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User Guide

CHOZN® GS$^{-/-}$ Platform

MilliporeSigma is the U.S. and Canada Life Science business of Merck KGaA, Darmstadt, Germany.

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Contents

Overview

The CHOZN® GS$^{-/-}$ Platform is a CHO (Chinese Hamster Ovary) mammalian cell expression system for rapid selection and scale-up of stable clones producing high levels of recombinant proteins (r-proteins). Central to the CHOZN® GS$^{-/-}$ Platform is the CHOZN® GS$^{-/-}$ CHO cell line, which requires glutamine in the medium because the endogenous glutamine synthetase (GS) gene was disrupted using our proprietary CompoZr™ zinc finger nucleases (ZFN) technology. Appendix 1 has details of the generation of the CHOZN® GS$^{-/-}$ CHO cell line.

Transfection with a plasmid containing a GS transgene allows metabolic selection in glutamine-free medium throughout r-protein production. The CHOZN® Platform also includes chemically defined (CD) media for expansion and cloning, and a media/feed platform that maximizes cell growth and r-protein production.

This user guide covers the cell line development process: culturing of cells, transfection, generation and screening of mini-pools, and subsequent derivation of single cell clones that express the target r-protein. If you have any questions, contact your technical representative. Services available for cell line development are listed in Appendix 3.

Precautions and Disclaimer

The CHOZN® GS$^{-/-}$ cell line and associated media and feed are for research and development use only, not for drug, household, or other uses. For other uses, contact your local customer representative.

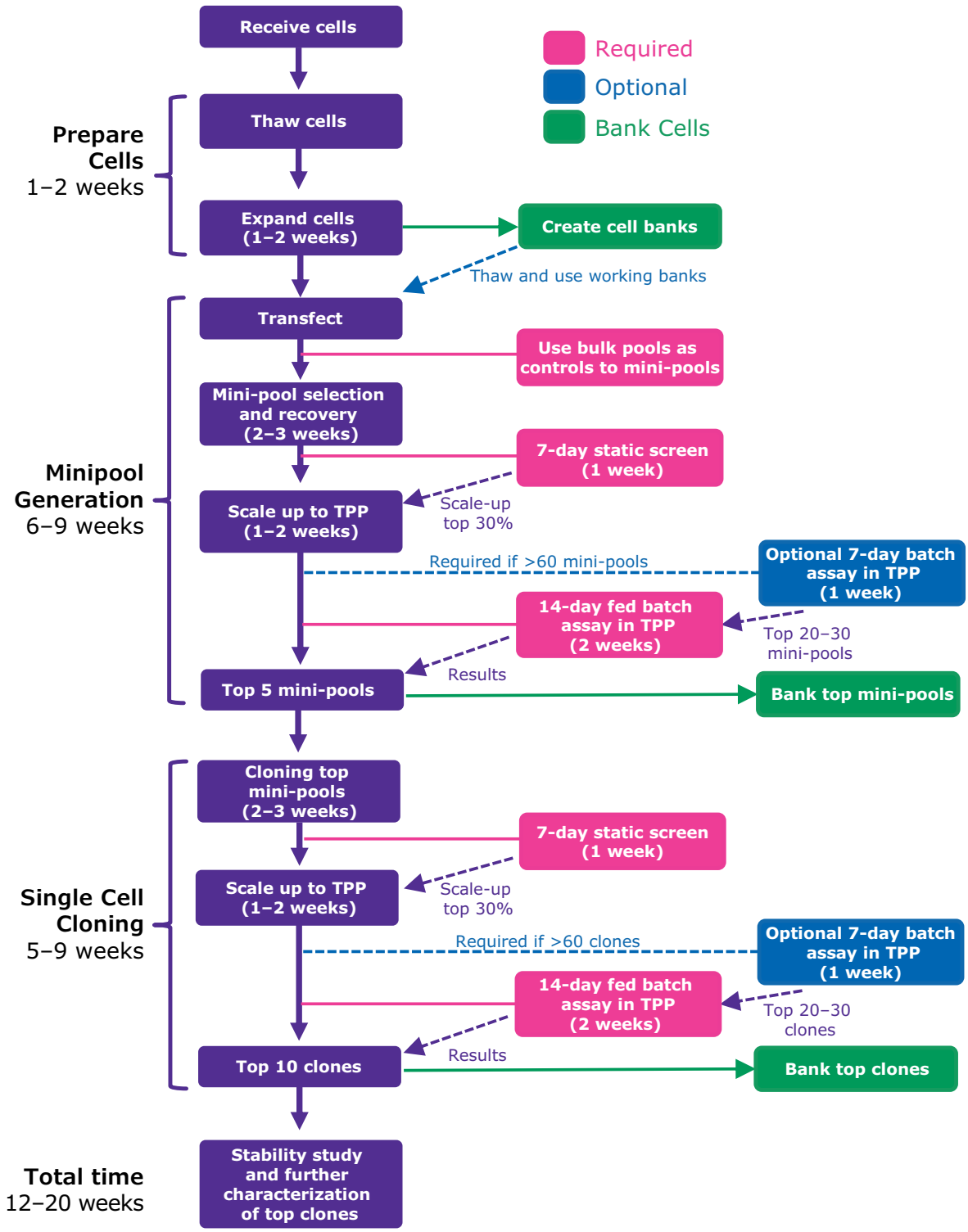
Consult the Safety Data Sheets for information regarding hazards and safe handling practices.

Storage and Stability

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

Interactive CHOZN® GS-/- Platform Flow Chart and Timeline

Click any step in flowchart below to navigate to protocol information:



Media, Feeds, Supplements and Reagents

This section describes EX-CELL® and Cellvento® media for culturing CHOZN® cells. These media have been developed for cell expansion, cell line development, and fed batch production with the CHOZN® platform. Contact your customer representative for information or documentation requests.

NOTE Refer to the Product Information Sheets for methods, storage and stability.

Cell Culture Reagents	Manufacturer	Catalog Number
CHOZN® GS-/- cells	SAFC	CHOGS
EX-CELL® CD CHO Fusion Medium	SAFC	14365C (liquid) 24365C (dry powder)
Cellvento® 4CHO-C Cloning Medium	SAFC	14390C (liquid)
EX-CELL® Advanced CHO Fed batch Medium	SAFC	14366C (liquid) 24366C (dry powder)
Cellvento® ModiFeed Prime COMP	SAFC	104132 (dry powder)
L-glutamine (200 mM)	Sigma-Aldrich	G7513
	SAFC	59202C (alternative for G7513)
D-(+)-Glucose (45% solution)	Sigma-Aldrich	G8769
HTST Treated Glucose (50% w/v, optional Emprove® alternative)	SAFC	58955C
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich	D2438
Sodium bicarbonate	Sigma-Aldrich	S5761
36-38% Hydrochloric acid	Sigma-Aldrich	H1758
50% Sodium hydroxide	Sigma-Aldrich	415413
Cell Culture grade water	Sigma-Aldrich	W3500
70% Isopropanol	Sigma-Aldrich	563935
Plasmid DNA encoding r-protein and GS cassette at 1 µg/µL	pCGS3.2 standard CHOZN® GS plasmid pCGS3.2 CHOZN® UCOE® GS plasmid	

Plasticware and Equipment

Equipment	Description/Recommendations
Micro pipettors and sterile tips	Rainin® Classic set or similar
Sterile serological pipettes	1 mL, 2 mL, 5 mL, 10 mL, 25 mL, 50 mL (CLS4485 to CLS4490)
25 cm^2 and T-75 cm^2 suspension culture flasks	Greiner Bio-one 690195 (C6731) and 658195 or similar
15 mL and 50 mL sterile conical centrifuge tubes	CLS430052 and CLS430290 or similar
50 mL TPP (TPP50) TubeSpin® tubes	Techno Plastic Products Z761028 or similar
125 mL sterile shake (E125) non-baffled, vented cap culture flask	CLS431143 or similar
96 well suspension plates	Greiner Bio-one 655185 (M3687) or similar
96 well adherent plates	CellBIND® CLS3300 or similar
24 well suspension plates	Greiner Bio-one 662102 or similar
1.5 mL sterile microtubes	T4816 or similar
4 mm electroporation cuvettes	Z706094 or similar
Sterile filtration unit 0.22 µm, 1000 mL capacity	Millipore® Stericup™ SCGPU10RE or similar
Sterile filtration unit 0.22 µm, 50 mL capacity	Millipore® Steriflip™ SCGP00525 or similar
1.5 mL sterile cryovials	Z359033 or similar
CO_2 incubator (5% CO_2, 37°C, 80% humidified)	Heracell™ VIOS 160i or similar
CO_2 shaker culture incubator system (50 mm throw, 200 rpm)	Kuhner ISF1-Z or similar
Automated cell counter or hemocytometer	Beckman Coulter Vi-CELL XR or similar
Refrigerated centrifuge with swinging bucket rotor (3000 rcf)	Capable of 3000 rcf
Electroporation instrument	Bio-Rad Genepulser® or similar

Equipment	Description/Recommendations
Glucose Monitor levels (accurate between 1 to 10 g/L)	Nova Biomedical BioProfile® FLEX2 or similar
Protein titer quantifier (high-throughput)	Sartorius Octet® or similar
Water bath (37°C)	
Biological safety cabinet (Class II; Type A2; ISO 5)	
Refrigerator (4°C)	
Freezer (-20°C)	
Ultra-cold freezer (-80°C)	
Liquid nitrogen (LN_2) freezer, Vapor phase (-150°C to -196°C)	
Controlled rate freezing vessel (1°C/minute cooling)	Nalgene Mr Frosty™ or similar

CHOZN® GS-/- Platform Handling Protocols

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

- Work with potentially biohazardous materials.
- Use universal precautions for biosafety (WHO Laboratory Biosafety Manual; 4th ed., 2020).
- Perform cell culture procedures using Biosafety Level 1 (BSL-1) containment and practices.
- Wear personal protective equipment.
- Use aseptic technique for all cell and media handling procedures. Spray all items entering biosafety cabinets with 70% isopropanol, as the addition of antibiotics or antimycotics 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.

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Protocol 1: Preparation of EX-CELL® CD CHO Fusion Medium Supplemented with L-glutamine

Purpose

Preparation of EX-CELL® CD CHO Fusion Medium supplemented with 6 mM L-glutamine used for thawing, expansion, and cryopreservation of CHOZN® GS-/- cells.

NOTES Refer to the product guides for recommendations on reconstitution, storage and stability information on all media and feeds.

GS selected, r-protein producing mini-pools and clones should always be grown in medium *without* L-glutamine (refer to Protocol 6).

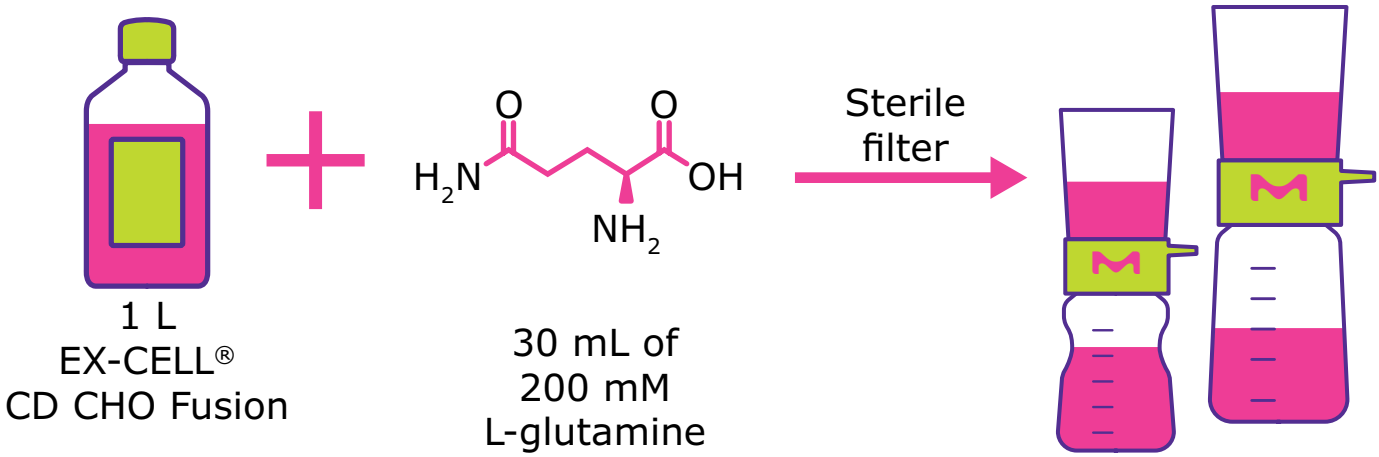

Figure 1: Preparing EX-CELL® CD CHO Fusion with 6 mM L-glutamine

Reagents and Equipment

- EX-CELL® CD CHO Fusion Medium (SAFC 14365C) for liquid or (SAFC 24365C) for dry powder.
- Sterile filtration unit 0.22 µm (1000 mL capacity, Millipore® Stericup™ SCGPU10RE or similar).
- L-glutamine; 200 mM (Sigma-Aldrich G7513 or SAFC 59202C as alternative).

Procedure

1. Thaw L-glutamine in a 37°C water bath until completely dissolved and warm. Store thawed L-glutamine at 2°C to 8°C for up to two weeks.
   - Warm L-glutamine in 37°C water bath if needed to dissolve.
2. Add 30 mL of 200 mM L-glutamine into 1 L of EX-CELL® CD CHO Fusion according to the Table 1.
3. Sterilize the complete medium using a 1000 mL, 0.22 µm filtration unit.
4. Mark the date of preparation on the container and store at 2°C to 8°C in the dark for up to one month.
5. Warm medium in 37°C water bath prior to each use.

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

   The medium is light-sensitive; do not place in direct sunlight.

Table 1: EX-CELL® CD CHO Fusion + 6 mM glutamine for culturing of CHOZN® GS-/- parental cells

Material	Product Number	Volume Needed	Final Concentration
EX-CELL® CD CHO Fusion	14365C or 24365C	1 L	1X
L-glutamine (200 mM)	G7513	30 mL	6 mM

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Protocol 2: Thawing CHOZN® GS-/- Cells

Purpose

Thawing of CHOZN® cells into EX-CELL® CD CHO Fusion Medium.

