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Technical Bulletin

CHOZN® Platform

Catalog NO. CHOGS (CHOZN® GS Cell Line)
Catalog NO. 14365C/24365C (EX-CELL® CD CHO Fusion)
Catalog NO 6366C (EX-CELL® CHO Cloning Medium)
Catalog NO. 14366C/24366C (EX-CELL® Advanced™ CHO Fed-batch Medium)
Catalog NO. 24367C/24368C (EX-CELL® Advanced™ Feed 1)

Product Description/Overview

The CHOZN® Platform is a CHO (Chinese Hamster Ovary) mammalian cell expression system for the fast and easy selection and scale up of clones producing high levels of recombinant proteins. Key to the CHOZN® Platform is the development of the CHOZN® ZFN Modified $GS^{-/-}$ CHO cell line that eliminates the endogenous Glutamine synthetase (GS) rendering the cells auxotrophic for the essential amino acid L-glutamine. The targeted gene modification within these cells allows for a more powerful and faster clone selection when isolating producing cell lines. The CHOZN® Platform also includes an optimized set of cGMP produced chemically defined (CD) growth and production media and feed that have been developed to maximize the growth and production of r-proteins from producing clones. The media and feed have been optimized to support both the initial cell line engineering process and stable cell line selection, through large scale growth and production. Media and feed formulations were developed using animal component free raw materials that have a proven track record for sourcing and robust manufacturability.

The CHOZN® ZFN-Modified $GS^{-/-}$ CHO cell line was created using Sigma's proprietary CompoZr® zinc finger nucleases (ZFN) technology. ZFNs are a class of engineered DNA-binding proteins which facilitate targeted genome editing by binding to a user-specified locus and causing a double-strand break (DSB). The cell then employs endogenous DNA repair processes, either non-homologous end joining (NHEJ) or homology-directed repair (HDR), to mend this targeted DSB. These repair processes can be channeled to generate precisely targeted genomic edits, resulting in an organism or cell lines with specific gene disruptions (knockouts), integrations, or modifications.

Glutamine synthetase (EC 6.3.1.2)[3] is an enzyme that plays an essential role in the metabolism of nitrogen by catalyzing the condensation of glutamate and ammonia to form glutamine. GS is one of the most commonly used selectable markers in the biopharmaceutical industry. Cells that lack a functional GS enzyme or have their enzyme inactivated by an inhibitor require that the medium be supplemented with L-glutamine in order to survive. L-Methionine sulphoximine (MSX) is a small molecule irreversible inhibitor of GS enzymatic activity when added into the medium. In cells containing a functional GS gene and enzyme, MSX can be used to inhibit the endogenous GS activity in order to select r-protein producing clones that have been transfected with an exogenous GS gene. However, MSX may pose certain hazards and its elimination from a biopharmaceutical cell culture process has many advantages (i.e. less regulatory scrutiny, lower production costs, less variability, etc). A cell line void of endogenous GS activity enables fast and easy clone selection and r-protein production to occur via a completely MSX-free process. SAFC's CHOZN® $GS^{-/-}$ cell line is the world's first CHO cell line that incorporates a specifically designed mutation that renders the gene product inactive. The cells are cGMP banked with full regulatory testing, show robust growth in culture, and allow for MSX-free selection processes for the generation and production of high producing recombinant cell lines.

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Precautions and Disclaimer

The CHOZN® GS$^{-/-}$ cell line and associated media and feed are for R&D use only, not for drug, household, or other uses. Please consult the Material Safety Data Sheets for information regarding hazards and safe handling practices.

Storage and Stability

Immediately upon receipt, store the cells in the vapor phase (approx -150C to -180C) of liquid nitrogen. Store all liquid media at 2-8C, protected from light, and store all dry powder medium at 2-8C, protected from light in a dry location.

Background: The CHOZN® GS$^{-/-}$ cell line

CHO cells are the preferred host expression system for the commercial-scale production of complex biopharmaceuticals (antibodies, enzymes, growth factors, etc.). The aneuploid, proline-requiring CHO-K1 line is a stable subclone of the parental CHO cell line derived from the ovary of an adult Chinese hamster (Puck et al, 1958). Creation, isolation, and characterization of high producing recombinant CHO-derived cell lines has been a long standing challenge for the pharmaceutical industry. Current methods to select high producing clones usually involve cell lines that have a growth requirement (i.e. DHFR) or inhibition of key metabolic enzyme (i.e.

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Glutamine synthetase). Recombinant clones are subsequently selected by growing transfectants under conditions in which only recombinant clones survive. The CHOZN® GS$^{-/-}$ cell line is the first CHO cell line that contains a mutation within the endogenous GS gene, making the cells dependent on exogenous supplementation of L-glutamine in the medium. These unique cells were generated using Sigma's CompoZr® ZFN technology to modify the CHO-K1 cell line (Sigma 85051005).

The suspension adapted CHO-K1 cell line was subsequently transfected with the CompoZr® GS ZFN pair (ZFNGSA9075/ZFNGSB9372, Sigma catalog number ZFNGS) (target sequence CCAAGCCCATTCCTGGGAactgGAATGGTGCAGGCT) (see Figure 1 below). The target sequence for this ZFN pair is located in exon 6 of the CHO GS gene. This is the sequence that codes for the substrate binding domain of the GS enzyme, therefore, mutations at this location result in a non-functional protein.

The ZFN transfected pool was single cell cloned, and the clones were screened for mutations at the ZFN target site. Several clones contained biallelic knockout mutations (see Figure 2 below) at the GS locus. After extensive characterization of the clones, one clone was identified as having more robust characteristics than the others. The clone ID for this CHOZN® GS$^{-/-}$ cell line is 2E3. Cells from clone 2E3 were subsequently banked under cGMP conditions in EX-CELL® CD CHO Fusion medium and the banks were thoroughly characterized according to FDA and EMEA testing requirements for master cell banks.

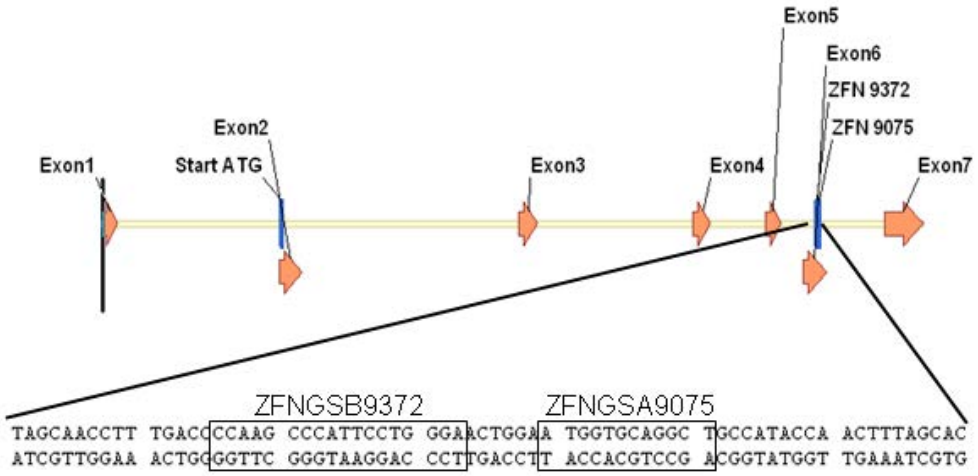

Figure 1. This is a schematic representation of the CHO GS gene and targeted sequence modification sites. The ZFN target site is indicated, with the ZFN binding sites identified by the highlighted sequences in the boxes. The ZFN9075/9372 pair target site is located within exon 6 of the GS gene, which codes for the substrate-binding domain of the protein.

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Allele	Sequence	Modification
WT Reference	CCAAGCCCATTCCTGGGAACTGGAATGGTGCAGGCT	Reference
Clone 2E3 Allele 1	CCAAGCCCATTCCTGGGAACT**CA**----------GCT	10bp deletion, 2bp substitution
Clone 2E3 Allele 2	CCAAGCCCATT----------CT	17bp deletion

Figure 2. This represents the sequence of the GS mutations within Clone 2E3 (CHOZN® GS$^{-/-}$ cell line) caused by ZFN transfection of the CHO-K1 cell line. The biallelic disruption of both copies of the GS gene are noted. Allele 1 has a 10 base deletion and a 2 base pair substitution within its allele, and allele 2 has a 17 base deletion. The flow chart below highlights the steps that are required to use the CHOZN® Platform. Relative timelines are noted within individual steps and major processes along the side.

