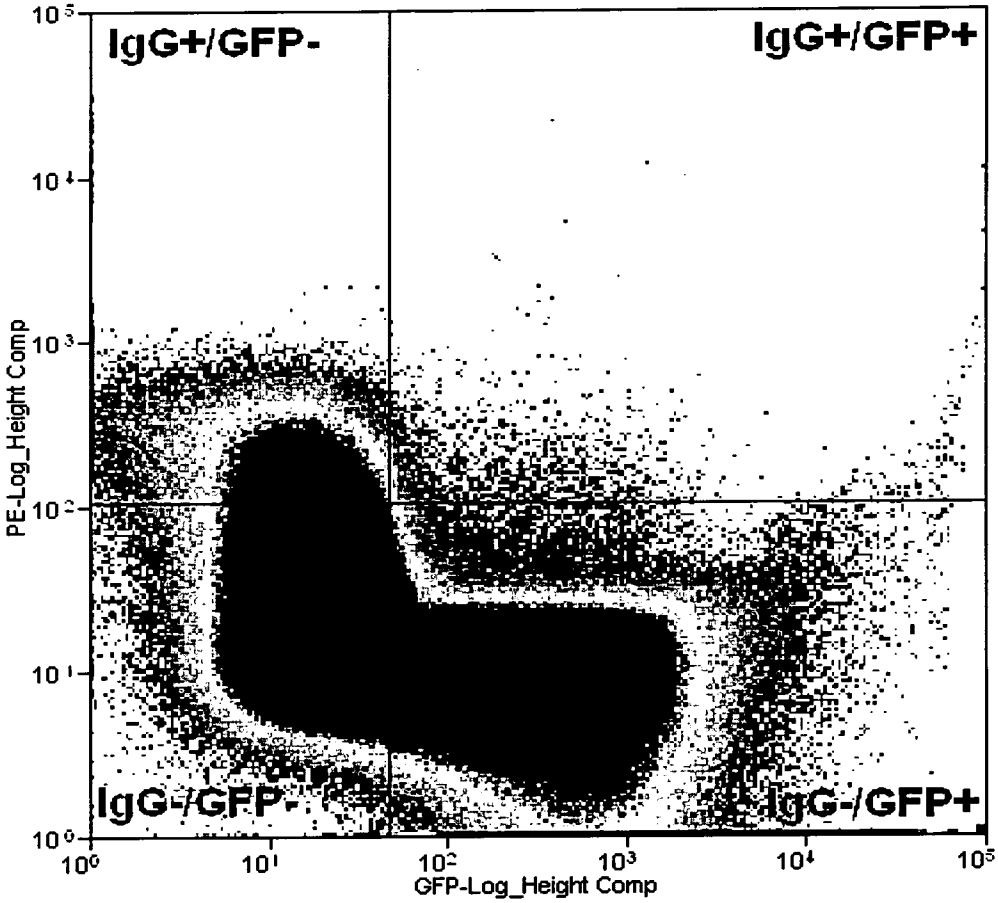
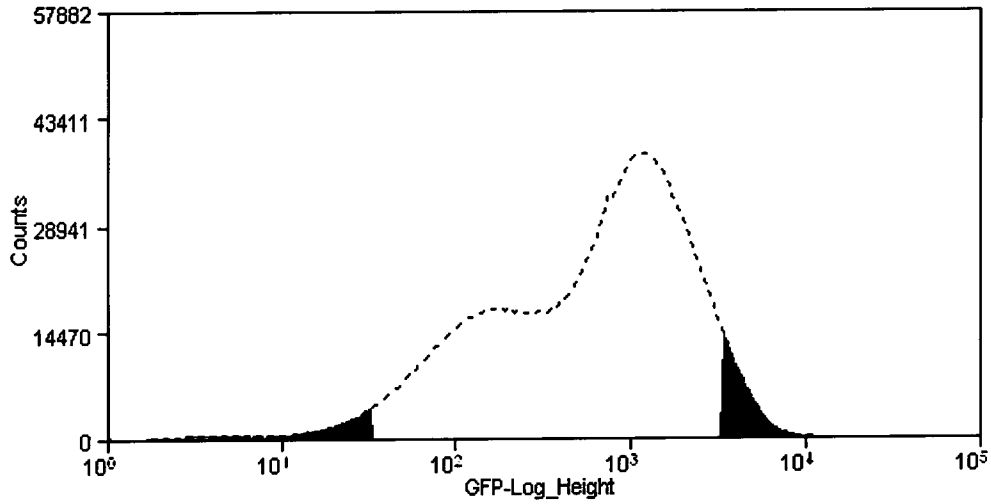