NOTE The original CHOZN® GS-/- cells require EX-CELL® CD CHO Fusion Medium *with* L-glutamine (refer to Protocol 1).

CHOZN® GS-/- cells transfected with plasmid containing GS gene cassette (mini-pools/clones) requires EX-CELL® CD CHO Fusion Medium *without* L-glutamine.

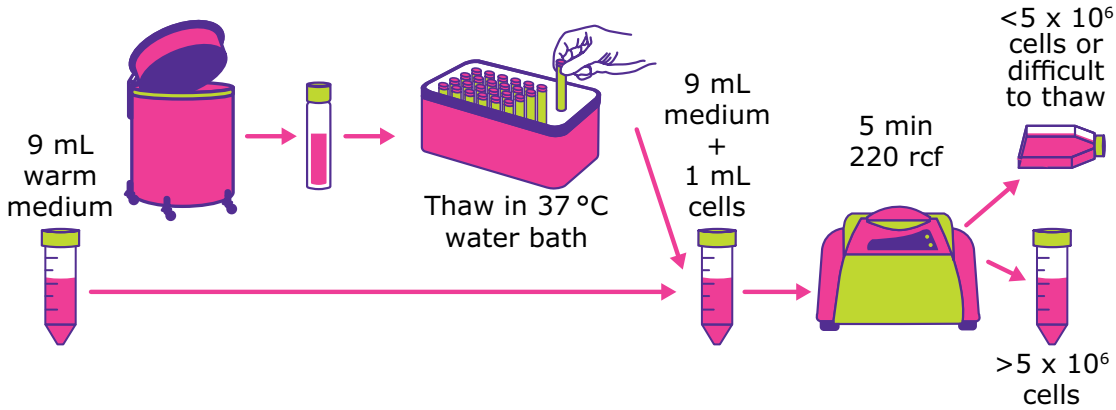

Figure 2: Thawing CHOZN® cells

Reagents and Equipment

- T-75 cm² suspension cell culture flasks (Greiner Bio-one 658195 or similar).
- 15 mL sterile conical centrifuge tube (CLS430052 or similar).
- 50 mL TPP (TPP50, Techno Plastic Products Z761028) TubeSpin® tubes or 125 mL sterile, non-baffled, vented cap shake culture flask (CLS431143 or similar).
- Frozen vial of CHOZN® GS-/- cells (SAFC CHOGS).

  NOTE CHOZN® GS-/- cells are cryopreserved at approximately 7.5 x 10⁶ cells/mL in 93% EX-CELL® CD CHO Fusion Medium *with* 6 mM L-glutamine and 7% Dimethyl sulfoxide (DMSO).
- EX-CELL® CD CHO Fusion Medium *with* 6 mM L-glutamine (refer to Protocol 1) for CHOZN® GS-/- cells.
- EX-CELL® CD CHO Fusion Medium *without* L-glutamine (SAFC 14365C) for mini-pools and clones.

Table 2: Recommended vessel and volume for initial thaw

Vessel	Cells	Volume (mL)	Orbital Shaker#
T-75 cm² suspension flask	<5x10⁶ cells or sensitive cells*	10 to 15	None
TPP 50 TubeSpin® Tube	>5x10⁶ cells and not sensitive	20	• 50 mm throw, 200 rpm • 25 mm throw, 285 rpm
125 mL shake flask	>5x10⁶ cells and not sensitive	22 to 50	• 50mm throw, 90 rpm • 25mm throw, 125 rpm

*Original CHOZN® GS-/- stock vial should always be thawed into T-75 cm² flask to increase success of thaw from 1 vial.

#Throw and recommended shake speed are underlined.

Procedure

1. Thawing the cells:
    a. Warm EX-CELL® CD CHO Fusion Medium in 37°C water bath for at least 30 minutes.
        i. Original CHOZN® GS-/- requires EX-CELL® CD CHO Fusion Medium *with* 6 mM L-glutamine.
        ii. Mini-pools and clones containing GS gene cassette require EX-CELL® CD CHO Fusion Medium *without* L-glutamine.

    NOTE Do not allow the medium to be warmed in the water bath for more than 1 to 2 hours.

    b. Transfer 9 mL of warm EX-CELL® CD CHO Fusion cell culture medium to a 15 mL conical tube and set aside.
    c. Obtain a vial of the CHOZN® GS cells (1 mL), mini-pool, or clone from the LN_2 freezer and put on dry ice.
    d. Rapidly thaw the vial by gently swirling the vial with the cap above the water line in a 37°C water bath until just thawed (approximately 1 minute).
    e. Remove vial from water bath, dry with paper, and decontaminate the outside of the vial with 70% isopropanol before placing in biological safety cabinet.
2. Removing DMSO from the cells:
    a. Carefully transfer cells from the cryovial to the 15 mL conical tube containing 9 mL of EX-CELL® CD CHO Fusion Medium in step 1b.
    b. Cap the 15 mL and invert conical tube slowly to mix.
    c. Centrifuge the cell suspension at 220 rcf for 5 minutes at room temperature (20°C to 22°C) to pellet the cells.
    d. Remove medium without disturbing the cell pellet.

    CAUTION
    Medium from this step contains DMSO. Dispose according to local regulations.

    e. Gently re-suspend cell pellet with 10 mL of EX-CELL® CD CHO Fusion Medium. Slowly pipette up and down to break up the cell clumps.
        i. Original CHOZN® GS-/- requires EX-CELL® CD CHO Fusion Medium *with* 6 mM L-glutamine.
        ii. Mini-pools and clones containing GS gene cassette require EX-CELL® CD CHO Fusion Medium *without* L-glutamine.
    f. Transfer the 10 mL cell suspension to T-75 cm^2 suspension flask or TPP50 guided by Table 2.
        i. If $<5x10^6$ cells or sensitive (difficult to thaw and recover), use T-75 cm^2 suspension flask at 10 mL to 15 mL, then proceed to step 3a.

        NOTE Original CHOZN® GS-/- stock vial should always be thawed into T-75 cm^2 flask to increase success of thaw from 1 vial.

        ii. If $>5x10^6$ cells use TPP50 containing 10 mL of EX-CELL® CD CHO Fusion Medium *with* L-glutamine (20 mL final volume), then proceed to step 3b.

3. Initial cell culture:

- **Part 3a (below)** for original CHOZN® GS$^{-/-}$ stock and cells sensitive to thaw and recovery.
- **Part 3b (below)** for >5x10^6 cells and are not sensitive to thaw and recovery.

a. T-75 cm^2 suspension cell culture flask:

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

ii. After 20 to 28 hours, determine viable cell density and viability.

NOTE Cells should recover within 24 hours and be >85% viable. If not and if another vial is available, check thawing conditions and thaw a new vial. If only 1 vial is available, wait until cells have recovered >85% viability before passaging.

iii. Prepare TPP50 containing 10 mL of EX-CELL® CD CHO Fusion Medium (20 mL final volume).

1. Original CHOZN® GS$^{-/-}$ requires EX-CELL® CD CHO Fusion Medium *with* 6 mM L-glutamine.
2. Mini-pools and clones containing GS gene cassette require EX-CELL® CD CHO Fusion Medium *without* L-glutamine.

iv. In the T-75 cm^2 flask (10 mL), pipette up and down once to dislodge cells from the bottom of the flask.

v. Transfer cells from the T-75 cm^2 flask (10 mL) to prepared TPP50 with media (20 mL final).

vi. Incubate TPP50 containing cells in a humidified 37°C shaker with 5% CO_2 shake set to 50 mm throw, 200 rpm.

NOTE If using 125 mL shake flask, adjust orbital shaker plate to 25 mm throw, 125 rpm (Table 3).

vii. Proceed to Protocol 3: Culturing CHOZN® Cells

b. TPP50 TubeSpin® Tube:

i. Incubate the cells in a humidified 37°C shaker with 5% CO_2 shake set to 50 mm throw, 200 rpm.

ii. After 20 to 28 hours, determine viable cell density and viability.

NOTE Cells should recover within 24 hours and be >85% viable. If not and if another vial is available, check thawing conditions and thaw a new vial. If only 1 vial is available, wait until cells have recovered >85% viability before passaging.

iii. Proceed to Protocol 3: Culturing CHOZN® Cells

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Protocol 3: Culturing CHOZN® GS-/- Cells

Purpose

Culturing CHOZN® cells to expand for subsequent protocols. Passaging of cells is required every 3 to 4 days.

NOTE The original CHOZN® GS-/- cells require EX-CELL® CD CHO Fusion Medium *with* L-glutamine (refer to Protocol 1).

CHOZN® GS-/- cells transfected with plasmid containing GS gene cassette requires EX-CELL® CD CHO Fusion Medium *without* L-glutamine.

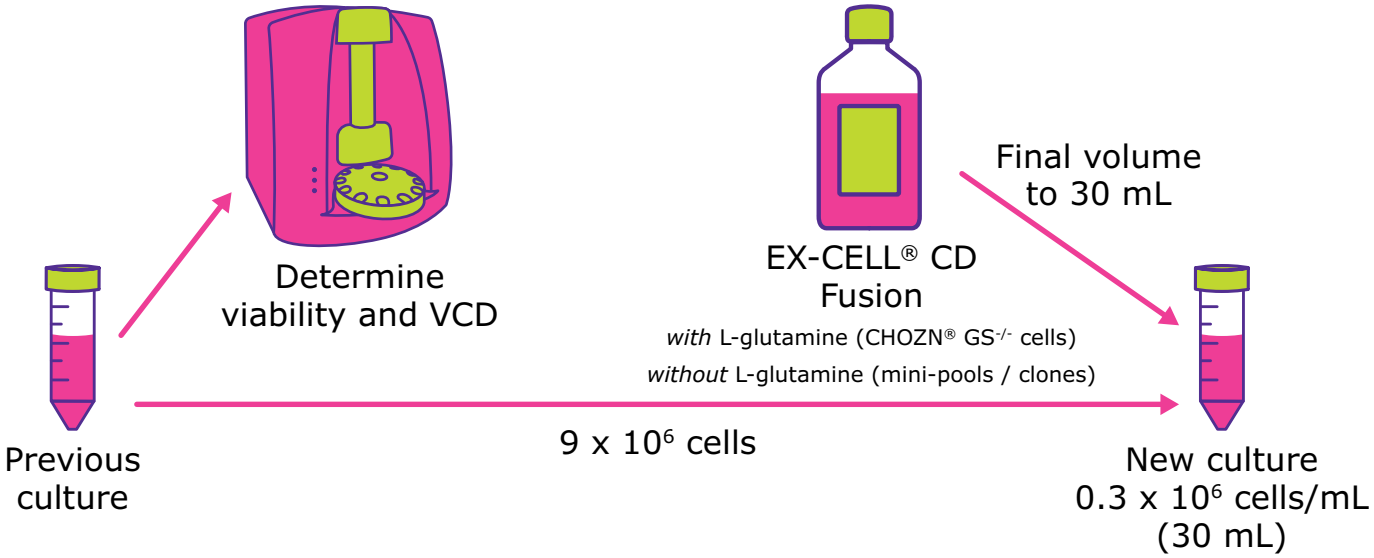

Figure 3: Culturing CHOZN® GS-/- cells

Reagents and Equipment

- 50 mL TPP (TPP50, Techno Plastic Products Z761028) TubeSpin® tubes or 125 mL sterile, non-baffled, vented cap shake culture flask (CLS431143 or similar).
- EX-CELL® CD CHO Fusion Medium *with* 6 mM L-glutamine (refer to Protocol 1) for CHOZN® GS-/- cells.
- EX-CELL® CD CHO Fusion Medium *without* L-glutamine (SAFC 14365C) for mini-pools and clones.

Procedure

Cells should be maintained between 0.3x10^6 to 8x10^6 cells/mL and passaged to fresh medium every 3 to 4 days.

1. Warm EX-CELL® CD CHO Fusion Medium in 37°C water bath for at least 30 minutes.
   a. Original CHOZN® GS-/- requires EX-CELL® CD CHO Fusion Medium *with* 6 mM L-glutamine.
   b. Mini-pools and clones containing GS gene cassette require EX-CELL® CD CHO Fusion Medium *without* L-glutamine.
2. Determine viable cell density and viability. Prepare to passage into new TPP50 at a density of 0.3x10^6 cells/mL in 20 mL to 30 mL of media. If using flasks, refer to Table 3 for working volumes.

   NOTE If cell viability is below 85%, check culture conditions and thaw another vial if available.

   a. **Example part 1**: if new TPP50 is to be seeded at 0.3x10^6 cells/mL in 30 mL of medium, then 9x10^6 cells are required.
      i. Calculation: 0.3x10^6 cells/mL x 30 mL = 9x10^6 cells
   b. **Example part 2**: if current culture viable cell density is 2x10^6 cells/mL, then 4.5 mL of previous culture is required.
      i. Calculation: 9x10^6 cells required / 2x10^6 cells/mL = 4.5 mL
   c. **Example part 3**: Medium volume to reach 30 mL in new TPP50 is 25.5 mL.
      i. Calculation: 30 mL required - 4.5 mL of previous cell culture = 25.5 mL
3. Add required volume of fresh EX-CELL® CD CHO Fusion Medium into a new TPP50.
4. Transfer the appropriate volume of cells to the new TPP50 ensuring final total volume in TPP50 is 20 mL to 30 mL.
5. Incubate culture in a humidified 37°C shaker with 5% CO_2, shake set to 50 mm throw, 200 rpm.

   NOTE If using shake flask, set orbital shaker to 25 mm throw, 125 rpm (Table 3).
6. Passage cells every 3 to 4 days and expand culture volume as necessary according to Table 3.