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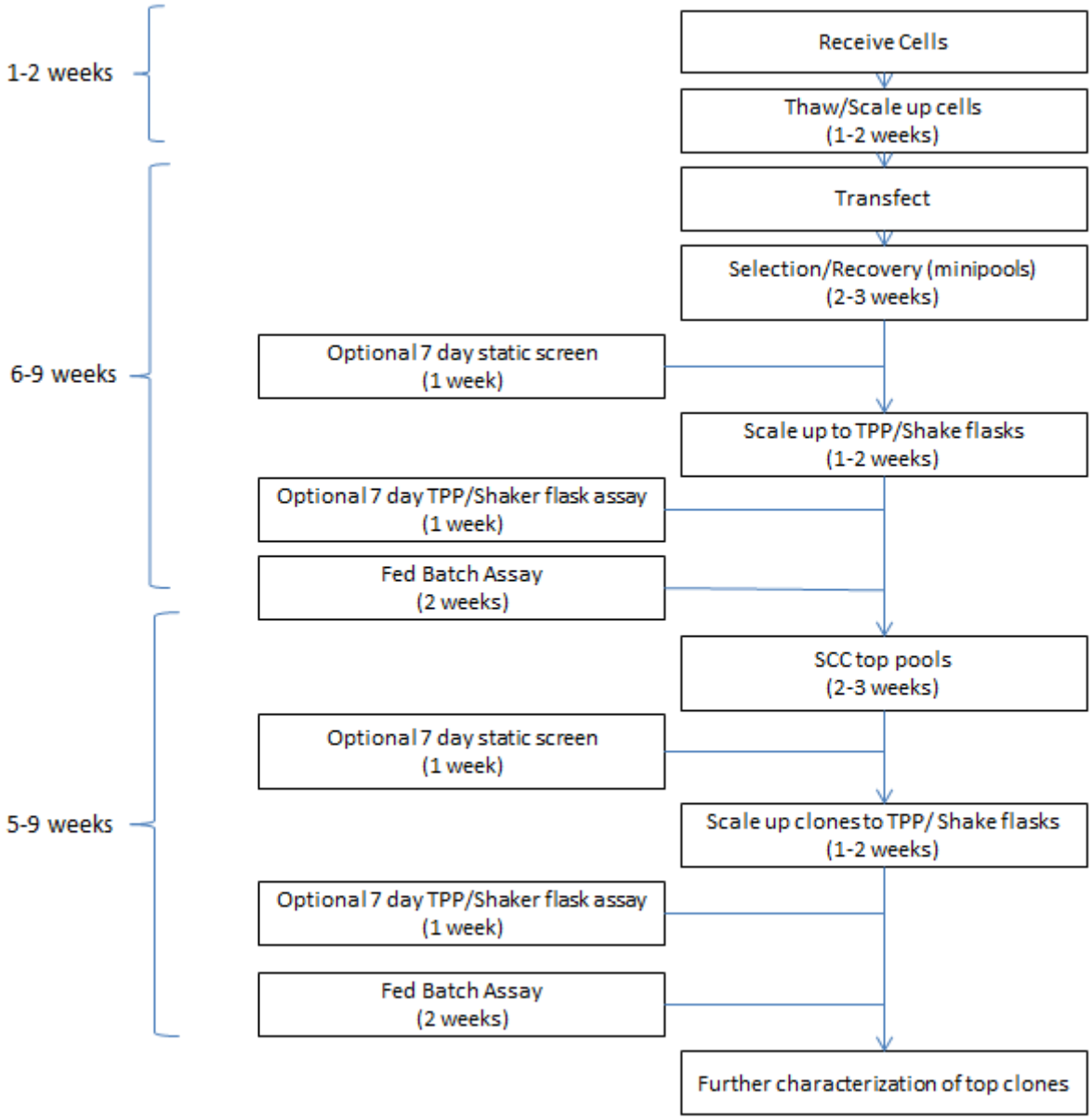
CHOZN GS Platform Workflow and Timeline



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Media, Feeds, and Supplements used with the CHOZN® Platform

Note: please refer to the Product Information Sheet/ Technical bulletin for each of these products for information on reconstitution techniques, storage and stability.

EX-CELL® CD-CHO Fusion Medium (#14365C-liquid) and (24365C-dry powder) without L-glutamine (cGMP)

EX-CELL® CD CHO Fusion Medium is a chemically defined, animal-component free medium developed for the long-term growth of CHO cells. The absence of any large macromolecules allows for streamlined isolation and purification of secreted proteins from the cells. This medium does not contain L-glutamine, which improves medium stability, eliminates L-glutamine degradation that causes ammonia build-up, and provides an appropriate medium for the culture of CHO cells that use GS selection. EX-CELL® CD CHO Fusion Medium is manufactured under cGMP production quality conditions with full raw material documentation and is available in liquid or powder format.

EX-CELL® CHO Cloning medium (C6366) without L-glutamine (cGMP)

EX-CELL® CHO Cloning medium is an animal-component free medium designed to support clonal survival and growth of Chinese Hamster Ovary (CHO) cell lines, with results comparable to the traditional method using 10% fetal bovine serum. Developed to meet the needs of the biotechnology industry, this medium is designed for single-cell cloning of recombinant CHO cell lines adapted to serum-free suspension culture. This medium does not contain L-glutamine, which improves medium stability, eliminates L-glutamine degradation that causes ammonia build-up, and provides an appropriate medium for the culture of CHO cells that use GS selection. This medium is manufactured under cGMP production quality conditions with full raw material documentation and is available in a liquid format.

EX-CELL® Advanced™ CHO Fed Batch medium Catalog NO. 14366C-liquid/24366C-dry powder

EX-CELL® Advanced™ CHO Fed-Batch Medium is a chemically defined, next generation media platform. The formulation was developed using multivariate analysis of 10,000+ data points that included performance, physical, regulatory and safety design specifications. This medium is designed to be used in conjunction with Advanced™ CHO Feed 1 for superior platform performance in fed-batch cultures.

EX-CELL® Advanced™ Feed 1Catalog NO. 24367C (w/ glucose) /24368C (w/out glucose)

EX-CELL® Advanced™ CHO Feed 1 is a single part, next generation feed with highly concentrated key critical raw materials. The formulation was developed using multivariate analysis of 10,000+ data points that included performance, physical, regulatory and safety design specifications. This feed is designed to be used in conjunction with Advanced™ CHO Fed-batch Medium for superior titer performance in fed-batch cultures.

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Required Cell Culture Reagents

Cell culture reagents	Manufacturer	Cat No.
CHOZN® GS$^{-/-}$ cells	SAFC	CHOGS
EX-CELL® CD CHO Fusion Medium	SAFC	14365C (liquid) 24365C (powder)
EX-CELL® CHO Cloning Medium	SAFC	C6366
EX-CELL® Advanced™ CHO Fed-batch Medium	SAFC	14366C (liquid) 24366C (dry powder)
EX-CELL® Advanced™ Feed 1	SAFC	24367C (w/ glucose) 24368C (w/out glucose)
L-glutamine (200mM)	Sigma-Aldrich SAFC	G7513 59202C
D-(+)-Glucose (45% solution)	Sigma-Aldrich	G8769
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich	D2438
L-methionine sulfoximine (MSX) optional	Sigma-Aldrich	M5379 or 76078 (cGMP grade)
Sodium Bicarbonate	Sigma-Aldrich	S5761
36-38% Hydrochloric Acid	Sigma-Aldrich	H1758
50% Sodium Hydroxide	Sigma-Aldrich	415413
Tissue Grade Water	Sigma-Aldrich	W3500

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Equipment/materials/reagents needed

- Expression vector (plasmid DNA) containing gene expression cassettes for the protein of interest and GS for metabolic selection (recommended plasmid concentration ~1µg/µl)
- Sterile filtration unit; 0.22µm, 1000ml capacity (Millipore® Stericup™ SCGPU10RE or equivalent)
- Low-volume pipettes and sterile tips (Rainin® Classic set or equivalent)
- Sterile pipettes (1ml, 2ml, 5ml, 10ml, 25ml, 50ml)
- T-25cm^2 and T-75cm^2 suspension cell (hydrophobic surface treated) culture flasks (Greiner Bio-one 690195 and 658195 or equivalent)
- 15ml and 50ml sterile conical centrifuge tubes (Corning 430052 and 430290 or equivalent)
- 125ml sterile Erlenmeyer non-baffled, vented cap culture flask (Corning 431143 or equivalent)
- 96 well suspension cell treated (hydrophobic surface) culture plates (Greiner Bio-one 655185 or equivalent)
- 24 well suspension cell treated (hydrophobic surface) culture plates (Greiner Bio-one 662102 or equivalent)
- 50ml TPP (Techno Plastic Products) TubeSpin® tubes
- Sterile microfuge tubes (1.5ml Eppendorf® or equivalent)
- 4mm Electroporation Cuvettes (Sigma Z706094 or equivalent)
- Bio-Rad Laboratories, Inc. (Genepulser® or similar electroporation instrument)
- Refrigerated centrifuge with swinging bucket rotor (capable of 3000x g forces)
- CO_2 incubator (5% CO_2, 37C, humidified)
- Automated cell counter or hemocytometer
- Orbital shaker plate or CO_2 shaker culture incubator system (ATR Multitron II or similar)
- Water bath set at 37C
- Biological safety cabinet (Class II; Type A2; ISO 5)
- Refrigerator (4C)
- Freezer (-20C)
- Ultra-cold freezer (-80C)
- Liquid nitrogen (LN_2) freezer; recommend vapor phase (-150C to -196C)
- Sterile cryovials (Nalgene 5000-1020 or equivalent)
- Cryovial labels (LN_2 resistant)
- LN_2 freezer boxes
- Controlled rate freezing vessel (Nalgene “Mr Frosty” or controlled rate freezer equipment capable of a cooling rate of approximately 1C/min.)
- 70% isopropanol (2-propanol; 70% in H_2O, Sigma 563935)

