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Technical Bulletin

CHOZN® ZFN-Modified DHFR$^{-/-}$ CHO Cell Line

CATALOG NO. CHODHFR

Product description/overview

The CHOZN ZFN-Modified DHFR$^{-/-}$ CHO cell line was created using Sigma's proprietary CompoZr® zinc finger nuclease (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 heal 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.

The dihydrofolate reductase (DHFR) protein catalyzes the reduction of 5,6-dihydrofolate to 5,6,7,8-tetrahydrofolate, an essential step in purine metabolism. Cells lacking a functional copy of the DHFR gene must be grown in culture medium supplemented with the purine precursors hypoxanthine and thymidine (HT). This metabolic selection process can be used to select for cells that have been transfected with a gene(s) of interest for recombinant protein expression along with an exogenous copy of the DHFR gene. Methotrexate (MTX) strengthens the selection process by inhibiting DHFR activity, thereby pushing the cells to express more DHFR and consequently more recombinant protein, presumably through gene amplification. Currently, the biopharmaceutical industry uses DuxB11 and DG44 host cells for DHFR selection of recombinant cell lines. In DG44 cells, both copies of the DHFR gene non-functional, enabling strong metabolic selection of recombinant cell lines. DuxB11 cells, however, have one functional and one-non functional copy of the DHFR gene. Therefore, DHFR metabolic selection strategies for recombinant cell line are less effective when using this host. These traditional DHFR deficient cell lines display some unfavorable growth and handling characteristics. They are known to be difficult to adapt to serum-free suspension growth conditions, and tend to be very clumpy in shaken cultures. For these reasons, SAFC developed a more robust, user friendly CHO DHFR deficient host cell line. Using the ZFN technology, SAFC has engineered a novel CHO K1 DHFR$^{-/-}$ cell line. This CHOZN DHFR null cell line is adapted to chemically-defined, suspension growth in EX-CELL CD CHO Fusion media (SAFC Cat. No. 14365C) and maintains many of the robust characteristics of CHO K1.

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Precautions and disclaimer

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

Storage and stability

Store the cells in the vapor phase of liquid nitrogen immediately upon arrival.

Kit components

Component	Cat. No.	Quantity
CHOZFN DHFR$^{-/-}$ cell line	CHODHFR-1VL	1 Vial of cells with >7.5e6 cells/mL

Background: CHOZN DHFR$^{-/-}$ cell line

The starting cell line was ECACC CHO K1 that had been adapted to suspension growth in an SAFC chemically defined (CD) media formulation (EX-CELL® CD CHO Fusion, Cat. No.14365C). The cell line was first transfected with ZFN pair 9461/9684 (target sequence cggagaccttccctggccaatgctcaggtactg). The transfected pool was single cell cloned, and the clones were screened for mutations at the ZFN target site. A heterozygous clone with a 7bp deletion on one of the alleles was isolated. This CHO DHFR$^{-/+}$ clone was then scaled up and re-transfected with DHFR ZFNs in order to target the remaining wild type allele and generate a DHFR KO cell line. In the second transfection, two different ZFN pairs targeting the DHFR gene, 9461/9684 and 9477/9476 (target sequence ccacccccggacttgcatgggtagccgct), were used. The binding sites for these two pairs of ZFNs are separated by 206 base pairs on the genomic DNA (see figure 1). ZFN activity by both of these ZFN pairs on the wild type would result in a deletion of close to this size, therefore simplifying the KO screening process. The transfected pool was single cell cloned, and the clones were screened for this deletion in the DHFR gene. A clone was isolated that contained the original 7bp deletion on one allele resulting from the first ZFN transfection and a 235bp deletion on the second allele resulting from the second ZFN transfection.

DHFR Gene Structure and ZFN Target Regions

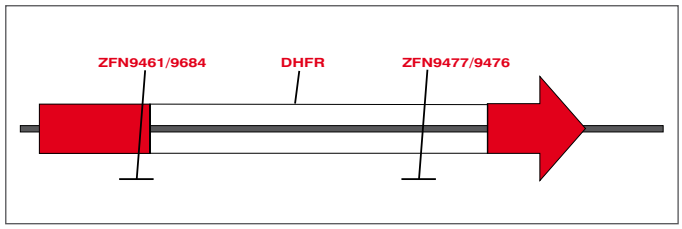

Figure 1. This is a schematic representation of the CHO DHFR gene. The ZFN target sites are indicated. The ZFN9461/9684 pair target site is located in exon 1, whereas the ZFN9477/9476 pair target site is located downstream in intron 1. The predicted ZFN cut sites are approximately 230bp apart.