Table 3: Working volume by flask size

Shake Flask	Volume Range (mL)	Orbital Shaker#
50 mL TPP® TubeSpin tube	20 to 30	• 50 mm throw, 200 rpm • 25 mm throw, 285 rpm
125 mL shake flask	22 to 50	• 50mm throw, 90 rpm • 25mm throw, 125 rpm
250 mL shake flask	60 to 100	
500 mL shake flask	125 to 200	
1L shake flask	250 to 400	

#Throw and recommended shake speed are underlined.

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Protocol 4: Cryopreservation of CHOZN® GS-/- Cells

Purpose

This protocol details procedures for establishing working cell banks of CHOZN® GS-/- cells. Cryopreserve cells in the log phase, 1 to 2 days after passage, for the highest possible viability and growth post-thaw.

NOTE The original CHOZN® GS-/- cells require EX-CELL® CD CHO Fusion Medium *with* L-glutamine (refer to Protocol 1).

CHOZN® GS-/- cells transfected with plasmid containing GS gene cassette requires EX-CELL® CD CHO Fusion Medium *without* L-glutamine.

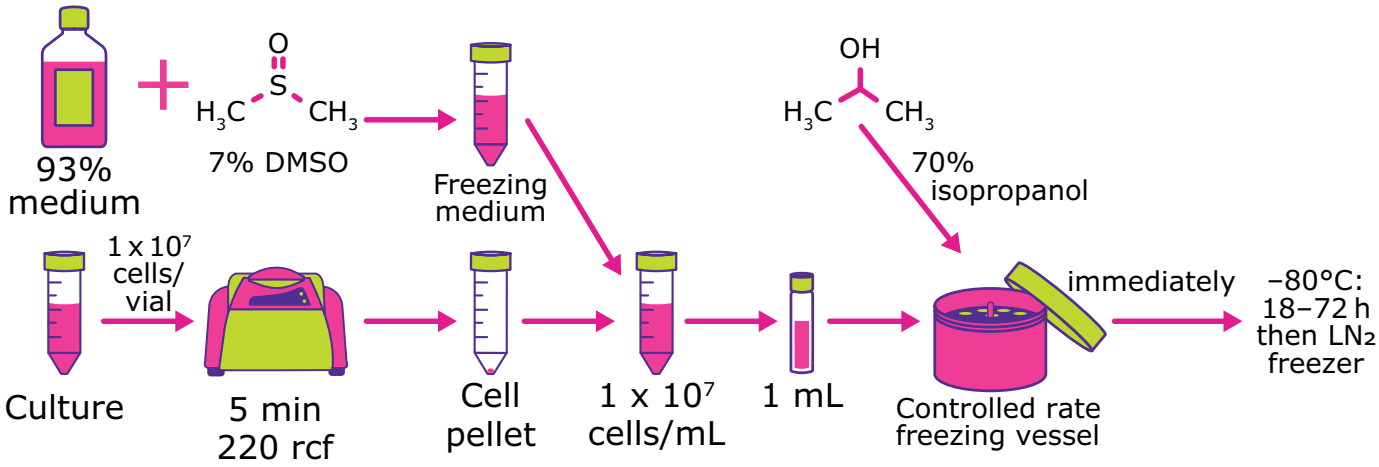

Figure 4: Cryopreservation of CHOZN® GS-/- cells

Reagents and Equipment

- EX-CELL® CD CHO Fusion Medium *with* 6 mM L-glutamine (refer to Protocol 1) for CHOZN® GS-/- cells.
- EX-CELL® CD CHO Fusion Medium *without* L-glutamine (SAFC 14365C) for transfected mini-pools and clones.
- Dimethyl Sulfoxide (DMSO, Sigma-Aldrich D2438).

  NOTE Use fresh DMSO from an unopened bottle for best results.
- 15 mL and 50 mL sterile conical centrifuge tubes (CLS430052 and CLS430290 or similar).
- 1.5 mL sterile cryovials (Z359033 or similar).
- Controlled rate freezing vessel or controlled rate freezer equipment.

Procedure

1. Follow manufacturer's instructions for preparation of freezing vessel.
2. Label the required number of cryovials.
3. For each vial to be frozen, 1x10^7 cells and 1 mL of freezing medium is required.
4. Prepare 1.5 times the freezing medium needed by mixing 93% EX-CELL® CD CHO Fusion Medium L-glutamine and 7% DMSO in a 15 mL or 50 mL conical tube.
    a. Example: For 10 cryovials, prepare 15 mL of freezing medium: 14 mL of EX-CELL® CD CHO Fusion Medium and 1 mL of DMSO.
    b. Original CHOZN® GS$^{-/-}$ requires EX-CELL® CD CHO Fusion Medium *with* 6 mM L-glutamine.
    c. Mini-pools and clones containing GS gene cassette require EX-CELL® CD CHO Fusion Medium *without* L-glutamine.
5. Determine culture's viable cell density and viability. Viability should be >90%.
    a. If viability is <90%, troubleshoot thawing conditions, equipment, and procedure. Return to Protocol 2 and Protocol 3 and start from a fresh thaw.

    NOTE Once cell preparation is initiated, work must proceed quickly. The total time from removing cells from the incubator to placing in the controlled rate freezing vessel and into the –80°C freezer should be less than 30 minutes.
6. Transfer the required number of cells to a 15 mL or 50 mL conical tube and centrifuge at 220 rcf for 5 minutes at room temperature (20°C to 22°C).
7. Carefully aspirate the supernatant, leaving the cell pellet undisturbed.
8. Gently resuspend the cells with freezing medium at 1x10^7 cells/mL.
9. Immediately aliquot 1.0 mL of the cell suspension into labeled cryovials and cap tightly.
10. Immediately transfer vials to the controlled rate freezing vessel and put vessel into a -80°C freezer.
11. After 18 to 72 hours, transfer frozen vials from freezing vessel to the vapor phase of a LN_2 freezer.

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Protocol 5: Transfection of CHOZN® GS$^{-/-}$ Cells

Purpose

This protocol describes transfection of the CHOZN® GS$^{-/-}$ cells via electroporation utilizing the pCGS3.2 plasmid. A transfection efficiency of 60% to 80% should be achieved. Figure 5 shows expression of a fluorescent reporter in a transfected pool. The number of mini-pools to be inoculated will determine how many cells and simultaneous transfection replicates are required. The number of transfections required is also determined by the transfection equipment. This protocol is optimized for Bio-Rad Gene Pulser® electroporator using 4 mm cuvettes. Two times the number of cells required for mini-pool plating should be transfected and if multiple transfections are performed for the same plasmid, they can be combined in a single pool before plating into mini-pools or bulk pools.

The pCGS3.2 plasmid is the plasmid for the CHOZN® GS platform and is designed for higher levels of expression of recombinant proteins, including monoclonal antibodies. The pCGS3.2 plasmid contains a glutamine synthetase cassette and transfected CHOZN® GS cells become glutamine independent after selection and integration of the plasmid into the genome.

Users of the CHOZN® & UCOE® Combined Platform will receive a second plasmid (catalog UCOEGS-1VL). This plasmid can be used in the same way as pCGS3.2 throughout this protocol. The UCOE® platform is not recommended for transient transfections.

NOTE Lipid-based transfection may be used with the CHOZN® GS cells. Mirus Bio CHOgro® High Yield Expression System (MIR 6200) is recommended to be used for transient transfection and while not recommended, it may be used also for stable expression. Transfections should be performed with the Mirus Bio CHOgro® expression medium and TransIT-PRO® transfection reagents before transition to EX-CELL® CD CHO Advanced medium for transient assay or EX-CELL® CD CHO Fusion medium for stable pool selection and recovery. This is because EX-CELL® CD CHO Fusion medium is not designed for lipid-based transfections.

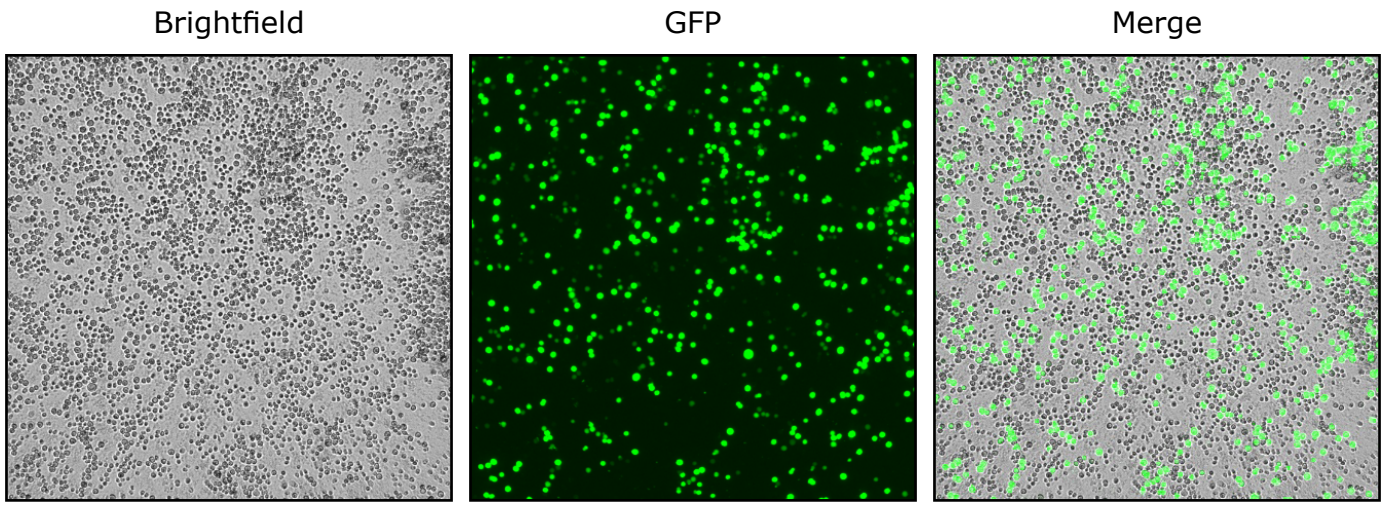

Figure 5: pCGS3.2-GFP transfected CHOZN® GS$^{-/-}$ cells

Images of pCGS3.2-GFP transfected CHOZN® GS$^{-/-}$ cells (pools) 24 hours post-transfection. FACS analysis quantifies the GFP-expressing cells to be 60% to 70% of the total cell population.

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Protocol 5a: Transfection

Purpose

Transfection of plasmid DNA into CHOZN® GS-/- cells.

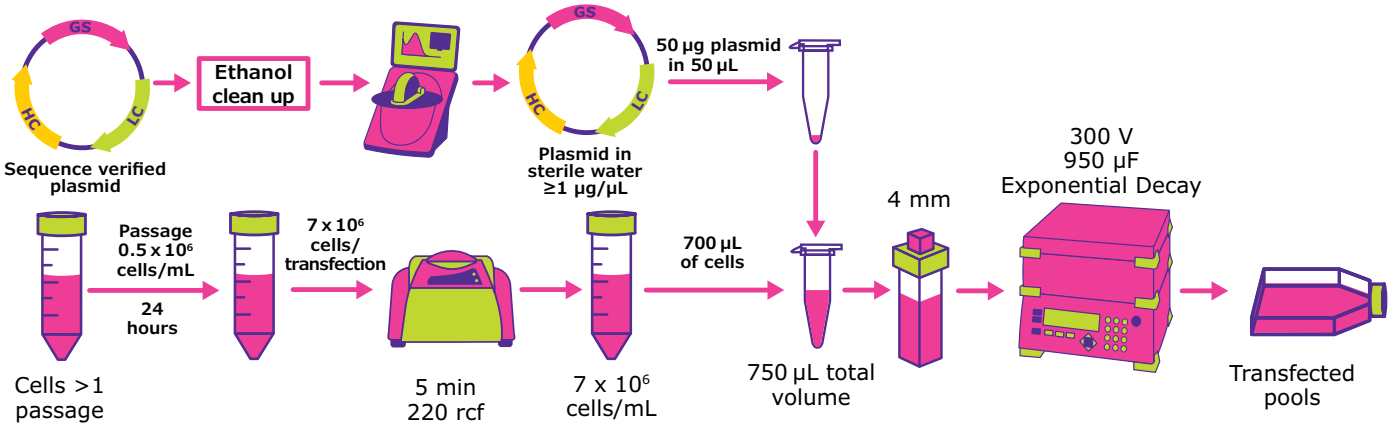

Figure 6: Transfection workflow

Reagents and Equipment

- pCGS3.2 Plasmid DNA encoding the protein of interest (plasmid concentration 1 µg/µL recommended).

  NOTE Plasmid DNA preparations should be sterilized by ethanol precipitation and resuspended in sterile water only.

- EX-CELL® CD CHO Fusion Medium *with* L-glutamine (prepared as in Protocol 1).
- 4 mm Electroporation Cuvettes (Z706094 or similar).
- T-25 cm^2 suspension cell culture flasks (C6731 or similar).
- 15 mL and 50 mL sterile conical centrifuge tubes (CLS430052 and CLS430290 or similar).
- 1.5 mL sterile microtubes (T4816 or similar).
- Bio-Rad Gene Pulser® or similar electroporation instrument (ThermoFisher Neon™ or Lonza Nucleofector®).

  NOTE If using electroporator other than Bio-Rad Gene Pulser, please contact manufacturer to optimize instrument and electroporation settings, including cell number and plasmid DNA amount. Recommendations below are specific to the Bio-Rad Gene Pulser.

Procedure

NOTE Transfect double the number of required cells to ensure there will be sufficient viable cells for mini-pool generation and control bulk pool.