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CHOZN® Platform User Protocols

NOTE: The following procedures should only be performed by personnel trained to:

- Work with potentially biohazardous materials.
- Handle all cell culture procedures under at least Biosafety Level 1 (BSL-1) containment and practices.
- Use Universal precautions for biosafety (WHO Laboratory Biosafety Manual; 3rd ed., 2004).
- Use aseptic technique for all cell and media handling procedures (the addition of antibiotics/antimycotics at any time during the following protocols is not recommended).

Note: All cell culture and media handling in these protocols must be carried out in a HEPA filtered (Class II) biological safety cabinet capable of creating an ISO Class 5 clean environment.

Part I: Media Preparation for Initial thaw and bank of CHOZN® GS$^{-/-}$ Cells

Note 1: Please refer to the product guides for recommendations on reconstitution, storage and stability information on all other media and feeds.

Note 2: GS selected producing clones and cell lines should always be grown in medium without L-glutamine (see Part VI).

I-A: EX-CELL® CD CHO Fusion Liquid Medium Supplemented with 6mM L-glutamine (referred to throughout the technical bulletin as "Growth Medium")

Reagents and Equipment

- EX-CELL® CD CHO Fusion medium (Sigma 14365C)
- Sterile filtration unit 0.22µm (1000ml capacity)
- Sterile pipettes
- Biological safety cabinet (Class II; Type A2; ISO 5)
- Freezer (-20C)
- L-glutamine; 200mM (Sigma G7513)
- Refrigerator (2-8C)
- Water bath set at 37C
- 70% isopropanol (in spray bottle for surface decontamination)

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Procedure

The following procedure describes the preparation of 1L of Growth Medium (EX-CELL® CD CHO Fusion supplemented with 6mM L-glutamine):

i) Prepare supplements
   a. L-glutamine: Thaw 200mM stock bottle of L-glutamine in a 37C water bath until completely dissolved. After thawing, store any unused L-glutamine at 2-8C for up to two weeks.

ii) Aseptically add 30ml of 200mM L-glutamine into 1L of EX-CELL® CD CHO Fusion according to the chart below.

iii) (Optional) Filter the complete medium through a 1000ml capacity sterile filtration unit to ensure sterility of the medium.

iv) Mark the date of preparation on the medium bottle and store medium at 2-8C in the dark until needed.

v) Discard any unused glutamine-supplemented medium after one month.

Growth Medium (EXCELL® CD CHO Fusion + 6mM glutamine) for CHOZN® GS$^{-/-}$ Parental Cells

Material	Product Number (Sigma-Aldrich)	Volume Needed	Final Concentration
EX-CELL® CD CHO Fusion	14365C	1L	1X
L-glutamine (200mM)	G7513	30ml	6mM

Part II: Stock Culture Initiation/Thawing and Sub-culturing of CHOZN® GS$^{-/-}$ Cells (cGMP banked).

Purpose

This protocol describes procedures for the stock initiation of CHOZN® GS$^{-/-}$ cells.

Note: Remember that the parental CHOZN® GS$^{-/-}$ cells are auxotrophic for L-glutamine and require that all media contain L-glutamine for growth.

Reagents and Equipment

- T-75cm^2 suspension cell (hydrophobic surface treated) culture flasks (Greiner Bio-one 690195 or equivalent)
- 15ml sterile conical centrifuge tube (Corning 430052 or equivalent)
- 125ml sterile, non-baffled, vented cap Erlenmeyer culture flask (Corning 431143 or equivalent)
- 50ml TPP (Techno Plastic Products) TubeSpin® tubes
- Sterile pipettes
- Frozen vial of CHOZN® GS$^{-/-}$ cells (Sigma CHOGS)

  Note: Cells are banked at approximately 7.5 x 10^6 cells/ml in 93% EX-CELL® CD-CHO Fusion Growth Medium (with 6mM L-glutamine) and 7% Dimethyl sulfoxide (DMSO).

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- EX-CELL® CD CHO Fusion cell culture Growth Medium (prepared as described in Part I-A containing 6mM L-glutamine)
- Water bath at 37C
- Biological safety cabinet (Class II; Type A2; ISO 5)
- Centrifuge
- CO_2 incubator (5% CO_2, 37C,humidified)
- Automated cell counter or hemocytometer
- Orbital shaker plate (set at 125 rpm for Erlenmeyer flasks, 200rpm if TPP® TubeSpin tube)
- 70% isopropanol in a spray bottle

Procedure

i) Thawing the cells

a. Adjust incubator settings to 37C and 5% CO_2. Humidify the unit by placing a shallow pan of sterile water near bottom of incubator or set to 80% if humidity control feature is available.

b. Pre-warm growth medium to 37C in a water bath.

Note: Do not allow the medium to be in the water bath for greater than 1-2 hours.

c. Obtain one sterile T-75cm^2 suspension cell (hydrophobic surface treated) culture flask.

d. Obtain a sterile 15 ml sterile conical centrifuge tube.

e. Aseptically transfer 8ml of pre-warmed, sterile EX-CELL® CD CHO Fusion cell culture Growth Medium (with 6mM L-glutamine) into the 15ml sterile conical centrifuge tube.

f. Obtain a frozen vial of the CHOZN® GS cells (1ml) from the LN_2 freezer.

g. Immediately thaw the vial by gently swirling the vial in a 37C water bath until just thawed (approximately 1 minute). Do not completely submerge the vial to avoid contamination.

h. Remove the vial from the water bath and spray the vial with a copious amount of 70% isopropyl alcohol to decontaminate the outside surfaces. Place vial in the biological safety cabinet.

i. Allow alcohol to completely dry from surface of vial before opening.

ii) Wash the cells

a. Aseptically transfer cells from the cryovial into the sterile 15ml conical centrifuge tube containing fresh EX-CELL® CD CHO Fusion cell culture Growth Medium (with 6mM L-glutamine)(step i-e above).

b. Centrifuge the cell suspension at 220 rcf for 5 minutes at 15-20C to pellet the cells.

c. Using a sterile pipet, carefully aspirate off the clarified medium taking care not to disturb the cell pellet. Discard the aspirate while retaining the cell pellet.

Note: Caution- Clarified medium contains DMSO. Dispose of properly according to local regulations.

iii) Sub-culturing the cells

a. Aseptically add 10ml of fresh EX-CELL® CD CHO Fusion cell culture Growth Medium (with 6mM L-glutamine) to the conical centrifuge tube containing the cell pellet. Gently re-suspend the cell pellet by pipetting up and down to break up the cell clumps.

b. Aseptically transfer the entire cell suspension (10ml) to the sterile T-75cm^2 suspension cell (hydrophobic surface treated) culture flask.

c. Incubate the cells in a 37C, humidified CO_2 incubator (non-shaking) for 20-28 hours.

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Note: The majority of the cells will not adhere to the flask. Use caution when transporting.

d. After 24 hours, determine and record cell density and viability.

Note: If stored under optimal storage conditions (vapor phase of liquid nitrogen, cells should recover within 24 hours and be >90% viable.

e. Passage the cells by transferring the 10ml of static culture from the T-75cm^2 flask into a 125ml sterile Erlenmeyer culture shaker flask (vented cap, non-baffled) containing an additional 10ml of fresh complete Growth Medium (total volume approximately 20ml). Place the flask on the orbital shaker plate, and shake at 125-130 rpm.

Note: If using TPP tubes in place of Erlenmeyer flasks, adjust shaker speed setting to 200rpm.

f. Once in shaker flask culture, maintain the cell culture stock by following the stock maintenance protocol described in Part III.

Part III: Stock Maintenance of CHOZN® GS$^{-/-}$ Cells

Purpose

This protocol describes procedures for stock maintenance of the parental CHOZN® GS$^{-/-}$ cells.