EX-CELL CD CHO Fusion media

EX-CELL CD CHO Fusion is a chemically defined, animal-component free medium developed for the long-term growth of Chinese Hamster Ovary (CHO) cells. The absence of any large macromolecules allows for isolation and purification of secreted proteins from the cells. This medium does not contain hypoxanthine or thymidine to allow for its use with dihydrofolate reductase (DHFR) gene amplification systems.

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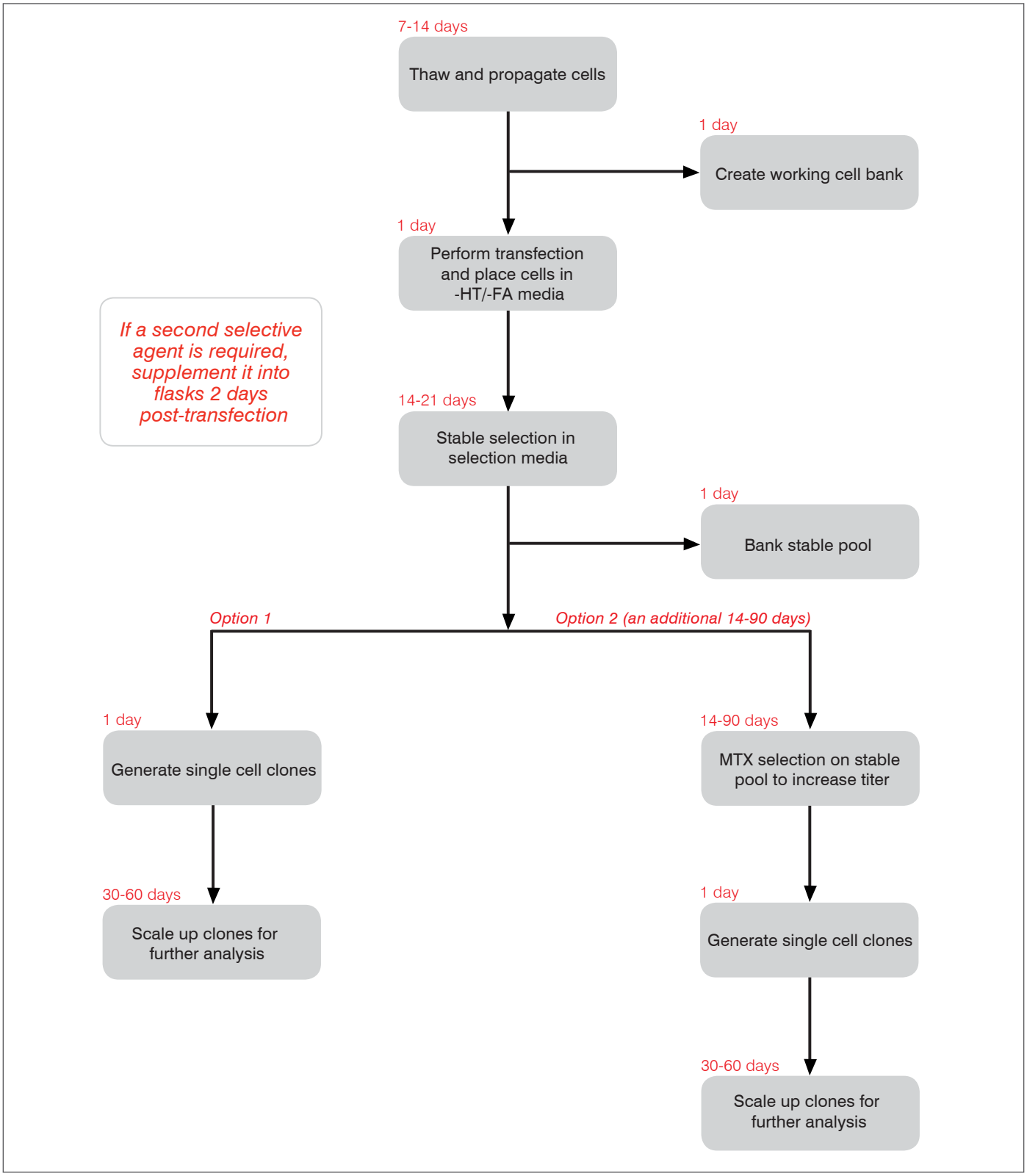
Experimental flow chart