1. Plasmid preparation and cell culture:
    a. Plasmid preparation (any day before transfection):
        i. Ethanol precipitate and resuspend plasmid DNA at ≥1 µg/µL in sterile water ensuring no salts or buffers that will interfere with transfection.
        ii. Measure plasmid DNA concentration and quality. *A260/A280 ratio for DNA should be 1.8-2.0.*

            NOTES Plasmid analysis by restriction digest and sequencing of the r-protein gene sequences to ensure correct r-protein sequences is recommended.
            Plasmid linearization is not required but will perform similarly to circular plasmid.
        iii. Calculate the amount of DNA required for the transfection protocol to ensure enough has been purified.
            1. For Bio-Rad Gene Pulser® use 50 µg per transfection.
            2. For other electroporators please follow manufacturer's recommendations for CHO-K1 cells.
        iv. Store plasmid at -20°C until ready for use.
    b. Cell culture preparation (24 hours before transfection)
        i. Inoculate TPP50 with $1.5x10^7$ CHOZN® $GS^{-/-}$ cells in 30 mL ($0.5x10^6$ cells/mL) of EX-CELL® CD CHO Fusion Medium *with* L-glutamine. Increase number of TPP50 as needed to have double the excess of cells required for transfection.
2. Electroporation set-up (day of transfection):
    a. Label electroporation cuvettes and chill on ice for at least 10 minutes.
    b. Label 1.5 mL sterile microtubes for mixing cell suspensions with DNA.
    c. Acquire EX-CELL® CD CHO Fusion Medium with L-glutamine from 4°C fridge. Do not warm to 37°C.

        NOTE EX-CELL® CD CHO Fusion Medium with 6 mM L-glutamine is used as the transfection and recovery medium.
    d. Label appropriate number of T-25 cm^2 suspension cell culture flasks (one per transfection).
    e. Add 5.0 mL EX-CELL® CD CHO Fusion Medium with L-glutamine to each flask.

3. Prepare cells for electroporation:
   a. Determine viable cell density and viability. >90% viability is required. If not, passage new cells in Protocol 3 until minimum viability is met.
   b. Use $7x10^6$ cells for each transfection and transfer to 15 mL or 50 mL conical tube.
      i. If using the same cells for multiple transfections of the same plasmid, they can be placed in the same conical tube.
      ii. A negative (no plasmid) and a positive control (plasmid encoding a fluorescent protein) for transfections are highly recommended.
   c. Centrifuge at 220 rcf for 5 minutes at room temperature (20°C to 22°C).
   d. Carefully aspirate the supernatant without disturbing the cell pellet.
   e. Resuspend cell pellet in 1.0 mL per transfection of room temperature EX-CELL® CD CHO Fusion Medium *with* L-glutamine.
4. Electroporation:

NOTE Methods described are for Bio-Rad Gene Pulser®. If utilizing a different electroporator, follow manufacturer's recommendations for CHO-K1 cells.

   a. For each electroporation, put 0.70 mL (about $5x10^6$ total cells) of cell suspension in a labeled and sterile 1.5 mL microtube.
   b. Add 50 µg of plasmid DNA to each 1.5 mL microtube with cells.
      i. For negative control, use 50 µL of water.
      ii. For positive control (plasmid encoding a fluorescent protein), use the same amount of plasmid DNA.
   c. Add sufficient EX-CELL® CD CHO Fusion Medium *with* L-glutamine for a final volume of 0.75 mL in each 1.5 mL microtube with cells and DNA.
   d. Transfer the total 0.75 mL of DNA/cell mixture to the chilled electroporation cuvette, and electroporate using the following settings:

Voltage (V)	Capacitance (µF)	Pulse
300	950	Exponential Decay

NOTE It is recommended to avoid transferring over foam from the electroporation cuvette into the T flask.

   f. Following electroporation, transfer the contents of cuvette to prepared T-25 cm^2 suspension cell culture flask with 5.0 mL EX-CELL® CD CHO Fusion Medium *with* L-glutamine (avoid transferring the white cell debris at the top of the cuvette).
   g. Incubate the T-25cm^2 flasks for 20 to 28 hours at 37°C and 5% CO_2 in a non-shaking incubator.
   h. Following recovery at 24 hours, place the transfected cells under selection as described in Protocol 6 to generate stably transfected pools.
   i. After 24 hours, if a fluorescent protein positive control was included, analyze by flow cytometry analysis or fluorescent imaging.
      i. If the cells are <30% fluorescently expressing, consider repeating the transfection.

Return to Flow Chart

Protocol 6: Mini-Pool Selection and Recovery

Purpose

Mini-pools increase the likelihood of isolating higher producing clones, because a limited number of transfected cells are included in each mini-pool. Clones are then isolated from those mini-pools with the highest average clonal expression. Mini-pools are inoculated the day after transfection along with one bulk pool from the same transfected cells. Bulk pools, inoculated at $0.2x10^6$ cells/mL, serve as a control for early indication of cell recovery during selection, because of the difficulty in accessing the viability and VCD in mini-pools. Bulk pools typically recover in 14 to 17 days, about 5 to 7 days earlier than mini-pools and if bulk pools do not recover, then mini-pools will not as well.

The number of mini-pools to plate is determined by the capacity of the user. Ideally, ten 96 well plates of mini-pools are established per plasmid. Each mini-pool begins with 5,000 cells/well before selection using medium *without* glutamine. Once mini-pools recover, over 10 to 21 days, they are assayed to identify those with the highest r-protein expression. The flowchart below outlines the methods as described in Protocol 8 to select the top mini-pools. Mini-pools with the highest expression of the r-protein are cryopreserved before proceeding to single cell cloning.

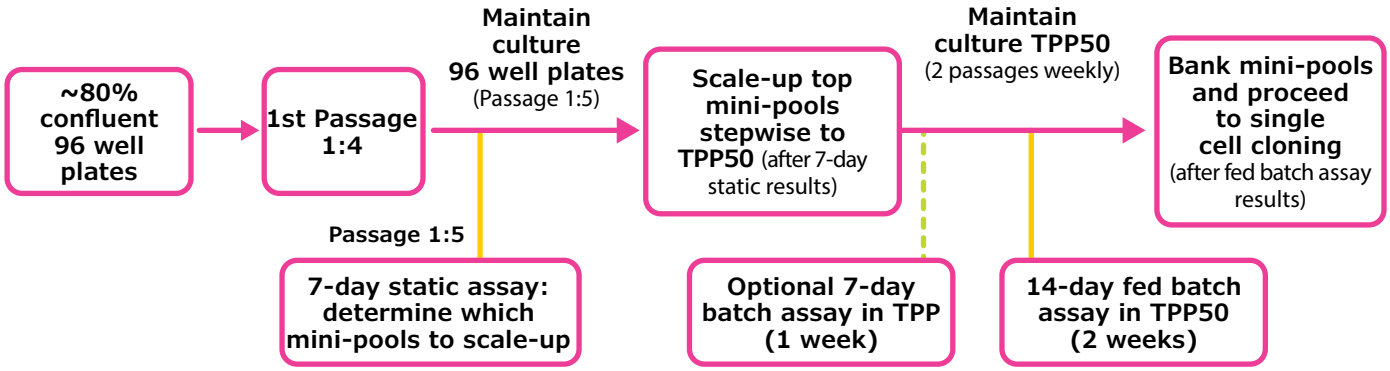

Figure 7: Mini-pool selection and recovery workflow

Return to Flow Chart

Protocol 6a: Plating Mini-Pools

Purpose

Mini-pool plating about 24 hours post-transfection.

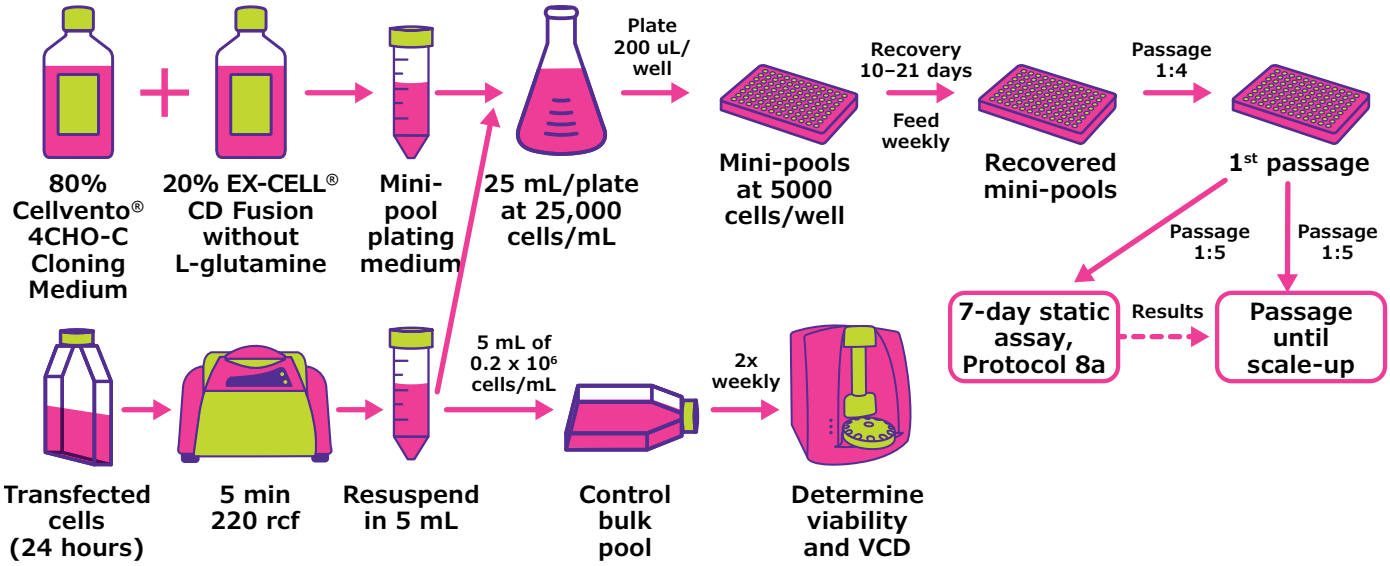

Figure 8: Mini-pool plating, selection, recovery, assay workflow

Reagents and Equipment

- Transfected cell culture (approximately 24 hours post-transfection, from Protocol 5a).
- EX-CELL® CD CHO Fusion Medium *without* L-glutamine (SAFC 14365C/24365C).
- Cellvento® 4CHO-C Cloning Medium (SAFC 14390C).
- 96 well suspension plates (Greiner Bio-one M3687 or similar).
- Multichannel micropipetter and reagent reservoirs.

Procedure

1. Mini-pool plating:

   a. 24 hours post-transfection, determine viability and viable cell density of each transfection in each T-25 cm^2 flask. Viability >60% is expected.

   b. Combine cells from replicate transfections and centrifuge together.

      - Centrifuge at 220 rcf for 5 minutes at room temperature (20°C to 22°C).

   c. Carefully aspirate the supernatant without disturbing the cell pellet.

   d. Resuspend the cell pellet in 5 mL EX-CELL® CD CHO Fusion Medium *without* L-glutamine.

      - If samples were pooled, resuspend in 5 mL of medium multiplied by the number of samples pooled.

   e. Determine viability and viable cell density of the resuspended transfected cultures.

   f. For each plasmid and cell line transfected, create a separate bulk pool control. Transfer $1x10^6$ transfected cells (5 mL of $0.2x10^6$ cells/mL) to 25 cm^2 flask and add EX-CELL® CD CHO Fusion Medium *without* L-glutamine to 5 mL total volume.

      - Incubate at 37°C and 5% CO_2 in a non-shaking incubator.

   g. For each plate of mini-pools, prepare 25 mL of mini-pool plating medium (80% Cellvento® 4CHO-C Cloning Medium and 20% EX-CELL® CD CHO Fusion) according to Table 4.

      NOTE Do not add L-glutamine to the mini-pool plating medium.

Table 4: Mini-pool Plating Medium

Material	Product Number (Sigma-Aldrich)	Final Concentration (volume/volume)
EX-CELL® CD CHO Fusion Medium	14365C	20%
Cellvento® 4CHO-C Cloning Medium	14390C	80%

   h. Dilute the cells to plate into mini-pools at 25,000 cells/mL into 25 mL of mini-pool plating medium per plate (Table 5).

      NOTE If serial dilutions are required, they can be performed in EX-CELL® CD CHO Fusion Medium, with the final dilution in plating medium as shown in Table 4 with final concentration of EX-CELL® CD CHO Fusion Medium at 20%.

Table 5: Mini-pool Seeding Density

Seeding Density (cells/well): 200 µL	Resuspension Density (cells/mL)	Cells per plate (25 mL)
5,000 cells	25,000 cells	625,000 cells

   i. Plate 200 µL per well of the diluted cells into 96 well plates using a multi-channel pipettor and reagent reservoir.

   j. Place the cells into a humidified CO_2 incubator at 37°C and do not disturb for 5 to 7 days. After 5 to 7 days, examine the plates for outgrowth.

2. Stable pool recovery:
    a. Use the control bulk pool as an indicator for mini-pool recovery. Bulk pools are inoculated from the same transfected cells as mini-pools and indicate if and when mini-pools are likely to recover, which is usually about 5 to 7 days before mini-pools. If bulk pools do not recover, then it is unlikely that mini-pools will and the transfection will need to be repeated.
        i. Determine viable cell density and viability twice weekly after day 5.
        ii. Add 1.5 mL of EX-CELL® CD CHO Fusion Medium *without* L-glutamine weekly.
        iii. Terminate cultures when recovered (>90% viability and stable doubling time).
    b. For the mini-pools in 96 well plates, once a week, add 25 µL to 50 µL of EX-CELL® CD CHO Fusion Medium *without* L-glutamine to each well to replace evaporated media. For edge wells, an additional 15 µL to 25 µL of medium per well is added to account for greater evaporation from these wells.
    c. If cells reach confluency in parts of a well at day 11 to 14, it can be triturated using a multi-channel pipette to disperse cells.
3. Mini-pool passaging, assay, scale-up, and cryopreservation:
    a. When wells are approximately 80% confluent, passage at a 1:4 dilution. Transfer 50 µL to a new 96 well plate containing 150 µL (200 µL total) of EX-CELL® CD CHO Fusion *without* L-glutamine. This should occur 10 to 21 days post-plating, depending on the plasmid design and original seeding density.
        NOTE Supernatant can be removed from the original plate to assess titer. However, mini-pools have different growth rates, and this initial screen is not an accurate indication of performance after scale-up.
    b. Passage mini-pools into new 96 well plates once a week at 1:5 dilution (40 µL) until scale-up. Avoid letting mini-pools become over-confluent.
    c. At the second passage, transfer to two 96 well plates: a culture plate and an assay plate for a 7-day static assay (refer to Protocol 8a).
    d. Using results from the 7-day static assay, scale-up the highest titer mini-pools to shaking cultures (refer to Protocol 7). A greater number of mini-pools selected for scale-up increases the chance of capturing the best clones. We recommend the top 30% or 100 mini-pools.
    e. Perform 7-day batch and 14-day fed batch assays to select mini-pools with highest expression (refer to Protocol 8b and Protocol 8c).
    f. Cryopreserve selected mini-pools using 93% EX-CELL® CD CHO Fusion *without* L-glutamine and 7% DMSO as described in Protocol 4.