Reagents and Equipment

- 125ml sterile Erlenmeyer culture flask (non-baffled, vented cap) Corning 431143 or equivalent
- 50ml TPP (Techno Plastic Products) TubeSpin® tubes
- Complete (contains 6mM L-glutamine) EX-CELL® CD CHO Fusion cell culture Growth Medium (prepared as described in Part I-A or I-B)
- Orbital shaker plate (set at 125-130 rpm in Erlenmeyer flasks; 200rpm if TPP)
- Water bath (set to 37C)
- CO_2 incubator (5% CO_2, 37C, humidified)
- Sterile pipettes
- Biological safety cabinet (Class II; Type A2; ISO 5)
- Automated cell counter or hemocytometer
- 70% isopropanol in a spray bottle

Procedure (to be performed every 3-4 days)

i) Verify that the incubator is set to 37C, 5% CO_2, and has water for humidity control (~80%).

ii) Pre-warm complete Growth Medium to room temperature or to 37C in a water bath.

iii) Aseptically remove a small volume of cell culture sample from the flask and count by trypan blue exclusion using a hemocytometer or an automated cell counter. Do not proceed if cell viability is less than 90%.

Note: If cell viability is below 90%, troubleshoot conditions prior to continuing.

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iv) Determine the correct volume of cell culture to inoculate a new flask at a starting cell density of 2.0-3.0 x10^5 cells/ml into the desired volume (see appropriate working volume for cell culture flasks in the table below).

v) Aseptically transfer the appropriate amount of cells to the new flask, and add pre-warmed growth medium up to the desired volume.

vi) Incubate flasks in a humidified 37C incubator with 5% CO_2 on an orbital shaker at 125-130 rpm.

Note: If using TPP tubes in place of Erlenmeyer flasks, adjust shaker speed setting to 200rpm.

vii) Passage cells by repeating the above steps at least twice weekly, and expand culture volume as necessary according to the chart below.

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Appropriate Working Volume by Flask Size

Shake Flask	Volume Range
50ml TPP® TubeSpin tube	25-30ml
125 ml shaker flask	17-35 ml
250 ml shaker flask	60-100ml
1L shaker flask	300-400ml

Part IV: Cryopreservation and Cell Banking of CHOZN® GS$^{-/-}$ Cells

Purpose

This protocol details procedures for establishing a working cell bank of CHOZN® GS$^{-/-}$ cells. In this example, a 10 x 1ml vial bank is described.

Reagents and Equipment

- Sterile cryovials (Nalgene 5000-1020 or equivalent)
- CHOZN® GS$^{-/-}$ stock cell culture
- EX-CELL® CD CHO Fusion Growth Medium (prepared as in Part I)
- DMSO (Sigma D2438) Note: use fresh solution from an unopened bottle for best results
- Cryovial labels (must be LN_2 resistant)
- 15ml and 50ml sterile conical centrifuge tubes (Corning 430052 and 430290 or equivalent)
- Sterile pipettes
- Biological safety cabinet (Class II; Type A2; ISO 5)
- Centrifuge
- Controlled rate freezing vessel (Nalgene “Mr Frosty”) or controlled rate freezer equipment
- 70% isopropanol
- Ultra-cold freezer (-80C)
- LN_2 freezer boxes
- LN_2 freezer
- Automated cell counter or hemocytometer

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Procedure

i) If using a manual controlled rate freezing system, fill the vessel with fresh 70% isopropanol and/or follow manufacturer's instructions.

ii) Label cryovials.

iii) Prepare Freezing Medium by aseptically adding fresh 100% DMSO to Growth Medium to achieve a final concentration of 7% DMSO. In this example, 20ml of Freezing Medium is prepared by adding 18.6 ml of EX-CELL® CD CHO Fusion Growth Medium plus 1.4 ml of fresh 100% DMSO to a sterile 50ml conical tube.

iv) Aseptically remove a small volume of cell culture from the flask and count by trypan blue exclusion using a hemocytometer or an automated cell counter. Do not proceed if cell viability is less than 90%.

v) Calculate the volume of cell stock and Freezing Medium needed to obtain $5x10^6$ – $1.0 x10^7$ cells per cryovial (1ml volume). In this example one would need approximately $1 x 10^8$ cells total.

Note: Once cell preparation is initiated, work must proceed quickly. It is recommended that the total time from removing cells from the stock culture to placing the controlled rate freezing vessel containing the banks into the –80C freezer take no more than 30 minutes.

vi) Aseptically transfer calculated volume of stock culture to an appropriately sized sterile conical centrifuge tube.

vii) Centrifuge at 220 rcf for 5 minutes at 15-20C.

viii) Carefully aspirate off the supernatant taking care not to disturb the cell pellet.

ix) Gently re-suspend the cells with calculated volume of Freezing Medium.

x) Mix thoroughly by gently pipetting the cell suspension.

xi) Immediately aseptically aliquot 1.0 ml of the cell suspension into labeled cryovials. Cap tightly.

xii) Quickly transfer the vials to the prepared controlled rate freezer system vessels (manual) and transfer into a -80C freezer, or transfer vials into a controlled rate freezer and follow recommended procedures for overnight freezing.

xiii) Transfer frozen vials to the vapor phase of a LN_2 freezer within 18-72 hours of freezing.

Part V: Transfection of CHOZN® $GS^{-/-}$ Cells

Purpose

This protocol describes procedures for transfection of the CHOZN® $GS^{-/-}$ cells via electroporation. The transfection conditions provided in this protocol have been optimized for the CHOZN® $GS^{-/-}$ parental cell line. When performed properly, a 60-80% transfection efficiency (determined by the expression of a fluorescent reporter construct in the transfected pool) should be achieved.

Note: While not recommended, lipid based transfection methodologies may also be used but lower transfection efficiencies may result. The usage of transfection reagents that are designed for suspension CHO cells are recommended (i.e. Mirus TransIT-PRO® Transfection Kit; MIR5700). Follow manufacturers recommended protocol when using such reagents.

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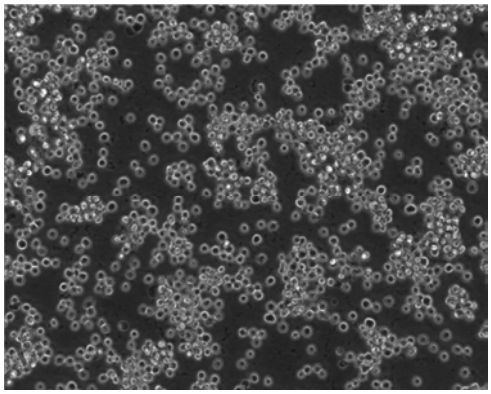

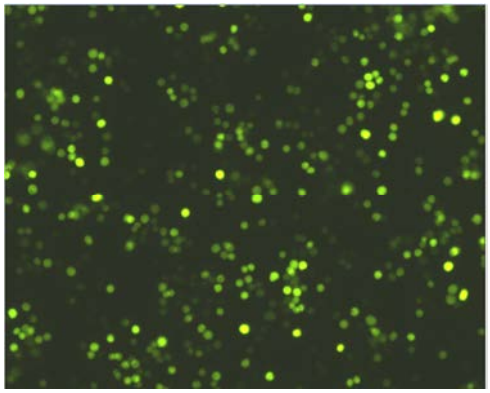

Images of pCMV-GFP transfected CHO cells (pool) 24 hours post-transfection.

Note: If the user plans to establish multiple stable pools from the transfected cultures, then it is recommended that at least 2-3 transfections are performed simultaneously and that the transfected cells are pooled together prior to initiating the selection process.