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Cell culture reagents needed

Cell culture reagents	Manufacturer	Cat. No.
CHOZN® DHFR-/- cells	SAFC	CHODHFR
EX-CELL® CD CHO Fusion	SAFC	14365C
L-Glutamine (200mM)	Sigma	G7513
50X HT Media Supplement	Sigma	H0137
CHO Cloning Media	Sigma	C6366
Folinic Acid	Sigma	F7878
Pluronic F68	Sigma	P5556
Dextran Sulfate	Sigma	D6924
DMSO	Sigma	D2650
MTX (optional)	Sigma	M8407
Folinic Acid	Sigma	F7878
Pluronic F68	Sigma	P5556
Dextran Sulfate	Sigma	D6924
7% DMSO	Sigma	D2650
MTX (optional)	Sigma	M8407

Equipment/materials needed

- Water bath at 37 ˚C
- T-75 cell culture flask
- Microcentrifuge tubes
- Sterile pipettes
- Biosafety cabinet
- T-25 cell culture flask
- Plasmid DNA
- Micro pipetter and sterile tips
- Centrifuge
- 15 mL sterile conical tube
- Electroporation cuvettes
- Sterile cryovials
- CO_2 incubator
- 50 mL sterile conical tube
- Electroporator
- Cryovial labels (LN2 resistent)
- Automated cell counter or hemocytometer
- 125 mL sterile shake flask
- -80 ˚C Freezer
- Isopropyl alcohol
- Orbital shaker plate
- 96-well tissue culture plates
- Liquid nitrogen freezer boxes
- Liquid nitrogen freezer

Protocols

NOTE: The following procedures are performed only by personnel trained to:

- *Work with biohazardous materials*
- *Use Universal Precautions*
- *Use aseptic technique*

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Part I: Stock culture initiation and maintenance

Purpose

This protocol describes procedures for the initiation of CHOZN® DHFR$^{-/-}$ cell culture.

Reagents and equipment

- T-75 cell culture flask
- 15 mL sterile conical tube
- 125 mL sterile shake flask
- Sterile pipettes
- Frozen vial containing CHOZN DHFR$^{-/-}$ cells (SAFC Cat. No. CHODHFR-1VL)
- Cell culture media (SAFC's EX-CELL® CD CHO Fusion media, Cat. No. 14365C)
- Cell culture media supplements (L-glutamine, hypoxanthine/thymidine and folinic acid- see below)
- Water bath at 37 ˚C
- Biosafety cabinet
- Centrifuge
- CO_2 incubator
- Automated cell counter or hemocytometer
- Orbital shaker plate

Procedure

Media preparation

The following procedure is for the preparation of 1 liter of complete growth media for CHOZN DHFR$^{-/-}$ parental cell line.

Growth media for CHODHFR parental cells

Product	Cat. No. (Sigma)	Final concentration
EXCELL CD CHO Fusion	14365C	1 x
L-Glutamine	G7513	4 mM
HT	H0137	1 x
Folinic Acid	F7878	2 uM

- Prepare supplements
  - HT: Add 10 mL of EX-CELL CD CHO Fusion to each vial of HT (2 vials needed for 1L of growth media). This creates a 50X solution. Discard any unused resuspended HT.
  - Folinic Acid: Resuspend folinic acid in sterile water to create a 1 mM stock solution. Sterile filter. Make 1 mL aliquots and store at -20 °C. Thaw prior to use.
  - Glutamine: Thaw glutamine in 37 °C water bath until completely dissolved. After thawing, glutamine may be stored at 4 °C for up to 2 weeks.
- Add media supplements to EX-CELL CD CHO Fusion to achieve the final concentrations listed in the table above.
- Filter sterilize the complete growth media.
- Store growth media at 4 ˚C when not in use. Discard any unused media after 1 month.