Return to Flow Chart

Protocol 7: Scale-Up of Mini-Pools or Clones

Purpose

Mini-pools or clones are expanded from static culture plates to shaking cultures. The scale-up strategy is outlined in Figure 9. Scaling up the cell cultures in this way will help prevent loss of stable mini-pools or clones that can result from a more aggressive scale-up strategy. The time required for stable mini-pools or clones to recover and adapt to shaking culture conditions will vary and will range from 10 to 21 days. It is essential that the mini-pools or clones are not overgrown or too sparse, as this may affect the final characteristics of the culture.

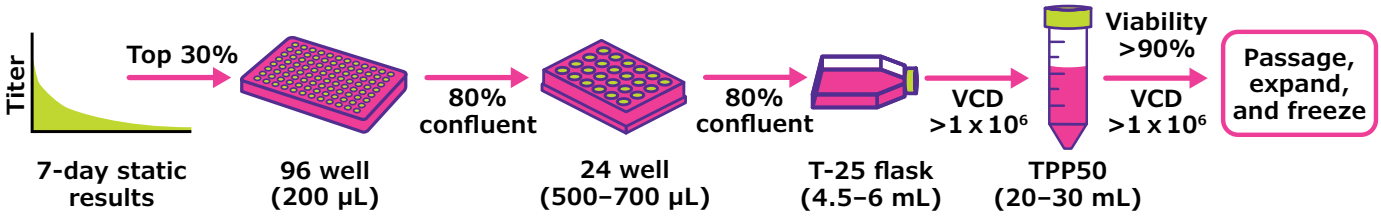

Figure 9: Mini-pool/clone scale-up workflow

Reagents and Equipment

- EX-CELL® CD CHO Fusion Medium *without* L-glutamine (SAFC 14365C/24365C).
- 24 well suspension cell culture plates (Greiner Bio-one 662102 or similar)
- T-25 cm^2 suspension cell culture flasks (Greiner Bio-one C6731 or similar)
- 50 mL TPP (TPP50, Techno Plastic Products Z761028) TubeSpin® tubes or 125 mL sterile, non-baffled, vented cap shake culture flask (CLS431143 or similar)

Procedure

1. Using the results of the 7-day static assay, expand selected mini-pools or clones following steps 2, 3, 4, and 5. Table 6 summarizes steps 2 to 4.
2. From 96 well to 24 well plate:
   a. Ensure individual wells are >80% confluent.
   b. Prepare the required number of 24 well plates with 500 µL of EX-CELL® CD CHO Fusion Medium *without* L-glutamine per well.
   c. Transfer contents of selected wells to 24 well plates. Pipette up and down to dislodge loosely attached cells.
3. From 24 well plate to T-25 cm^2 flask:
   a. Ensure individual wells are >80% confluent.
   b. Prepare the required number of T-25 cm^2 flasks with 5 mL of EX-CELL® CD CHO Fusion Medium *without* L-glutamine.
   c. Transfer each well of the 24 well plate to a T-25 cm^2 flask. Pipette up and down to dislodge loosely attached cells.

4. From T-25 cm^2 flasks to TPP50 or shake flasks:
    a. Maintain selected mini-pools or clones in T-25 cm^2 flasks until VCD >1x10^6 cells/mL.
    b. Prepare required number of TPP50 with 25 mL of EX-CELL® CD CHO Fusion Medium *without* L-glutamine.
    c. Transfer entire contents of T-25 cm^2 flasks into prepared TPP50 (about 30 mL final volume).
    d. Optional: in some cases, individual minipools may have difficulty adapting to shaken conditions. In order to preserve the culture if this occurs, the T-25 cm^2 flasks may be backfilled with 5 mL of EX-CELL® CD CHO Fusion Medium *without* L-glutamine and incubated until growth in TPP is observed.
        i. This is maintained in case the cells have difficulty adapting to shake conditions and requires higher VCD before transfer to shake conditions. Backfilled T-25 cm^2 flasks can be discarded once TPP50 is established.
5. Culture maintenance, expansion for batch assays, and cryopreservation
    a. Once cultures are in TPP50, passage every 3 to 4 days in EX-CELL® CD CHO Fusion Medium *without* L-glutamine, replacing medium as indicated in Protocol 3.
    b. Expand cultures as needed for 7-day batch and 14-day fed batch assays (refer to Protocol 8b and Protocol 8c).
    c. When mini-pools or clones are selected, cryopreserve the selected mini-pools or clones in EX-CELL® CD CHO Fusion Medium (*without* L-glutamine) and 7% DMSO (refer to Protocol 4, replacing medium as indicated).

Table 6: Recommended scale-up strategy

Culture Plate/Flask Size	Product Number	Medium Volume	When to Scale Up	Number of Days Normally Required
96 well plate	Greiner-one 655185	200 µL per well	80% confluent	2 to 4 after cells have recovered
24 well plate	Greiner-one 662102	500 to 750 µL per well	80% confluent	3 to 5
T-25 cm^2 flask	Greiner-one 690195	4.5 to 6 mL	VCD >1x10^6 cells/mL	3 to 5
TPP50 TubeSpin tube	MilliporeSigma Z761028	20 to 30 mL	Passage twice weekly to 0.3x10^6 cells/mL	3 to 4

Return to Flow Chart

Protocol 8: Recombinant Protein Assays for Screening Mini-Pools and Clones

Purpose

Protein assays identify mini-pools or clones expressing the highest levels of recombinant protein. The assays chosen, and the selection criteria, are project dependent. The assay flow chart describes at which stage of mini-pool or clone generation the assays should be performed to select mini-pools or clones to reduce number to screen. Table 7 describes the number of mini-pools or clones to select for the subsequent assay or activity. These assays are increasingly predictive of manufacturing performance but involve increasing effort. We use a high throughput assay initially to reduce the number of mini-pools or clones that have low production.

Protocol 8a: 7-Day Static Assay
A relatively high-throughput assay performed in 96 well plates. This assay does not precisely predict the potential rank order of mini-pools' or clones' r-protein expression. Therefore, the top 30% or 60 to 100 mini-pools or clones are expanded for further assessment.

Protocol 8b: 7-Day Batch Assay
An assay in TPP50 or shake flasks to more precisely predict mini-pools or clones that will express higher levels of r-protein. This assay can reduce the candidates from the 7-day static assay to be assessed by the 14-day fed batch assay but can be omitted if there are less than 60 mini-pools or clones.

An alternative to this assay is to perform a sample reduction 14-day fed batch assay (refer to Protocol 8c). All scaled-up mini-pools or clones are seeded for the 14-day fed batch assay, then 20 to 30 candidates are selected on day 7 of the assay while the rest are discarded. This method requires performing high-throughput r-protein analysis on day 7 and acquiring the results on the same day.

Protocol 8c: 14-Day Fed Batch Assay
A suspension culture assay in TPP50 or shake flasks that most precisely predicts mini-pool or clone performance under manufacturing conditions. At least 60 candidates from the 7-day static assay or 30 candidates from the 7-day batch assay should be selected for this assay. The 14-day fed batch protocol uses Cellvento® ModiFeed Prime, a chemically defined formulation that is highly concentrated, one-part and pH neutral, and is process controlled with decreased complexity.

For customers who use EX-CELL® Advanced CHO Feed 1 for 14-day fed batch instead of Cellvento® ModiFeed Prime, the feed schedule is described in Appendix 4.

With these assay results, mini-pools are identified from which to clone, and/or clones are finally identified for further assessment.

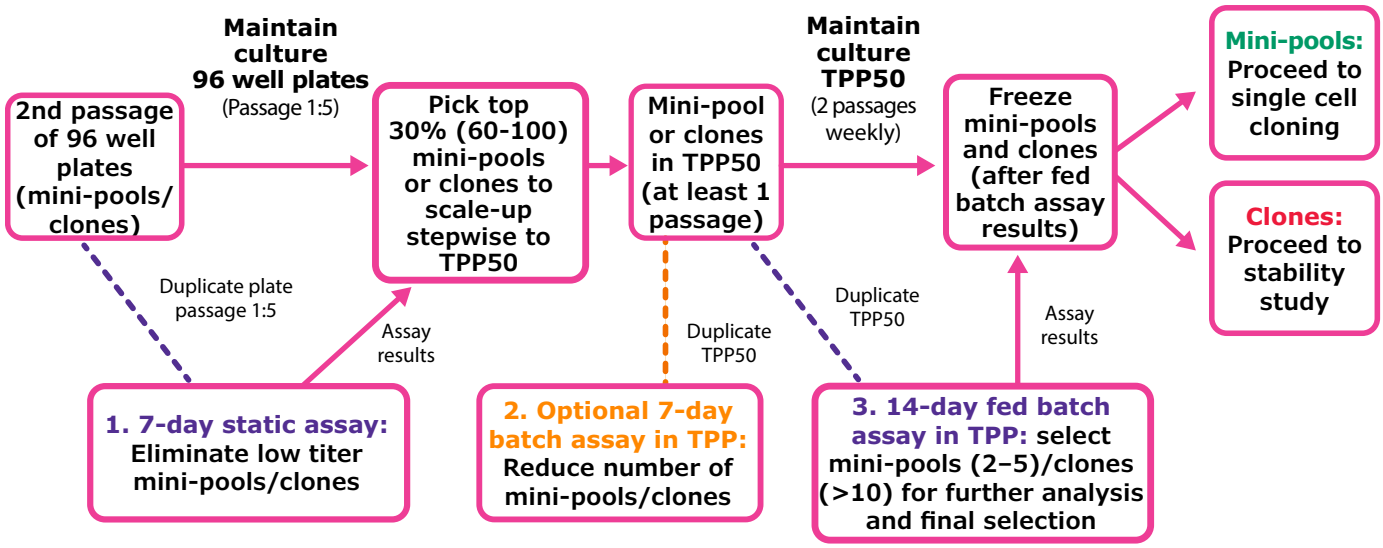

Figure 10: R-protein assays and decisions workflow for mini-pools and clones

Table 7: Summary of input and selection for mini-pool and clones for screening assays

Assay	Input Number of Mini-pool/Clones into Assay	Number of Candidates from Assay	Next Assay/Activity
7-day static	All plates	Top 30% or 60 to 100	7-day batch
7-day batch	>60	20 to 30	14-day fed batch
14-day fed batch	<60, recommended to perform assay in duplicate	Mini-pools: 2-5	Cell cryopreservation and single cell cloning
		Clones: >10	Cell cryopreservation and stability study

Return to Flow Chart

Protocol 8a: 7-Day Static Assay

Purpose

First round assessment of r-protein production in static plates.

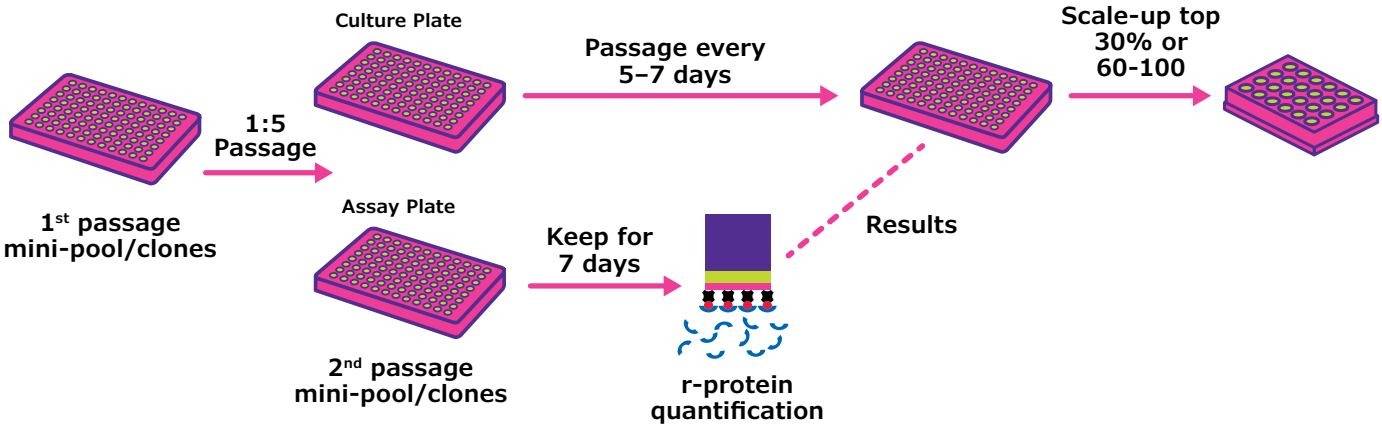

Figure 11: 7-day static assay workflow

Reagents and Equipment

- Cells: Stable mini-pools or clones in 96 well plates post-glutamine selection and recovery.
- EX-CELL® CD CHO Fusion Medium *without* L-glutamine (SAFC 14365C/24365C).
- 96 well suspension cell culture plates (Greiner Bio-one M3687 or similar).
- Assay for protein measurements, high-throughput capabilities recommended (Sartorius Octet® or similar).