Reagents and Equipment

- Biological safety cabinet (Class II; Type A2; ISO 5)
- CHOZN® GS$^{-/-}$ stock cell culture
- EX-CELL® CD CHO Fusion Growth Medium (prepared in Part I)
- 4mm Electroporation Cuvettes (Sigma Z706094)
- T-25cm^2 suspension cell (hydrophobic surface treated) culture flasks (Greiner Bio-one 690195 and 658195 or equivalent)
- Plasmid DNA containing a gene expression cassette for the protein of interest, as well as an expression cassette for GS for metabolic selection (recommended plasmid concentration ~1µg/µl)

  Note: Plasmid DNA preparations should be sterile (via ethanol precipitation) and re-suspended in sterile water with no salts or buffers.
- 15ml and 50ml sterile conical centrifuge tubes (Corning 430052 and 430290 or equivalent)
- Sterile pipettes
- Centrifuge
- 70% isopropanol
- Sterile microfuge tubes (1.5ml Eppendorf® or equivalent)
- Automated cell counter or hemocytometer
- CO_2 incubator (5% CO_2, 37C, humidified)
- Bio-Rad Gene Pulser® or similar electroporation instrument

Procedure

i) (24 hours before transfection) Cell culture preparation.

a. Inoculate a flask of CHOZN® GS$^{-/-}$ cells (from the stock cell culture) to a final cell density of 0.5x10^6 cells/ml.

ii) Electroporation set-up (day of transfection).

a. Prepare Complete Growth Medium (see Part I)

Note: Complete EX-CELL® CD CHO Fusion Growth Medium (containing 6mM L-glutamine) is used as the transfection and recovery medium.

b. Label the appropriate number of T-25cm^2 suspension cell culture flasks (one per transfection).

c. Aseptically add 5.0 ml complete EX-CELL® CD CHO Fusion Growth Medium to each flask.

iii) Label and place electroporation cuvettes on ice to chill.

iv) Label sterile microfuge tubes for mixing cell suspensions with DNA.

v) Prepare cells for electroporation as follows:

a. Aseptically remove a cell culture sample from the flask and count by trypan blue exclusion using a hemocytometer or an automated cell counter. A minimum viability target of >90% must be achieved to proceed with electroporation.

b. Calculate the appropriate volume of stock culture needed for the transfections (6.25x10^6 cells/transfection).

c. Aseptically transfer calculated volume of stock culture to an appropriately sized sterile conical centrifuge tube.

d. Centrifuge at 220 rcf for 5 minutes at 15-25C.

e. Carefully aspirate off the supernatant without disturbing the cell pellet.

f. Re-suspend cell pellet in the Complete EX-CELL® CD CHO Fusion Growth Medium (1.0 ml medium per transfection).

vi) Electroporation

a. For each electroporation, mix 0.8ml of cell suspension (~5x10^6 total cells) with desired amount of DNA in a sterile microfuge tube. 30-50µg of plasmid DNA is recommended for each transfection (the volume of DNA in an individual transfection should not exceed 50µl).

b. Transfer the DNA/cell mix to the chilled electroporation cuvette, and electroporate using the following settings:

Voltage	Capacitance	Pulse
300 V	950 µF	Exponential Decay

c. Transfer ~0.6ml of each electroporation condition to the 5ml Growth Medium in the prepared T-25cm^2 suspension cell culture flask (try not to transfer the white cell debris).

d. Incubate the T-25cm^2 flasks for 24 (+/- 4) hours at 37°C and 5% CO_2.

e. To generate stably transfected pools, place the cells under selection as described in Part VI.

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Part VI: Stable Pool Selection and Recovery

Purpose

Described are two options for the creation of stable pools: 96-well minipool technique, and the bulk selection technique.

In the minipool selection method, the goal is to create small pools of stably transfected cells. Since each pool began with a small original population, there will be more variation between the pools. By screening a number of different minipools, the chance of rapidly isolating high producing and robust stable pools increases. The bulk selection technique yields information on the recovery of the cells, and an early indication if the transfection was successful. Stable pools developed using the bulk pool method typically yield lower titers than stable pools generated in the minipool method. It is recommended to perform the bulk pool method as a control.

Note: The bulk selection is performed in chemically defined media, whereas the 96-well minipool process is performed using two media, one of which is chemically defined, and one that is animal-component free, but not chemically defined. The EX-CELL® CHO Cloning Medium used in the 96-well minipool process contains plant hydrolysates.

A) Stable Pool Selection via Bulk Selection (Chemically-defined Process)

Pools selected in a bulk selection method are likely to survive and tend to have shorter recovery timelines compared to the minipool selection methods. However, there will likely be less variation between different bulk pools when compared to the variation between different minipools. The overall final titers of the top bulk pools are likely to be lower than those generated following the two minipool methods described below. This process is performed using chemically-defined medium.

Reagents and Equipment

- Transfected cell culture (24hrs post-transfection)
- GS Selection Medium (EX-CELL® CD CHO Fusion Medium ***without*** L-glutamine)
- T-25cm^2 and T-75cm^2 suspension cell (hydrophobic surface treated) culture flasks (Greiner Bio-one 690195 and 658195 or equivalent)
- Automated cell counter or hemocytometer
- 15ml sterile conical centrifuge tubes
- Sterile pipettes
- Centrifuge
- Biological safety cabinet (Class II; Type A2; ISO 5)
- CO_2 incubator (5% CO_2, 37C, humidified)
- 70% isopropanol

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Procedure

i) 24 hours post-transfection, aseptically transfer the transfected cells from the T-25cm^2 flask to a sterile 15ml conical tube.

ii) Centrifuge at 220 rcf for 5 minutes at 15-25C.

iii) Carefully aspirate off the supernatant taking care not to disturb the cell pellet.

iv) Re-suspend the cell pellet in 10.0 ml EX-CELL® CD CHO Fusion ***without*** L-glutamine (GS Selection Medium) (see table below).

v) Transfer the re-suspended cells to a T-75cm^2 suspension cell culture flask.

vi) Perform a complete medium exchange on the pool once a week during the selection process.

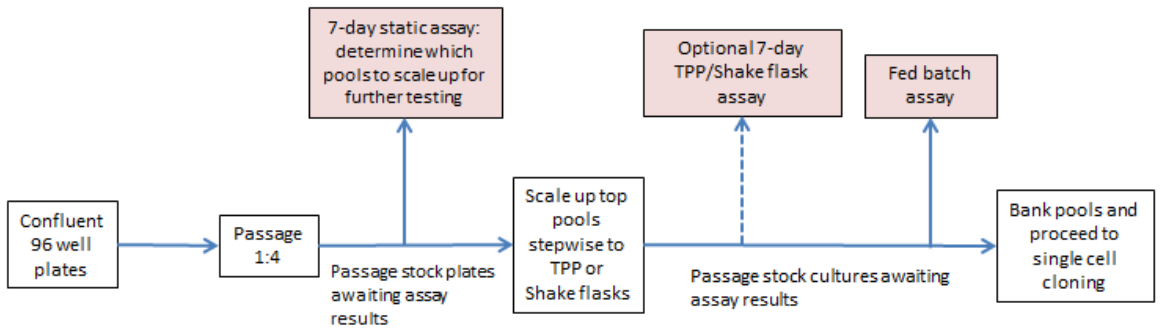
   a. Transfer the entire cell culture from the T-75 cm^2 flask to a sterile 15ml conical centrifuge tube.
   b. Centrifuge at 220 rcf for 5 minutes at 15-25C.
   c. Carefully remove the supernatant taking care not to disturb the cell pellet.
   d. Re-suspend the cell pellet in 10-12ml of fresh GS Selection Medium (EX-CELL® CD CHO Fusion ***without*** L-glutamine), and place the re-suspended cells back into the original flask.
   e. Incubate the flask at 37C 5% CO^2, humidified.

vii) Count the pool twice weekly.

viii) After the pool recovers from selection (viability >90% and stabilized doubling time, approximately 10-21 days post transfection), passage the pool to 0.3x10^6 viable cells/ml twice weekly. The stable pools may be scaled up to shake flasks for further characterization at this point.

B) Stable Pool Selection via 96-well Protocol (Animal-Component Free Process)

In this option, the cells are plated into 96-well plates in a blend of 80% EX-CELL® CHO Cloning Medium (Sigma C6366) and 20% GS Selection Medium (EX-CELL® CD CHO Fusion ***without*** L-glutamine). 5000 cells/well is the recommended plating density.



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Reagents and Equipment

- Transfected cell culture (approximately 24 hours post-transfection)
- GS Selection Medium (EX-CELL® CD CHO Fusion Medium ***without*** L-glutamine)
- EX-CELL® CHO Cloning Medium (Sigma C6366)
- 96 well suspension cell treated (hydrophobic surface) culture plates (Greiner Bio-one 655185 or equivalent)
- Automated cell counter or hemocytometer
- 15ml sterile conical centrifuge tubes
- Sterile pipettes
- Centrifuge
- Biological safety cabinet (Class II; Type A2; ISO 5)
- CO_2 incubator (5% CO_2, 37C, humidified)
- Multichannel micropipetter and sterile tips
- 70% isopropanol
- Sterile 0.2µm Millipore Steriflip® filter apparatus (or similar)

Procedure

i) 24 hours post-transfection, transfer the transfected cells from the T-25cm^2 flask to a sterile 15ml conical centrifuge tube.

ii) Centrifuge at 220 rcf for 5 minutes at 15-20C.

iii) Carefully aspirate off the supernatant taking care not to disturb the cell pellet.

iv) Re-suspend the cell pellet in 10.0ml GS Selection Medium (EX-CELL® CD CHO Fusion ***without*** L-glutamine)

v) Aseptically remove a small volume sample and count by trypan blue exclusion using a hemocytometer or an automated cell counter.

vi) Prepare the 96 well minipool plating medium (80% cloning medium and 20% EX-CELL® CD CHO Fusion mix) according to the table below.

Note: Do not add L-glutamine to the Plating Medium.