IgG+/GFP-
IgG+/GFP+
IgG-/GFP-
IgG-/GFP+
PE-Log_Height Comp
GFP-Log_Height Comp
10⁰
10¹
10²
10³
10⁴
10⁵

FIG. 10

A


57882
43411
28941
14470
0
Counts
10^0
10^1
10^2
10^3
10^4
10^5
GFP-Log_Height

B

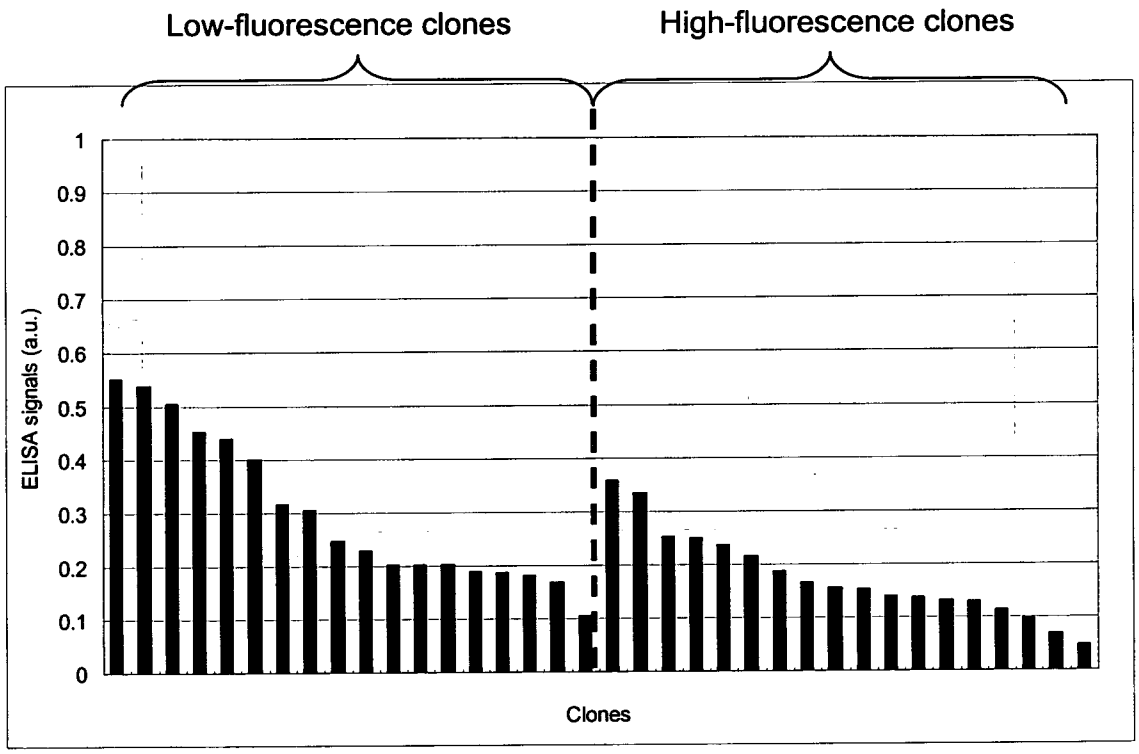

Low-fluorescence clones
High-fluorescence clones
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
ELISA signals (a.u.)
Clones