Procedure

This protocol is the screening assay referred to in the following protocols:

- Mini-pools: Protocol 6a, step 3c
- Clones: Protocol 9b, step 7b

1. After 5 to 7 days, when the plates are 80% confluent, passage plates 1:5 into two new 96 well plates by transferring 40 µL cells to 160 µL of EX-CELL® CD CHO Fusion Medium *without* glutamine in each well. One plate is for continued culture and the other for the 7-day static r-protein assay. Incubate plates at 37°C, 5% CO_2 in a static incubator.
    a. *Culture plate* is maintained by passaging 1:5 when 80% confluent (every 5 to 7 days) by transferring 40 µL cells from each well to 160 µL of EX-CELL® CD CHO Fusion Medium *without* glutamine in a fresh well.
    b. *Assay plate* is for the 7-day static r-protein assay and should be undisturbed.
2. After 7 days, harvest the supernatant of the *Assay plate*. Centrifuge at 330 rcf for 5 minutes, transfer the supernatant to a fresh 96 well plate, and quantify r-protein.
3. From the culture plate generated in step 2, the top 30% or 60 to 100 mini-pools or clones with the highest secreted r-protein concentration are expanded to 24 well plates and then to T-25 cm^2 flasks (refer to Protocol 7).

Return to Flow Chart

Protocol 8b: 7-Day Batch Assay

Purpose

This is a second assessment of r-protein production in TPP50 to further reduce the number of mini-pools or clones under consideration. If fewer than 60 samples are still in contention or if performing the sample reduction during the 14-day fed batch, proceed to Protocol 8c.

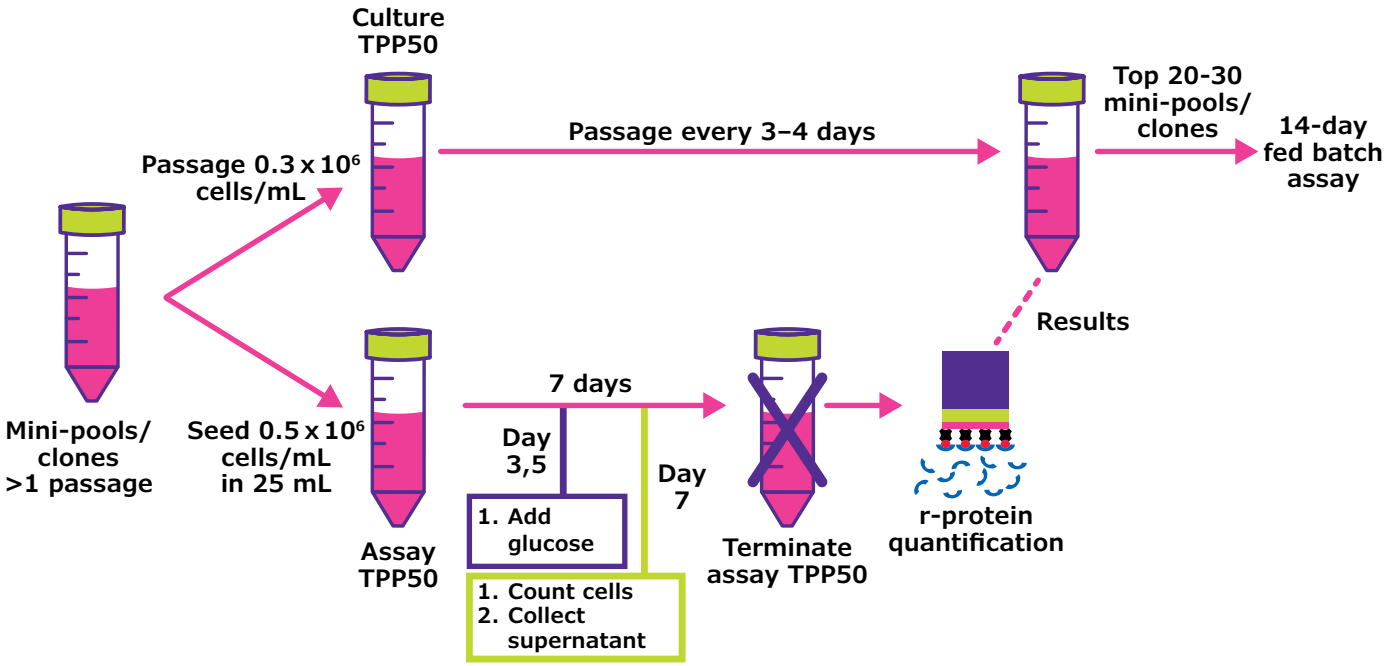

Figure 12: 7-day batch assay workflow

Reagents and Equipment

- Cells: Mini-pools or clones in TPP50 spin tubes.
- EX-CELL® CD CHO Fusion Medium *without* L-glutamine (SAFC 14365C/24365C)- to maintain cells in TPP50.
- EX-CELL® Advanced CHO Fed Batch Medium (SAFC 14366C/24366C)- to Assay r-protein in TPP50.
- D-(+)-Glucose (45% solution) Sigma-Aldrich G8769.
- Emprove® alternative: HTST Treated Glucose (50% w/v) SAFC 58955C
- 50 mL TPP (TPP50, Techno Plastic Products Z761028) TubeSpin® tubes or 125 mL sterile, non-baffled, vented cap shake culture flask (CLS431143 or similar).
- Instrument for measuring 1 to 10 g/L glucose in cell culture (Nova Biomedical BioProfile® FLEX2 or similar).
- Assay for protein measurements, high-throughput capabilities recommended (Sartorius Octet® or similar).

Procedure

1. The 7-day batch assay is used if there are >60 mini-pool or clones and reduces the number of samples for the 14-day fed batch assay. It is performed when mini-pools/clones have been in TPP50 for at least 1 passage.
2. For each mini-pool or clone, centrifuge $9x10^6$ cells for continued culture and $12.5x10^6$ cells for assay at 220 rcf for 5 minutes at room temperature and resuspend in TPP50s.
    a. *Culture TPP50*: Resuspend in 30 mL ($0.3x10^6$ cells/mL) of EX-CELL® CD CHO Fusion Medium *without* L-glutamine.
        i. The *culture TPP50* is maintained and passaged as needed for the duration of the assay and used to seed the 14-day fed batch assay. Refer to Protocol 3, replacing medium as indicated.
    b. *Assay TPP50* (can be performed in duplicates): Resuspend in 25 mL ($0.5x10^6$ cells/mL) of EX-CELL® Advanced CHO fed batch medium *without* L-glutamine for each mini-pool/clone and incubate in a shaking incubator at 37°C and 5% CO_2.
3. On days 3 and 5:
    a. Add 400 µL of 45% glucose to each sample (6 g/L final).
4. On day 7:
    a. Count cells and assess viability.
    b. Collect 0.5 mL from *Assay TPP50* and centrifuge at 1000 rcf for 5 minutes to remove cells. Transfer supernatant to a new 1.5 mL microtube.
    c. Store supernatant at -20°C until ready for r-protein assay.
    d. Terminate *Assay TPP50*.
5. Quantify r-protein in supernatants.
6. Select 20 to 30 candidates with highest titer for inclusion in the 14-day fed batch assay (refer to Protocol 8c).

NOTE Alternatively, the first 7 days of the 14-day fed batch can be performed instead of the batch assay. After the first 7 days, assay r-protein titer and continue only with 20 to 30 mini-pools or clones with the highest r-protein expression levels.

Return to Flow Chart

Protocol 8c: 14-Day Fed Batch Assay

Purpose

This is the final and most predictive of the r-protein assays. The number of candidate mini-pools or clones can be reduced by assaying r-protein at day 7 and eliminating all but the 30 that produce the most r-protein. High-throughput r-protein quantification is required for day 7 sample reduction.

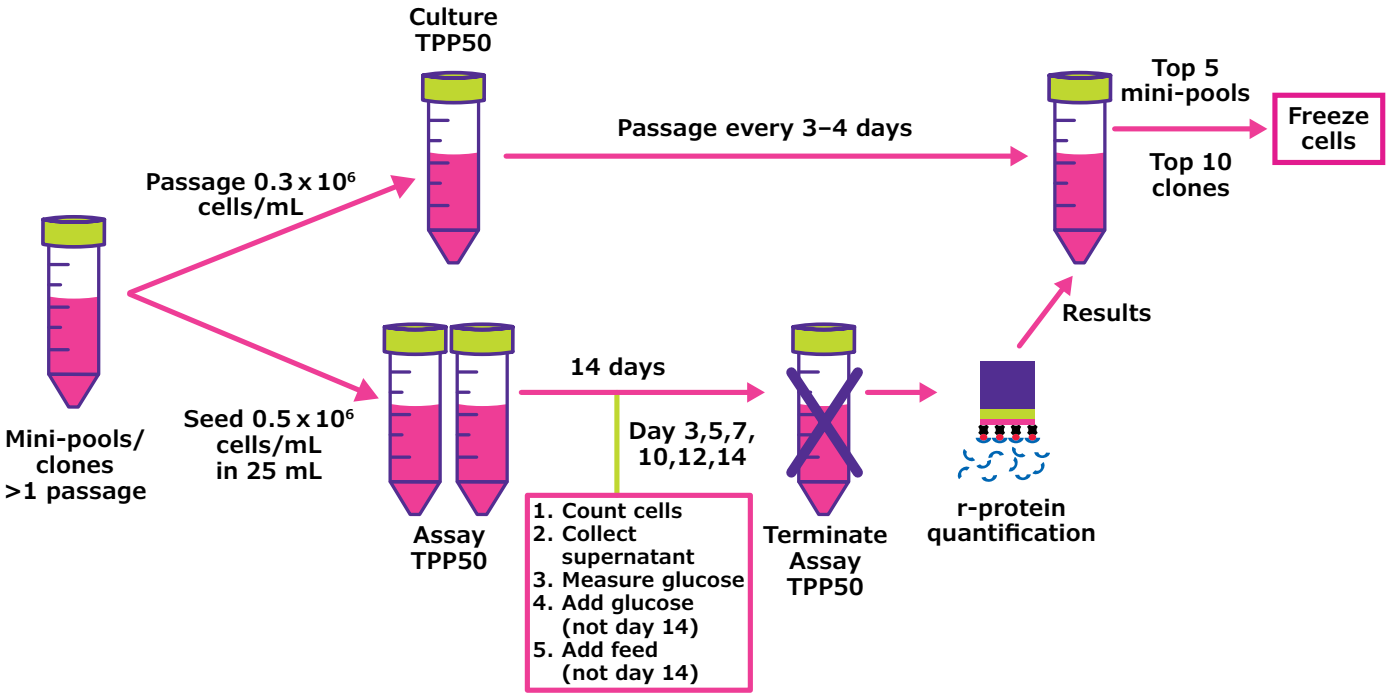

Figure 13: 14-day batch assay workflow

Reagents and Equipment

- Cells: Mini-pools or clones in TPP50 spin tubes.
- EX-CELL® CD CHO Fusion Medium *without* L-glutamine (SAFC 14365C/24365C)- to maintain cells in TPP50.
- EX-CELL® Advanced CHO Fed Batch Medium (SAFC 14366C/24366C)- to assay r-protein in TPP50.
- Cellvento® ModiFeed Prime COMP (104132).
- D-(+)-Glucose (45% solution) (G8769).
- Emprove® alternative: HTST Treated Glucose (50% w/v) SAFC 58955C
- 50 mL TPP (TPP50, Techno Plastic Products Z761028) TubeSpin® tubes or 125 mL sterile, non-baffled, vented cap shake culture flask (CLS431143 or similar).
- Instrument for measuring 1 to 10 g/L glucose in cell culture (Nova Biomedical BioProfile® FLEX2 or similar).
- Assay r-protein quantification, high-throughput capabilities recommended (Sartorius Octet® or similar).

Procedure

1. The 14-day fed batch assay should be performed after mini-pools or clones have been in TPP50s for at least 1 passage. To avoid weekend feed and glucose addition, start the 14-day fed batch assay on a Friday. Set up TPP50s for both assay and continued culture.
2. For each mini-pool or clone, collect $9x10^6$ cells for *Culture TPP50* and $12.5x10^6$ cells for *Assay TPP50* by centrifugation at 220 rcf for 5 minutes at room temperature.
    a. *Assay TPP50*: (can be performed in duplicates): Resuspend $12.5x10^6$ cells of each mini-pool/clone in 25 mL EX-CELL® Advanced CHO fed batch medium *without* L-glutamine ($0.5x10^6$ cells/mL). Incubate shaking at 37°C and 5% CO_2.
    b. *Culture TPP50*: Resuspend $9x10^6$ cells of each mini-pool/clone in 30 mL EX-CELL® CD CHO Fusion Medium *without* L-glutamine ($0.3x10^6$ cells/mL).
        i. The Culture TPP50 is maintained and passaged as needed for the duration of the assay and used for cell cryopreservation or single cell cloning. Follow Protocol 3, replacing medium as indicated.
3. Feed and sample on days 3, 5, 7, 10, 12 and 14, guided by Table 8:
    a. Assess viable cell density and viability at each time point.
    b. Collect supernatant for glucose and r-protein assay. Collection for glucose assay is required at each time point. Supernatant for r-protein assay is collected starting from day 7.
        i. Clarify 1.0 mL from each *Assay TPP50* at 1000 rcf for 5 minutes.
        ii. Assay glucose using half the clarified supernatant.
        iii. On day 7 and after, transfer half the supernatant to a new 1.5 mL microtube or 96 deep well plate for r-protein assay to be performed on day 7 or store at -20°C for r-protein assay at the end of the fed batch assay.
    c. After determining glucose levels in samples, add glucose to 6 g/L.
        i. On day 7, add glucose to 9 g/L to avoid weekend maintenance.
        ii. If glucose is consistently <0.5 g/L, add 1 g/L more glucose to the final concentration (example: if adding to 6 g/L previously, then add glucose to 7 g/L).
        iii. Day 14 does not require glucose addition since *Assay TPP50* will not be continued.
    d. Add 0.75 mL (3% v/v) Cellvento® ModiFeed Prime COMP to each *Assay TPP50*. This follows the "Low" feed schedule for Cellvento® ModiFeed Prime COMP.
        i. On day 7, add 1.38 mL (5.5% v/v) Cellvento® ModiFeed Prime COMP to avoid weekend maintenance.
        ii. Day 14 does not require feed addition since *Assay TPP50* will not be continued.
    e. Terminate any *Assay TPP50* with viability <70%.
    f. On day 14, *Assay TPP50* can be discarded or clarified supernatant can be stored at -20°C for other analysis such as protein quality attributes.