96 Well Minipool Plating Medium

Material	Product Number (Sigma-Aldrich)	Final Concentration
EX CELL® CD CHO Fusion Medium	14365C	20%
EX CELL® CHO Cloning Medium	C6366	80%

vii) Determine the total volume needed to inoculate the desired number of wells at 5000 cells/well. Each well requires 200µl of total volume (see guide chart below).

Seeding Density (cells/well)	Resuspension Density (cells/ml)
5,000	25,000

viii) Dilute the cells into the appropriate amount of 96-well minipool Plating Medium achieving the desired cell density as determined above.
   a. Note: It may be necessary to achieve these concentrations through serial dilutions of the cells.

ix) Plate the diluted cells into 96 well plates (200µl per well) using a multi-channel pipetter.

x) Place the cells into a humidified CO_2 incubator at 37C and do not disturb for 5 days. After 5 days, remove and examine the plates for outgrowth.

xi) Once a week, replace any evaporated medium with GS Selection Medium (EX-CELL® CD CHO Fusion medium ***without*** L-glutamine).

xii) Once the cells are approximately 80% confluent and appear healthy when examined under the microscope, split the cells into new 96 well plate (first passage plate), with a 1:4 dilution into 200µl of GS Selection Medium (EX-CELL® CD CHO Fusion ***without*** L-glutamine). This should occur 10-21 days post-plating, depending on the expression vector design and original seeding density.

Note: If desired, supernatant can be removed from the original plate for a preliminary titer screen. However, since the pools have different growth rates, this initial screen may not be an accurate indication of which pools will be top performers after scale up.

xiii) Titer screening method (OPTIONAL). This screen can be used to identify the top producing stable pools early on in the scale up process in order to reduce the total number of pools scaled up into shake cultures. If it is not necessary to reduce the number of stable pools taken to the next step then proceed to Part VII. This static 7-day assay does not predict the rank order of the minipools, but can be used to determine the top 20-30% of pools to scale up for further analysis.
   a. Once the cells in the second passage plate are 80% confluent, split them 1:5 into 200µl of selection medium into two new 96-well plates. One plate will be a propagation plate, and the other plate will be used for a terminal assay to screen the minipools for productivity.
   b. Passage the propagation plate as described above, splitting the cells with a 1:5 dilution when they become 80% confluent, 1-2 times per week.
   c. Incubate the terminal assay plate for 7 days, and harvest supernatants for titer quantitation (ForteBio or similar).

xiv) Scale up the selected pools for further characterization (see Part VII).

Part VII: Scale Up and Characterization of Stable Minipools

Purpose

Described is the scale up of pools from the propagation plates.

Procedure

The recommended scale up strategy is listed below (Table 1) and is outlined via the flowchart. Scaling up the cell cultures in the recommended order will help prevent loss of stable pools resulting from a more aggressive scale up strategy. When scaling up from static plates or flasks, be sure to pipette medium across the bottom of the vessel to dislodge any cells that may be semi-attached to the plastic. When scaling up from static T-75cm^2

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flasks to shaker flasks, it is recommended to retain a small population of cells in a static environment in case the pool has difficulty adapting to a shaken environment.

The amount of time it takes for a specific stable pool to recover and adapt to shaker culture conditions will vary depending on expression vector design and pool characteristics. Some pools may scale up more quickly than others. It is important not to let the pools become overgrown or too sparse, as each of these environments may have an effect on the final characteristics of the pool.

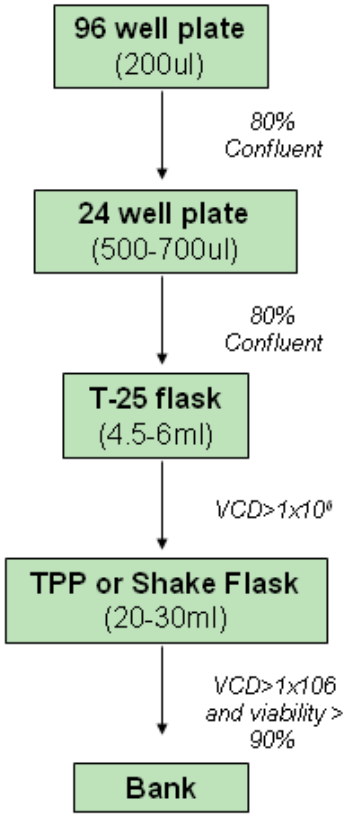

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Table 1: Recommended Scale up Strategy

Culture plate/flask size	Product Number	Medium Volume	When to scale up	Amount of days normally required
96-well plate	655185 (Greiner-one)	200µl per well	80% confluent	2-4 days after cells have recovered
24-well plate	662102 (Greiner-one)	500-750µl per well	80% confluent	3-5 days
T-25cm^2 flask	690195 (Greiner-one)	4.5-6 ml	VCD above 0.5x10^6	3-5 days
T-75cm^2 flask	658195 (Greiner-one)	9-15 ml	VCD above 0.5x10^6	2-4 days
125ml Erlenmeyer shaker flask	431143 (Corning)	20-30 ml	Passage twice weekly to 0.3x10^6	2-4 days

Screening Protocols in TPP® TubeSpin tubes /Shake Flasks

Optional 7- day TPP® TubeSpin tube /Shake Flask assay

Once the pools are adapted into shaker flasks or TPP® TubeSpin tubes, the number of minipools can be narrowed down to fewer pools via a 7-day terminal assay in TPP® TubeSpin tubes or Shake flasks. Similar to the static 7-day assay, the cells are inoculated and cultured for 7 days, and titer analysis is performed at the end of the assay. This assay better predicts the rank order of the minipools, and can be used to narrow down to the top 20-30 pools to be screened in a fed batch assay. Alternatively, this assay can be skipped and all minipools can be placed into the Fed Batch Assay.

To perform the 7-day TPP® TubeSpin tube /Shaker Flask assay, inoculate a flask at 0.3e6 cells/ml in GS Selection Medium (EX-CELL® CD CHO Fusion ***without*** L-glutamine). Allow the culture to grow for 7 days. Add 3g/L glucose on days 3 and 5. On day 7, remove supernatant for titer analysis.

Fed Batch Assay

The Fed Batch Assay is used to determine from which pools to single cell clone. This assay yields information on growth and productivity characteristics of the stable pools (which may predict the growth and productivity characteristics of clones derived from those pools).

Please see section XII for description of the Fed Batch Assay.

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Part VIII: Optional MSX Selection of Stable Pools

Purpose

If desired, higher selection pressure can be applied to the stable pools to try to improve the pool titer. In this process, GS selection stringency is increased via the addition of L-methionine sulfoximine (MSX), an inhibitor of GS, to the cell culture medium. We have observed that some pools can achieve higher titers when exposed to MSX post-recovery. Not all pools respond to this method, and some pools are not able to recover from the additional selection pressure.

Reagents and Equipment

- Stable GS selected pools
- GS Amplification Medium (EX-CELL® CD CHO Fusion Medium ***without*** L-glutamine + 25µM MSX)
- T-75cm^2 suspension cell treated culture flasks
- Automated cell counter or hemocytometer
- 15ml sterile conical tubes
- Sterile pipettes
- Centrifuge
- Biological safety cabinet (Class II; Type A2; ISO 5)
- CO_2 incubator (5% CO_2, 37°C, humidified)
- L-methionine sulfoximine (MSX) M5379 or 76078 (cGMP grade)

  Note: MSX may be hazardous if inhaled or ingested. Use standard hazardous material handling precautions when preparing and using concentrated stocks.
- 70% isopropanol

Procedure

i) After the pools have recovered from the no-glutamine selection, aseptically remove a cell culture sample and count by trypan blue exclusion using a hemocytometer or an automated cell counter.

ii) Transfer 5x10^6 cells from the stock culture to a sterile 15ml conical tube.

iii) Centrifuge at 220 rcf for 5 minutes at 15C to 25C.

iv) Carefully aspirate off the supernatant taking care not to disturb the cell pellet.

v) Re-suspend the cell pellet in 10 ml EX-CELL® CD CHO Fusion with 25uM MSX (GS Amplification Medium, see table below).

GS Amplification Medium

Material	Product Number (Sigma)	Final Concentration
EX-CELL® CD CHO Fusion Medium	14365 C	1X

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L-methionine slufoximine (MSX)	M5379 or 76078 (cGMP grade)	25µM

vi) Place the re-suspended cells in a T-75cm^2 flask (10ml).

vii) Aseptically remove a small volume from the flask and count by trypan blue exclusion using a hemocytometer or an automated cell counter.

viii) Perform a complete medium exchange on the pool once a week.

a. Transfer the entire cell culture from the T-75cm^2 flask to a sterile 15mL conical centrifuge tube.

b. Pellet the cells at 220 rcf for 5 minutes at room temperature.

c. Carefully remove the supernatant taking care not to disturb the cell pellet.

d. Re-suspend the cell pellet in 10-12mL of fresh GS Amplification Medium, and transfer the re-suspended cells back into the original flask.

ix) Count the pool twice weekly.