Vial thaw for stock culture initiation and maintenance

- Adjust incubator settings to 37 ˚C and 5% CO_2.
- Prepare growth media as described above.
- Obtain sterile T-75 cell culture flask.
- Obtain 15 mL conical tube. Place 8 mL prepared media in conical.
- Obtain frozen vial from LN2 freezer.
- Immediately thaw vial by gently swirling in 37 °C water bath until just thawed.
- Spray the vial liberally with 70% isopropyl alcohol and place the vial in the biosafety cabinet.
- Transfer cells to the 15 mL conical containing fresh growth media.
- Centrifuge cell suspension for 5 minutes at 220 X g.
- Remove supernatant without disturbing the cell pellet.
- Add 10 mL fresh growth media to suspension and mix gently by pipetting.
- Transfer suspension to T-75 cell culture flask.
- 24 hours post-thaw, remove a cell culture sample and count the cells by trypan blue exclusion.
- If culture viability is >80%, then passage into subculture 1 (see below). If culture viability is <80%, then leave in static T-75 culture. Cells may remain in static culture for up to 3 days post-thaw.

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Subculture 1

- Set agitation on orbital shaker to 125 rpm.
- Remove cell culture sample and count by trypan blue exclusion.
- Transfer the entire culture from the T-75 flask to a 125 mL shake flask.
- Transfer 10 mL fresh pre-warmed growth media to 125 mL shake flask, incubate on the orbital shaker.
- Maintain cultures in 37 ˚C and 5% CO_2 incubator.

Stock maintenance (every 3-4 days)

- Remove cell culture sample and count by trypan blue exclusion.
- Determine volume of cells needed to inoculate a new culture at 300,000 cells/mL.
- Transfer the appropriate volume of cells from the existing stock culture to a new flask, and add pre-warmed growth media to bring up to the desired culture volume.

Part II: Cell Banking

Purpose

This protocol details procedures for establishing a working cell bank of CHOZN® DHFR$^{-/-}$ cells.

Materials and equipment

- Stock cell culture
- Freezing media components (EX-CELL® CD CHO Fusion (SAFC Cat. No. 14365C) + 7% DMSO (Sigma-Aldrich Cat. No. D2650)
- Sterile cryovials
- Cryovial labels (must be LN2 resistant)
- Sterile centrifuge tubes
- Sterile pipettes
- Centrifuge
- Freezer buddy or controlled rate freezer
- 70% isopropanol
- -80 °C freezer
- LN2 freezer boxes
- LN2 freezer

Procedure

- If using freezer buddies, fill with fresh 70% isopropanol.
- Label cryovials.
- Prepare freezing media: Growth Media + 7% DMSO.
- Remove 600 µL sample from stock cell culture and count by trypan blue exclusion.
- Calculate the volume of cell stock and freezing media needed to obtain a $5x10^6$ to $5x10^6$ cells per mL (cryovial)

Once cell preparation is initiated, work must proceed quickly to get vials into freezer. It is recommended that the total time from removing cells from the stock culture to transferring to the freezer does not exceed 30 minutes.

- Transfer calculated volume of stock culture to appropriately sized centrifuge tube.
- Centrifuge at 220 X g for 5 minutes at room temperature.
- Carefully aspirate supernatant.
- Gently resuspend pelleted cells with calculated volume of freezing media. Mix thoroughly by pipetting.
- Immediately aliquot cell suspension to labeled cryovials. Cap tightly.
- Quickly transfer the vials to prepared freezer buddies and store in -80 ºC freezer or transfer vials to a controlled rate freezer for overnight freezing.
- Transfer vials to LN2 freezer within 18-72 hours of freezing.

Part III: Transfection and selection of stable pools

Purpose

This protocol describes procedures for transfection of the CHOZN® DHFR$^{-/-}$ cell line and the subsequent selection of the transfected pool.

For successful selection of stable pools using the DHFR system, the expression plasmid must contain an exogenous DHFR expression cassette.

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Materials and equipment

- Stock cell culture
- T-25 cell culture flask
- 15 mL sterile conical tube
- Sterile pipettes
- Cell culture media (SAFC EX-CELL® CD CHO Fusion, Cat. No. 14365C)
- Cell Culture media supplements (see table below)
- Biosafety cabinet
- Centrifuge
- CO_2 incubator
- Automated cell counter or hemocytometer
- Orbital shaker plate
- Electroporation cuvettes (Sigma-Aldrich Cat. No. Z706094; 0.4 cm gap width)
- Eppendorf 1.5 mL microcentrifuge tubes
- Plasmid DNA (30-50 µg per transfection)

Procedure

Electroporation

The day before transfection, pass stock cell culture to 500,000 cells/mL. Prepare 500 mL transfection/selection/recovery media with the following reagents.