4. Quantify r-protein in supernatant.
5. If working with mini-pools:
    a. Select mini-pools to clone from, considering titer, specific productivity, growth characteristics and protein quality attributes, if available. Typically, two to five mini-pools are selected. Cloning is guided by Protocol 9.
    b. Cryopreserve cells from the selected mini-pools. Guided by Protocol 4, freeze cells from the *Culture TPP50s*.
6. If working with clones:
    a. Select clones for further analysis, considering titer, specific productivity, growth characteristics and protein quality attributes if available. Typically, ten clones or greater are selected for further analysis, such as stability assessment, guided by Protocol 10.
    b. Cryopreserve cells from the selected clones. Guided by Protocol 4, freeze cells from the *Culture TPP50s*.

Table 8: Activity, Feed addition, and Glucose addition schedule

	Day					
	3	**5**	**7**	**10**	**12**	**14**
Cell counting	Y	Y	Y	Y	Y	Y
Store supernatant for titer analysis	-	-	Y	Y	Y	Y
Measure glucose	Y	Y	Y	Y	Y	Y
Add glucose	To 6 g/L	To 6 g/L	To 9 g/L	To 6 g/L	To 6 g/L	-
Feed using Cellvento® ModiFeed Prime COMP (v/v)	3%	3%	5.5%	3%	3%	-

Return to Flow Chart

Protocol 9: Clone Isolation by Limiting Dilution

Purpose

Single cell clones (SCC) are isolated from mini-pools. Cloning from multiple mini-pools increases the likelihood of finding a clone that meets project criteria. Clones are isolated from each mini-pool separately since this increases the probability of independent clones with diverse phenotypes being captured.

This protocol describes clone isolation by limiting dilution, in which cells are seeded in 96 well plates at an average of 0.5 cells per well. At this density between 10 and 40 clones are expected per 96 well plate. Other methods of cloning include seeding by flow cytometry or cell printing.

Importantly, for all methods, clonality should be demonstrated by imaging to demonstrate cell growth in a well arose from a single cell.

Protocol 9a describes preparation of cloning medium using 20% conditioned medium to increase cloning efficiency.

Protocol 9b describes cloning by limiting dilution.

Figure 14 summarizes cell cloning and assays to identify clones expressing the most r-protein. After cloning and expansion, cells will be cryopreserved and used to further characterize stability of productivity (refer to Protocol 10), protein quality, and process development.

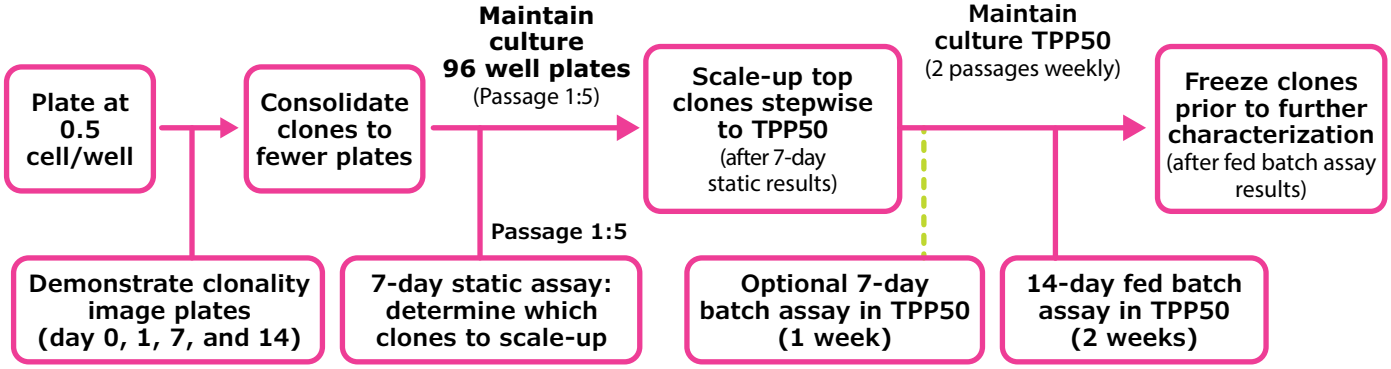

Figure 14: Clone isolation, recovery, and assay workflow

NOTE CHOZN® is compatible with other methods of cloning (i.e. FACS). Other methods should still use the 80/20 cloning plating medium.

Return to Flow Chart

Protocol 9a: Cloning Medium Preparation

Purpose

Preparing clone plating medium for clone isolation.

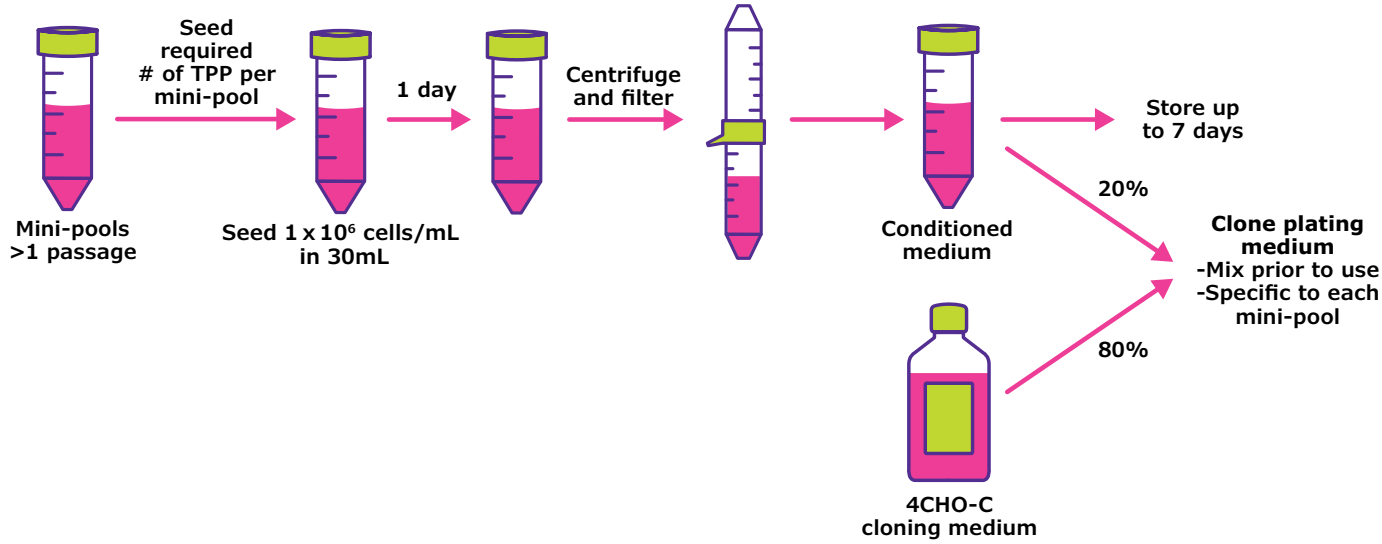

Figure 15: Clone plating medium preparation

Reagents and Equipment

- 50 mL TPP (TPP50, Techno Plastic Products Z761028) TubeSpin® tubes or sterile shake flask.
- 15 mL and 50 mL sterile conical centrifuge tubes (CLS430052 and CLS430290 or similar).
- Sterile 0.2 µm Millipore Steriflip® filter apparatus or similar.
- EX-CELL® CD CHO Fusion Medium *without* L-glutamine (SAFC 14365C).
- Cellvento® 4CHO-C Cloning Medium (SAFC 14390C)

Procedure

1. Prepare conditioned medium (CM) 24 to 48 hours in advance:
    a. Each mini-pool to be cloned requires matched CM from that mini-pool. So, if 3 mini-pools are to be single cell cloned from, 3 separate CM are needed; 1 from each mini-pool.
    b. Make 2 times excess CM: 5 mL of CM is required for every 96 well plate but make 10 mL per plate.
    c. Set up the appropriate number of TPP50s assuming a yield of 30 mL of CM, or use shake flasks and a volume guided by Table 3 in Protocol 3.
    d. For each TPP50, collect $3.0x10^7$ cells from that mini-pool by centrifugation (220 rcf, 5 minutes, 20°C). Seed each TPP50 with $1.0x10^6$ cells/mL in 30 mL of EX-CELL® CD CHO Fusion Medium *without* L-glutamine.
    e. Culture for 24 hours (±4 hours) in a humidified 37°C shaking incubator with 5% CO_2 shake set to 50 mm throw, 200 rpm.
        NOTE It is recommended to count the culture prior to clarification in order to confirm that they doubled (approximately) and maintained a high viability.
    f. Clarify conditioned medium by centrifugation at 2440 rcf for 5 minutes at room temperature.
    g. Filter the clarified medium using a 0.2 µm Steriflip® filter device or equivalent.
    h. Label and use immediately (refer to Protocol 9b) or store at 2°C to 8°C for up to 7 days. Do not freeze conditioned medium.
2. Prepare the clone plating medium immediately before cloning:
    a. Each mini-pool to be cloned from requires clone plating medium specific to that mini-pool.
        NOTE *Do not* mix the conditioned medium and the cloning medium more than 2 to 4 hours prior to cloning.
    b. For each plate, mix 5 mL conditioned media and 20 mL Cellvento® 4CHO-C Cloning Medium (Table 9). 25 mL of clone plating medium is required for every 96 well plate.
    c. Clone plating medium is used in Protocol 9b.

Table 9: Clone Plating Medium

Material	Product Number (Sigma)	Final Concentration
Conditioned Medium	(refer to Protocol 9a, step 1)	20%
Cellvento® 4CHO-C Cloning Medium	14390C	80%

Return to Flow Chart

Protocol 9b: Single-Cell Cloning by Limiting Dilution

Purpose

Clone isolation by limited dilution averaging 0.5 cells/well.

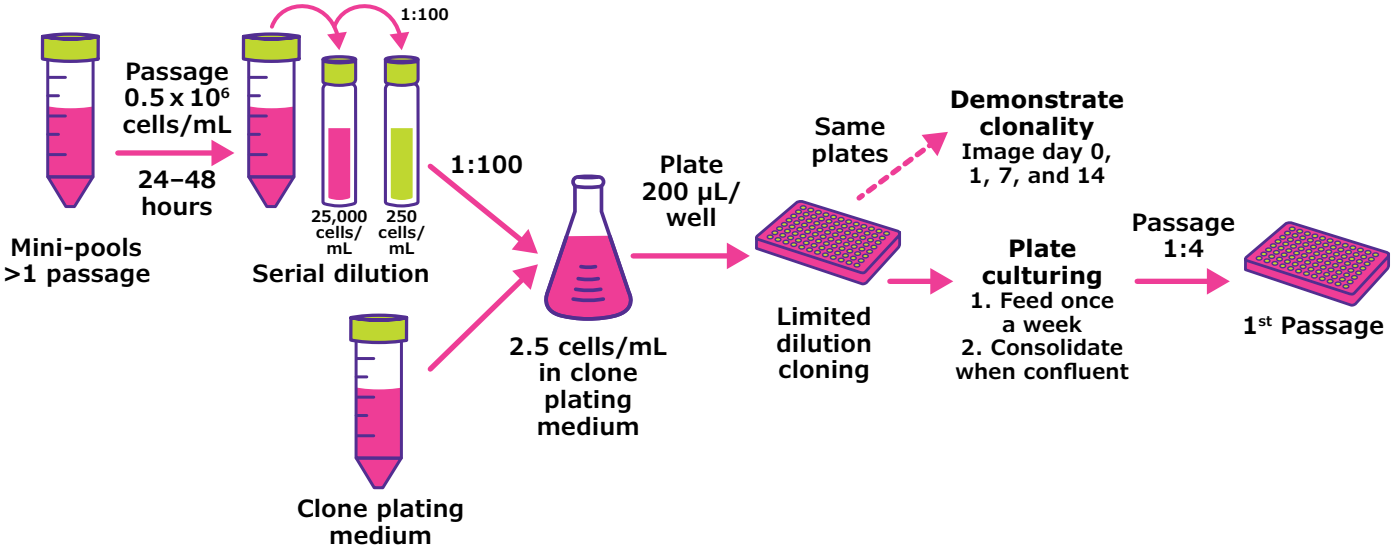

Figure 16: Limited dilution cloning workflow

Reagents and Equipment

- Mini-pools generated and characterized in Protocol 6 and Protocol 8.
- 96 well suspension cell culture plates (recommended: Corning® CellBIND® 96 well plates, Product Number 3300).
- 50 mL Reagent Reservoirs (CLS4870).
- Clone Plating Medium (refer to Protocol 9a).
- EX-CELL® CD CHO Fusion Medium *without* L-glutamine (SAFC 14365C).

Procedure

1. Prepare mini-pools (24 to 48 hours before single cell cloning):
    a. Passage the cells at 0.5x10^6 cells/mL in TPP50 24 to 48 hours prior to cloning to ensure cells are in the exponential growth phase and at high viability at the time of cloning.
2. Prepare clone plating medium
    b. For each mini-pool (refer to Protocol 9a), 25 mL of clone plating medium is required for each plate.
3. Limiting dilution cloning:
    a. Determine the viable cell density and viability of the mini-pools to be cloned from. Viability above 95% is required. If cloning from a recently thawed mini-pool, passage until >95% viability.
    b. Serially dilute each mini-pool separately to a final cell density of 2.5 cells/mL in clone plating medium. Initial dilutions can be in EX-CELL® CD CHO Fusion Medium *without* L-glutamine. A recommended dilution strategy is described below in Table 10.