FIG. 11

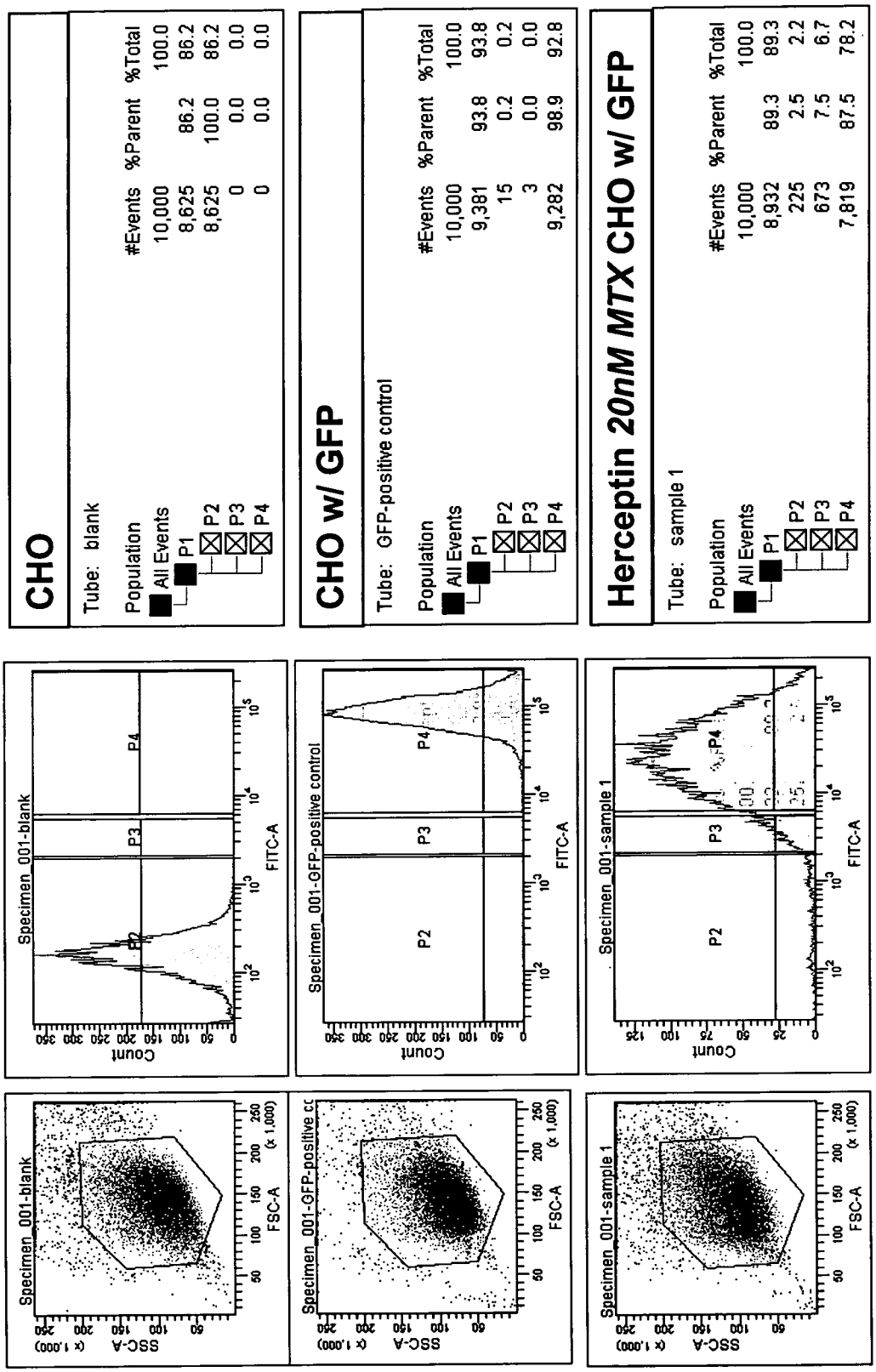

CHO
Tube: blank
Population
All Events
P1
P2
P3
P4
#Events %Parent %Total
10,000 100.0
8,625 86.2 86.2
8,625 100.0 86.2
0 0.0 0.0
0 0.0 0.0
CHO w/ GFP
Tube: GFP-positive control
Population
All Events
P1
P2
P3
P4
#Events %Parent %Total
10,000 100.0
9,381 93.8 93.8
15 0.2 0.2
3 0.0 0.0
9,282 98.9 92.8
Herceptin 20nM MTX CHO w/ GFP
Tube: sample 1
Population
All Events
P1
P2
P3
P4
#Events %Parent %Total
10,000 100.0
8,932 89.3 89.3
225 2.5 2.2
673 7.5 6.7
7,819 87.5 78.2
Specimen_001-blank
Specimen_001-GFP-positive control
Specimen_001-sample 1
SSC-A (x 1,000)
FSC-A (x 1,000)
Count
FITC-A

FIG. 12