CHODHFR transfection, selection and recovery media

Product	Cat. No. (Sigma)	Final concentration
EXCELL CD CHO Fusion	14365C	1 x
L-Glutamine	G7513	4 mM

- Label the appropriate number of T-25 cell culture flasks (one per electroporation), and add 5 mL transfection/selection/recovery media to each.
- Label and place electroporation cuvettes (one per electroporation) on ice to chill.
- Label sterile microcentrifuge tubes for mixing cell suspensions with DNA.
- Transfer 30-50 µg of sterile plasmid DNA to each microcentrifuge tube
- Prepare cells for electroporation as follows:
  - Count stock cell culture by trypan blue exclusion and determine the appropriate volume to pellet (6.25 million cells per transfection).
  - Remove calculated volume of stock and place in a 15 mL sterile conical tube.
  - Centrifuge at 220 X g for 5 min at room temperature.
  - Carefully aspirate supernatant from pellet.
  - Resuspend cell pellet in the transfection/recovery media (1 mL media per transfection).
- For each electroporation, mix 0.8 mL of cells with the plasmid DNA in the microcentrifuge tube.
- Transfer the DNA/cell mix to the chilled electroporation cuvette, and electroporate using the following settings:

Electroporation settings

Voltage	Capacitance	
300 V	950 uF	Exponential decay

- Transfer approximately 0.6 mL of each electroporation condition (do not transfer the white cell debris) to the 5 mL transfection/recovery media in the prepared T-25 cell culture flask.
- 2-4 hours post –transfection, count the cells by trypan blue exclusion and record the culture viability.
- Incubate the T-25 flasks for 48 hours +/- 4 hrs at 37 °C and 5% CO_2.

Selection and recovery of stable pools

Note: The transfection/recovery media immediately places the cells under selection, as it does not contain HT. If a double vector expression system is being used, such as is commonly used for two-subunit recombinant protein expression, then an additional selective agent is recommended to select for cells expressing elements from both vectors. It is recommended that the second selective agent be added to the selection media 48 hours post-transfection.

- On day 2 post-transfection
  - Count cells by trypan blue exclusion
  - Pellet all cells and resuspend in 5 mL of fresh selection media.
  - Pipette cells back into the original T-25 cell culture flask.
- Once per week, count the cells, pellet and resuspend in fresh selection media. Transfer the suspension back into the original flask.
- Once the cells are >60% viable and over 0.5 x 10^6 cells/mL, place the cells in 10 mL media in a T-75 cell culture flask.

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- After the cells have grown to at least 1 x 10^6 cells/mL and are >80% viable, transfer cells into 20 mL selection media supplemented with 1500 ug/mL Pluronic F-68 and 200 ug/mL dextran sulfate (see formulation below) and place in a 125 mL shake flask. Shake cultures at 120-130 rpm in 37 °C CO_2 incubator.

Note: It is recommended that the stable pools are banked at both the T-75 stage and also following the first subculture in shake flasks

Growth media for stable pools in shake flasks

Product	Cat. No. (Sigma)	Final concentration
EX-CELL® CD CHO Fusion	14365C	1 x
L-Glutamine	G7513	4 mM
Pluronic F-68	P5556	1500 µg/mL
Dextran sulfate	D6924	200 µg/mL
Second selective agent if required		

Gene amplification by MTX (optional)

MTX can be used to amplify the gene copy number of DHFR, which often results in co-amplification of the transgene(s) for the recombinant protein of interest and may enhance overall protein productivity. MTX amplification can be done in a single round or in multiple rounds by progressively increasing the concentration of MTX added to the selection media. Recommended concentrations of MTX for sequential rounds of amplification are 50 nM, 100 nM, 250 nM, 500 nM, 1 µM, and 2 µM.

- Remove cell culture sample from the stable pool and count by trypan blue exclusion.
- Determine volume of cells needed to inoculate a new culture at 300,000-500,000 cells/mL.
- Remove calculated volume of stock and place in a 15 mL sterile conical tube.
- Centrifuge at 220 X g for 5 min at room temperature.
- Carefully aspirate supernatant from pellet.
- Resuspend cell pellet in growth media containing the appropriate concentration of MTX and transfer to a shake flask containing the appropriate volume of growth media containing MTX (recommended 20-30 mL in a 125 mL shake flask).
- Every three to four days, perform a complete media change on the culture by centrifuging the cells, aspirating off the spent media, and resuspending in fresh growth media containing the appropriate concentration of MTX.
- Once the culture viability is >90%, recombinant protein productivity should be assessed.
- Proceed with additional rounds of MTX amplification or single cell cloning.