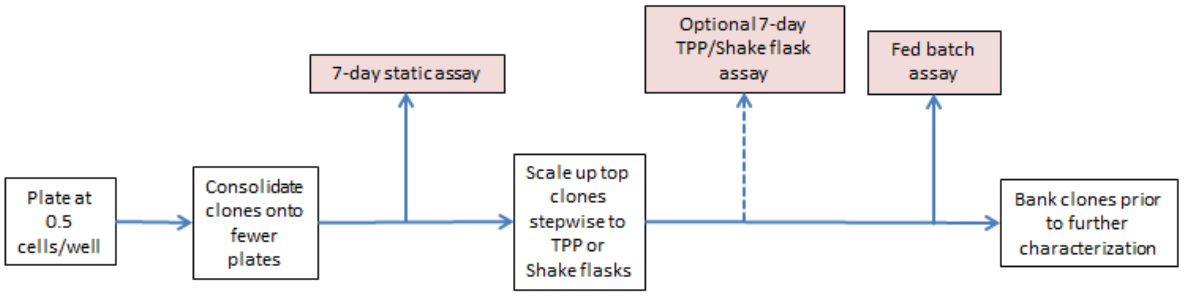
x) After the pool recovers from MSX selection (viability >90% and stabilized doubling time, approximately 10-21 days post transfection), passage the pool to 0.3x10^6 viable cells/ml twice weekly. The stable pools may be scaled up to shake flasks for further characterization at this point.

xi) Bank the top stable pools following the protocol in Part IV substituting GS Amplification Medium for the GS Growth Medium.

Part IX: Single Cell Clone (SCC) Isolation from Stable Pools by Limiting Dilution and Subsequent Scale Up

Purpose

Once a stable pool has been generated, the pool can be single cell cloned to isolate high producing clones. If minipools were generated, we recommend single cell cloning from the top two or more producing pools.



Reagents and Equipment

- GS stable pool(s)
- 96-well suspension cell culture plates

- EX-CELL® CHO Cloning Medium
- Conditioned Medium from stable pool (see Part X)
- GS Selection Medium (EX-CELL® CD CHO Fusion Medium ***without*** L-glutamine)
- Multichannel micropipetter and sterile tips
- Sterile pipettes
- Biological safety cabinet (Class II; Type A2; ISO 5)
- CO_2 incubator (5% CO_2, 37°C, humidified)
- Centrifuge
- Automated cell counter or hemocytometer
- 70% isopropanol

Procedure

i) Plate the cells

a. Determine the number of 96-well plates to be plated. This number will be dependent upon the number of clones desired. Approximately 10-40 clones per plate can be expected using this protocol.

b. Aseptically remove a cell culture sample from the stable pool stock culture and count by trypan blue exclusion using a hemocytometer or an automated cell counter.

c. Calculate the volume of cell culture and medium needed for plating. Each well will contain 200µL and 0.5 cells per well (final concentration 2.5 cells/mL).

d. Make the appropriate volume of Clone Plating Medium: 80% EX-CELL® CHO Cloning Medium + 20% Conditioned Medium (see Part X).

Clones Plating Medium

Material	Product Number (Sigma)	Final Concentration
Conditioned Medium	(see Part X)	20%
EX-CELL® Cloning Medium	C6366	80%

e. Remove the appropriate volume of cells from the stable pool stock culture and inoculate into the appropriate amount of Clone Plating Medium, achieving a final cell density of 2.5 cells/mL. It may be necessary to achieve this concentration through serial dilutions of the cells.

f. Plate the diluted cells into 96-well plates (200µl per well) using a multi-channel pipetter.

g. Place the plates in the incubator (37C, 5% CO_2, humidity 80%). For maximal cloning efficiency, do not remove the plates from the incubator until plates are ready to be screened (around day 6 or 7 post plating).

ii) Screen for single cell clones

a. Around day 6 or 7, and day 14 post-plating, visualize all wells under a microscope, checking for wells with single colonies (clonal). Feed with 20µL of GS Selection Medium (EX-CELL® CD CHO Fusion ***without*** L-glutamine) on both of these days.

b. Track all wells determined to be clonal.

c. When the clones are 70-100% confluent. Consolidate the clones onto fewer plates for ease of screening.

iii) Titer screening method (OPTIONAL). This screen can be used to identify the top producing clones on in the scale up process in order to reduce the total number of clones scaled up into shake cultures. If it is not necessary to reduce the number of clones taken to the next step then proceed to step xv. This static 7-day assay does not predict the rank order of the clones, but can be used to determine the top 20-30% of clones to scale up for further analysis.

a. Once the cells in the second passage plate are 80% confluent, split them 1:5 into 200µl of selection medium into two new 96-well plates. One plate will be a propagation plate, and the other plate will be used for a terminal assay to screen the minipools for productivity.

b. Passage the propagation plate as described above, splitting the cells with a 1:5 dilution when they become 80% confluent, 1-2 times per week.

c. Incubate the terminal assay plate for 7 days, and harvest supernatants for titer quantitation (ForteBio or similar).

iv.) Scale up and characterize the selected clones (please follow the scale-up and characterization procedures detailed in Part VII)

v.) Once the top clones have been identified via the screening outlined in section X, Bank single cells clones via cryopreservation following the procedure described in Part IV, substituting GS Selection Medium (EX-CELL® CD CHO Fusion ***without*** L-glutamine) for GS Growth Medium.

a. Note: In order to maintain selection pressure, use GS Selection Medium (EX-CELL® CD CHO Fusion without glutamine) to expand production clones prior to banking.

Part X: Conditioned Medium Production for Single Cell Cloning

Purpose

This protocol describes procedures for the production of conditioned cell culture medium for single cell cloning by limiting dilution. Conditioned medium has been shown to be beneficial in single cell cloning processes by providing the clones with the beneficial nutrients and/or factors that are secreted by exponentially growing healthy cells.

Reagents and Equipment

- Cells: Stock culture from a GS stable transfectant pool (in exponential growth phase)
- GS Selection Medium (EX-CELL® CD CHO Fusion Medium ***without*** L-glutamine)
- Sterile shaker flask (determine appropriate volume)
- Sterile centrifuge tubes (determine appropriate volume)
- Sterile 0.2µm Millipore Steriflip® filter apparatus or equivalent
- Sterile pipettes
- Water bath at 37C
- Centrifuge
- Automated cell counter or hemocytometer

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- Biological safety cabinet (Class II; Type A2; ISO 5)
- CO_2 incubator (5% CO_2, 37°C, humidified)
- Orbital shake plate
- 70% isopropanol

Procedure

i) Set-up of production culture

a. Determine desired volume of production culture and prepare an appropriately size shaker flask.

b. Aseptically remove a cell culture sample from the stable pool stock culture and count by trypan blue exclusion using a hemocytometer or an automated cell counter.

c. Calculate amount of cells required from the stock culture and the volume of fresh GS Selection Medium required to achieve an initial cell density of $1.0x10^6$ cells/mL.

d. Aseptically transfer the calculated volume of cells from the stock culture into to a sterile centrifuge tube.

e. Centrifuge cells at 220 X g for 5 minutes at 15C-20C.

f. Carefully aspirate off the supernatant ***without*** disturbing the cell pellet.

g. Gently re-suspend the cell pellet in the appropriate volume of fresh GS Selection Medium.

h. Aseptically remove a cell culture sample from the production culture and count by trypan blue exclusion using a hemocytometer or an automated cell counter.

i. The initial viable cell density should be 0.9-1.2 x 10^6 cells/mL.

j. Place the culture in the incubator (37C, 5% CO_2, 80% humidity) on an orbital shaker set at 125 rpm, for 24 hours (+/- 4 hours).

ii) Production Culture Harvest (24 hours post culture initiation)

a. Aseptically remove a sample from the conditioned medium production culture and count by trypan blue exclusion using a hemocytometer or an automated cell counter.

b. Transfer culture to centrifuge tube(s).

c. Centrifuge at 2440 X g for 5 minutes.

d. Transfer the clarified medium (conditioned medium) to sterile 50mL conical tube(s). Be careful not to disturb cell pellet.

e. Filter the clarified medium using a 0.2µm Steriflip® filter device or equivalent.

f. Label filtered conditioned medium with Cell Line ID, “Conditioned Medium”, and expiration date (one week from the harvest date).

iii) Storage

a. Conditioned medium may be stored for up to 7 days at 2-8C.

b. Do NOT freeze conditioned medium.