Note: It is recommended that the stable pools are banked after recovery from each round of MTX amplification.

Part IV: Conditioned media production for single cell cloning

Purpose

This protocol describes procedures for the production of conditioned cell culture media for single cell cloning by limiting dilution.

Studies have shown that the addition of conditioned media to cloning media enhances the outgrowth of many CHO cell lines from low-density cell culture.

Materials and equipment

- Sterile shake flask (determine appropriate volume)
- Sterile centrifuge tubes (determine appropriate volume)
- Sterile 0.2 µm Steriflip® filter apparatus
- Sterile pipettes
- Stock culture of stable pool d3 or d4 following last passage (exponential growth phase)
- Cell culture media appropriate for cell line
- Water bath at 37 °C
- Centrifuge
- Biosafety cabinet
- Incubator
- Orbital shake plate

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Set-up of culture for conditioned media production

- Determine the necessary volume of conditioned media and prepare an appropriate size shake flask.
- Remove cell culture sample and count by trypan blue exclusion.
- Calculate amount of stock culture and fresh media required to achieve a final density of 1.0 x 10^6 cells/mL in desired volume of production culture.
- Transfer calculated volume of inoculum to centrifuge tube.
- Centrifuge at 220 X g for 5 min.
- Remove supernatant without disturbing the cell pellet.
- Resuspend gently with calculated volume of fresh media.
- Count the conditioned media production culture following inoculation.
- Viable cell density must be 0.9-1.2 x 10^6 cells/mL.
- Incubate at conditions appropriate for cell line.
- Production culture will be harvested after 24 hours (+/- 4 hours).

Conditioned media harvest

- Count the culture by trypan blue exclusion and record results.
- Transfer culture to centrifuge tube(s).
- Centrifuge at 2440 X g for 5 min.
- Transfer supernatant (conditioned media) to clean 50 mL conical tube. Be careful not to disturb cell pellet.
- Filter with 0.2 μm Steriflip® filter device.

Storage

- Conditioned media may be stored up to 7 days at 4 °C.
- Conditioned media may NOT be frozen.

Part V: Single cell cloning to isolate high producing clones

Purpose

This protocol describes the isolation of single cell clones from stable pools.

Materials and equipment

- Stable pool of producing cells
- 96-well tissue culture plates
- CHO Cloning media (Sigma-Aldrich Cat. No. C6366)
- Conditioned media from stable pool (See Part IV: Conditioned media protocol)
- L-glutamine (Sigma-Aldrich Cat. No. G7513)
- Cell culture media appropriate for cell line
- Biosafety cabinet
- CO_2 incubator
- Centrifuge

Procedures

Single cell cloning

- Determine the number of 96-well plates to be set up. This number will be dependent upon the number of clones desired. Approximately 10-40 clones per plate can be expected using this protocol.
- Count the stable pool by trypan blue exclusion to determine viable cell density.
- Calculate the volume of cell culture and media needed for plating.
  - 200 uL per well at 0.5 cells per well (Final concentration 2.5 cells/mL).
- Prepare the appropriate volume of plating media (see table below).
- Remove the calculated volume of cells from the transfected stock. If a very small volume of cells is required, serial dilutions can be made prior to adding the cells to the plating media.
- Add 200 μL of the cell/plating media mixture to the 96-well plates and return plates to the incubator (37 ˚C, 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).

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Plating media for limited dilution single cell cloning

Product	Cat. No. (Sigma)	Final concentration
CHO Cloning Media	C6366	80%
Conditioned Media	Prepared above	20%
L-Glutamine	G7513	4 mM

Media for feeding and scale-up of clones

Product	Cat. No. (Sigma)	Final concentration
EXCELL® CD CHO Fusion	14365C	1 x
L-Glutamine	G7513	4 mM
MTX (optional, if used pool amplification)	M8407	Concentration used for stable pool

Screening for single cell clones

- Six days post-plating, visually screen the plates under a microscope, identifying all wells with single colony outgrowth (clonal populations). Feed all clonal populations with 20 ìl of growth media (see table below) on day 6 and day 14 post-plating.
- Track all wells determined to be clonal.
- When the wells are 70-100% confluent, then scale up the clones for further characterization.

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