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Part XI: Fed Batch Assay

Purpose

This section describes the basic Fed Batch assay technique. This assay can be used to screen minipools and clones (see sections VII and IX). The parameters described in this assay are to be used as a basic guideline; further optimization may benefit some clones or minipools.

Reagents and Equipment

- Cells: Stock culture from a GS stable clones (in exponential growth phase)
- GS Selection Medium (EX-CELL® CD CHO Fusion Medium ***without*** L-glutamine)
- EX-CELL® Advanced™ CHO Fed Batch Medium **(**14366C/24366C)
- EX-CELL® Advanced™ Feed 1 (24367C/24368C)
- D-(+)-Glucose (45% solution) Sigma-G8769
- Antibody quantitation system (Forte´Bio® Octet or equivalent)
- Sterile shaker flasks (determine appropriate volume)
- Sterile centrifuge tubes (determine appropriate volume)
- Sterile pipettes
- Centrifuge
- Automated cell counter or hemocytometer
- Biological safety cabinet (Class II; Type A2; ISO 5)
- CO_2 incubator (5% CO_2, 37C, humidified)
- Orbital shake plate
- 70% isopropanol

Procedure

Fed Batch Assay

Clones and minipools can be screened for r-protein production via a Fed Batch assay. This assay is performed using the EX-CELL® Advanced™ CHO Fed Batch medium and feed system. No adaptation is required.
A titration (2.5-10%) of EX-CELL® Advanced™ CHO Feed 1 is strongly recommended to determine the optimal concentration for the specific process. For best results, it is recommended to initiate feeding only after reaching mid to late exponential phase.

i.). Once the cells (clones or stable pools) have been scaled up and adapted into suspension culture, inoculate 30ml of EX-CELL Advanced CHO Fed-batch Medium (14366C) in a 125 mL Erlenmeyer shake flask or TPP® TubeSpin tube at an initial starting cell density of $0.3x10^6$ cells/ml.

ii.). Feed cultures by aseptic addition of both sterile glucose (G8769) up to 4g/L of glucose (final concentration) and 5% of starting volume with hydrated EX-CELL Advanced CHO Feed 1 (see above) on days 3, 5, 7, 9 and 11 post-inoculation.

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iii.)Monitor growth and productivity characteristics regularly during the course of the assay
a.) Harvest a small sample of cell culture to count via automated cell counter
b.) Harvest a small sample of cell culture, pellet cells via centrifugation, and analyze supernatant via inferometry, HPLC or ELISA

iv.) Terminate the assay once the viability of the cultures drops below 70%.

Part XII: Bench-top Bioreactor Scale Protein Production

Purpose

Once the top 2 or 3 clones have been identified, the performance of each clone in a controlled environment can be determined by using microbioreactors or traditional bench-top bioreactors.

The valuation of 2 or 3 clones is recommended, especially when a specific protein profile characteristic or a specific process is desired.

Reagents and Equipment Specific for Bench-top Bioreactor Scale

- Cells: stock culture from a GS stable single cell isolate in exponential growth phase
- GS Selection Medium (EX-CELL® CD CHO Fusion Medium ***without*** L-glutamine)
- EX-CELL® CHOZN® Advanced Medium (liquid; ***without*** L-glutamine)
- EX-CELL® CHOZN® Advanced Feed (liquid as prepared according to the product information sheet) ***without*** L-glutamine
- D-(+)-Glucose (45% solution) (Sigma G8769)
- Bioreactor designed for animal cell culture
- Bioreactor pH Adjustment (Sigma B1185) or alternative base solutions such as sodium bicarbonate, sodium carbonate or sodium hydroxide

Procedure

* It is essential that cells are passaged during exponential growth phase. Days to passage can be modified by varying the seeding density.

Small Scale (Shake Flask Scale-up Method)

For the inoculation of microbioreactors or a small number of small scale bioreactors scaling up the cells in shake flasks can be a suitable strategy. Table x shows the recommended working volumes for different flask sizes.

1. Thaw a vial from the clone bank following procedure described in Part 2 (substitute GS Selection Medium: EX-CELL® CD CHO Fusion ***without*** L-glutamine for Growth Medium).

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2. As soon as the cell line is in suspension, start scaling up cells in shake flasks by seeding the cultures at 0.3-0.4x10^{6}vc/mL in GS Selection Medium (EX-CELL® CD CHO Fusion ***without*** L-glutamine). Increase the shake flask size with each passage, following the proportions indicated in the following table.

Recommended Scale Up Strategy in Shake Flasks

Culture flask size	Product Number	Medium Volume	Seed density	Amount of days normally required
125ml Erlenmeyer flask	431143 (Corning)	20-30 ml	0.3x10^{6}vc/mL	3-4 days*
250mL Erlenmeyer flask	431144 (Corning)	50-100mL	0.3x10^{6}vc/mL	3-4 days*
500mL Erlenmeyer flask	431145 (Corning)	150-200mL	0.3x10^{6}vc/mL	3-4 days*
1000mL Erlenmeyer flask	431147 (Corning)	300-400mL	0.3x10^{6}vc/mL	3-4 days*

Large Scale (Wave Bag Scale-up Method)

For the inoculation of larger size bioreactors or multiple small size bioreactors, scaling cells up in a rocking bag system may be a better option than using multiple shake flasks.
CHOZN® cells can be grown in rocking bags following standard recommendations from the bioreactor bag vendor. An example of a process that can be used with rocking bag systems can be found on following table.

1. Thaw a vial from the clone bank following procedure described in Part 2 (substitute GS Selection Medium for Growth Medium).
2. As soon as the cell line is in suspension, start scaling up cells in shake flasks by seeding the cultures at 0.3-0.4x10^{6}vc/mL in GS Selection Medium (EX-CELL® CD CHO Fusion ***without*** L-glutamine). Increase the shake flask size with each passage, following the proportions indicated in the following table.
3. Once the cells have been in suspension culture for at least 3 passages, the rocking system can be inoculated using GS Selection Medium (EX-CELL® CD CHO Fusion ***without*** L-glutamine) and seeding it at 0.3-0.4x10^{6}vc/mL. Inoculate the bag at a low volume (follow vendor instructions for minimum working volume). While the cells are still in exponential growth phase, similarly to the process followed in shake flasks, dilute the cell concentration down to 0.3-0.5x10^{6}vc/mL by adding fresh GS Selection Medium (EX-CELL® CD CHO Fusion ***without*** L-glutamine) to the bag. Increase the rocking speed as the working volume in the bag is increased. During this process, always maintain cells in exponential growth phase. Repeat this process until the require volume to inoculate the production bioreactor/s is reached.

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Recommended Parameters for Wave Bag Scale-up Method

Parameter	Application
Working volume	1-10L in 20L bags
Temperature	37°C
Rocking angle	8°
Rocking speed	10-25rpm
Gas flow rates	100ccm air + 5ccm CO_2

Production Protocols: Fed-Batch Production Culture Assay (with EX-CELL® CHOZN® Advanced Medium and Advanced Feed) in Bench-top Scale Bioreactors

1. Inoculate bioreactor by seeding cells directly into EX-CELL® CHOZN® Advanced medium at the desired inoculation density. Typical inoculation densities for a production bioreactor are 0.3 – 1x10^6vc/mL. Higher inoculation densities are suitable, however, it is not recommended for the inoculum to be more than 20% of the initial working volume. Initial working volume should be set between 60-80% of final working volume.
2. Bioreactor parameters such as pH, temperature and DO may need to be optimized for individual clones. Other bioreactor parameters such as agitation speed or gas flow rate will vary depending on the specific design of the bioreactors. The following table shows an example of parameters used with CHOZN® clones as a reference for a starting process.

   Note: Cells expanded in the EX-CELL® CD CHO Fusion medium can be inoculated directly in the production medium EX-CELL® CHOZN® Advanced Medium without any adaptation.
3. Maintain glucose above 2g/L by supplementing appropriate volumes to increase concentration up to 6g/L.
4. Add 5-15% EX-CELL® CHOZN® Advanced Feed starting at 2x10^6vc/mL or day 3, whichever comes first. Continue adding feed every other day until the end of the run.
5. Collect samples as appropriate for metabolite, production and product quality analysis.

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Parameter		Vessel 1.2L	Vessel 5.5L
Working volume		0.8-1L	4-5L
Agitation	Impeller	Pitched 48mm	Pitched/Marine 70mm
	Speed	150-300rpm 0.4-0.7m/s	150-250rpm 0.6-1m/s
	Power input	8-67W/m^3	21-124W/m^3
Aeration	Sparger	Open tube 0.5mm	Ring sparger 100um
	Gasses sparged	Air, oxygen, CO_2	Oxygen/CO_2
	Flow rates sparger	0.1-0.15vvm (Open pipe)	0.05-0.1vvd (Ring)
	Gasses overlay	Air	Air
	Flow rates overlay	0.01vvm	0.01vvd