Table 10: Dilution strategy

First Dilution	Second Dilution	Final Dilution
25,000 cells/mL	250 cells/mL	2.5 cells/mL
Cell culture diluted into EX-CELL® CD CHO Fusion Medium *without* L-glutamine	1:100 from 25,000 cells/mL into EX-CELL® CD CHO Fusion Medium *without* L-glutamine	1:100 from 250 cells/mL into clone plating medium

    c. Seed 200 µL of the diluted cells into 96 well plates from reagent reservoir with multi-channel pipettor. Corning® CellBIND® 96 well plates are recommended for optimal image clarity to demonstrate clonality. Plating density averages 0.5 cell/well.
    d. Incubate plates in static incubator 37°C, 5% CO_2. Avoid removing the plates from the incubator unless for feeding or imaging.
4. Assess wells for clonality:
    a. Clonality is demonstrated by confirming that cells in a well are derived from a single cell. Imaging analysis platform from Advanced Instruments' Solentim Cell Metric® or similar is recommended.
    b. Image wells on the day of plating (day 0) to identify those with a single cell. Ensure cells can be detected at the bottom of the well, by imaging at least an hour after plating or following brief centrifugation (200 rcf for 5 minutes).
    c. Image each well on days 1, 7 and 14 post-plating.
    d. Analyze images to validate clonality: only a single cell should be clearly identifiable on day 0. Later images such as day 7 and 14 will help indicate where to look for the single cell progenitor on day 0 images. Day 1 should show a doubling of the single cell or 1 cell if clones are growing and dividing slowly.

5. Clone maintenance (first 2 to 3 weeks):
    a. Depending on evaporative loss, feed 25 µL to 50 µL EX-CELL® CD CHO Fusion *without* L-glutamine to all wells once per week. It may be necessary to feed an additional 15 µL to 25 µL of EX-CELL® CD CHO Fusion *without* L-glutamine to the wells located around the edge of the plate, due to increased evaporation in these wells.
        i. Image before feeding if imaging is scheduled.
        ii. While feeding the plates, minimize handling that could cause individual cells to move within the well, as this can complicate the image analysis and clonality verification.
6. Clone consolidation and expansion:
    a. Optional: Each well can be triturated using a multi-channel pipette after imaging on day 14. This can break up cell clumps, increase confluence for colonies, and outgrowth.
    b. When the clones are 70% to 100% confluent, consolidate the confirmed clonal wells into fewer plates for ease of handling and screening.
        i. Prepare sufficient 96 well plates to accommodate clones with 150 µL of EX-CELL® CD CHO Fusion *without* L-glutamine per well.
        ii. Passage 1:4 (50 µL) from each clonal well of the original plate to a well in the new plate. (Note mini-pool of origin, location of original well, and new well)
7. Clone passaging, assay, scale-up:
    a. Passage clones into new 96 well plates once a week at 1:5 dilution (40 µL) until scale-up. Avoid letting clones become over-confluent.
    b. At the second passage, transfer to two 96 well plates: a culture plate and an assay plate for a 7-day static assay (refer to Protocol 8a).
    c. Using results from the 7-day static assay, scale-up the highest titer clones to shaking cultures (refer to Protocol 7). We recommend the top 30% or 100 clones.
    d. Perform 7-day batch and 14-day fed batch assays to select clones with highest expression (refer to Protocol 8b and Protocol 8c).
8. Cryopreserve the clones (refer to Protocol 4). These vials are the passage 0 or population doubling level 0 (PDL) and used for used in the stability study (refer to Protocol 10). These may also be needed for additional testing or characterization and process development.

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Protocol 10: Clone Stability Assessment

Purpose

The r-protein expression stability of clones is determined by comparing titer after clone isolation to titer after continuous expansion, modeling time to and during manufacturing process. Performing stability assessment is highly recommended because potential adverse events on the r-protein gene and its regulation may occur during continuous long cell culturing and may affect r-protein production. The productivity retention and the length of the stability assessment required is determined by your manufacturing process. We recommend comparing protein expression after 60 population doubling levels (PDL) and prefer clones that have at least 70% of initial r-protein productivity. Other clone performance criteria, such as growth, protein quality attributes, and genetic stability may also be considered.

The high PDL clones are generated from clones expressing most r-protein (refer to Protocol 8c) by continuous passage (refer to Protocol 3) and r-protein productivity is compared in cells before and after continuous passaging (refer to Protocol 8c).

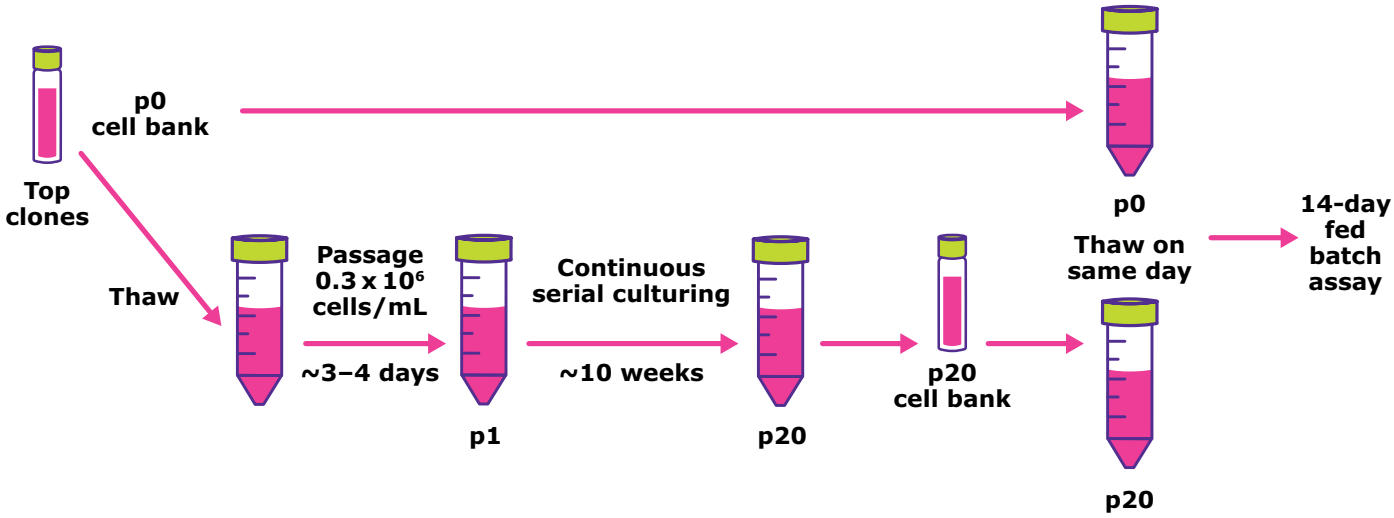

Figure 17: Clone stability assessment

Reagents and Equipment

- EX-CELL® CD CHO Fusion Medium *without* L-glutamine (SAFC 14365C).
- TPP50 TubeSpin® tubes (Z761028) or Sterile shake flask of appropriate volume.

Procedure

1. Prepare clones for stability assessment:

   NOTE Clones contain the GS coding cassette and should be maintained in EX-CELL® CD CHO Fusion Medium *without* L-glutamine.

   a. Thaw (refer to Protocol 2) "Passage 0" cells created from step 8 of Protocol 9b**.**

   b. Serially passage (refer to Protocol 3) the clones until the desired population doubling levels (PDL).

      i. The number of passages required is based on the PDL required by your manufacturing process.

      ii. 20 passages over 70 days is about 60 PDL.

   c. Cryopreserve passaged clones using 93% EX-CELL® CD CHO Fusion Medium *without* glutamine and 7% DMSO (refer to Protocol 4). Label banks with "Passage 20".

2. Compare r-protein expression at early and late passage.

   NOTE Clones contain the GS coding cassette and should be maintained in medium *without* L-glutamine.

   a. Thaw a "Passage 0" and "Passage 20" vial for each clone (refer to Protocol 2).

   b. Maintain clones from Passage 0 and Passage 20 for at least 2 passages (refer to Protocol 3) ensuring >90% viability is achieved.

   c. Compare r-protein production by Passage 0 and Passage 20 pairs for all clones using 14-day fed batch assay (refer to Protocol 8c).

   d. The ratio of the titer of the Passage 20 to the titer of the Passage 0 indicates stability of each clone.

   e. Clones maintaining 70% of the initial titer after extensive passaging are deemed suitably stable.

      NOTE Other criteria, such as growth, protein quality attributes, and genetic stability may also be considered.

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Protocol 11: Recombinant Protein Production in Bench-Top Bioreactors

Purpose

The assessment of clones in bench-top bioreactors is the best predictor of performance in manufacturing.

The number of clones evaluated in bioreactors will depend on the capacity of the facility. In addition to r-protein titer, specific protein quality attributes might be important and will influence the number of clones assessed. At a minimum, three clones should be assessed to give a good probability of identifying one with appropriate characteristics.

Protocol 11a: Small and large scale-up strategy

Protocol 11b: 14-day fed batch in a bench-top bioreactor

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Protocol 11a: Scale-Up to Bench-Top Bioreactor

Purpose

This seed train describes expansion of cell number and volume sequentially from TPP50 to bench-top bioreactor scale.

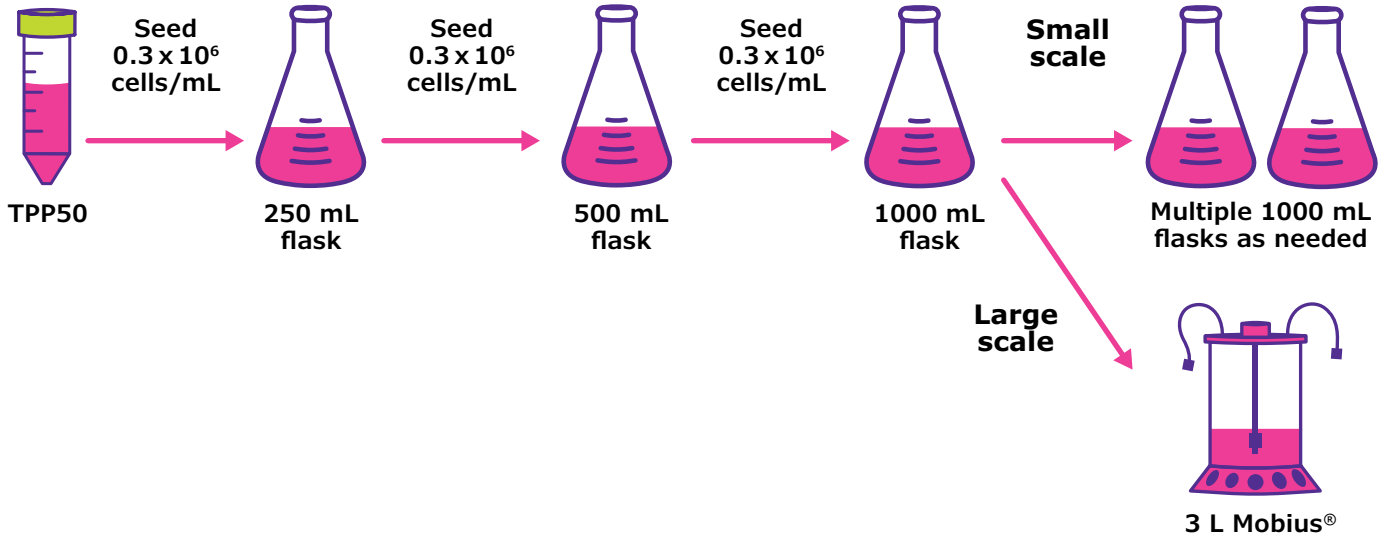

Figure 18: Scale-up to bioreactor workflow

Reagents and Equipment

- EX-CELL® CD CHO Fusion Medium *without* L-glutamine (SAFC 14365C /24365C).
- Sterile shake non-baffled vented cap culture shake flask (determine appropriate volume, Table 11).
- Bioreactor for animal cell culture (Mobius® 3L single use benchtop bioreactor CR0003L200 or similar).

Procedure

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

1. Small scale (shake flask scale-up method)
   Scale up cells in shake flasks for inoculation of microbioreactors or small-scale bioreactors. Table 11 shows the recommended working volumes for different flasks.

   a. Thaw a vial of each clone into EX-CELL® CD CHO Fusion *without* L-glutamine (refer to Protocol 2).

   b. Passage cells at 0.3x10^6 cells/mL EX-CELL® CD CHO Fusion *without* L-glutamine to shake flasks of sequentially increasing volume until sufficient cells for bioreactor seeding.

      NOTE After the initial passage, it may be possible to use EX-CELL® Advanced CHO Fed Batch Medium (SAFC 14366C/24366C) to scale-up to bioreactor. Testing is required to determine if this will affect the performance of clones.

Table 11: Recommended scale-up strategy in shake flasks

Culture Flask Size	Product Number	Medium Volume (mL)	Seed Density	Number of Days Normally Required
TPP50 *or* 125 mL shake flask	Z761028 CLS431143	20 to 30	0.3x10^6 cells/mL	3 to 4
250 mL shake flask	CLS431144	50 to 100	0.3x10^6 cells/mL	3 to 4
500 mL shake flask	CLS431145	150 to 200	0.3x10^6 cells/mL	3 to 4
1000 mL shake flask	CLS431147	300 to 400	0.3x10^6 cells/mL	3 to 4

2. Larger volume scale-up in a Mobius® bioreactor
   For the inoculation of larger bioreactors or multiple small bioreactors, scaling cells up in a Mobius® bioreactor may be a better option than using multiple shake flasks.

   a. Follow the Small Scale (Shake Flask Scale-up Method)- Protocol 11a, step 1 using EX-CELL® CD CHO Fusion *without* L-glutamine.

   b. Once the cells have been in suspension culture for at least 3 passages, inoculate using EX-CELL® CD CHO Fusion *without* L-glutamine and seeding at 0.3x10^6 cells/mL. Guidelines for the use of the Mobius® bioreactors for scale-up are available in the Scalability of the Mobius® Single-use Bioreactors documentation found at https://www.emdmillipore.com/.

   c. CHOZN® cells can be scaled up using an N-1 perfusion seed train as described in Preparing CHO Cells for Higher Productivity by Optimizing a Perfused Seed Train.

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Protocol 11b: Bench-Top Bioreactor Fed Batch

Purpose

14-day fed batch assay in bench-top bioreactor.

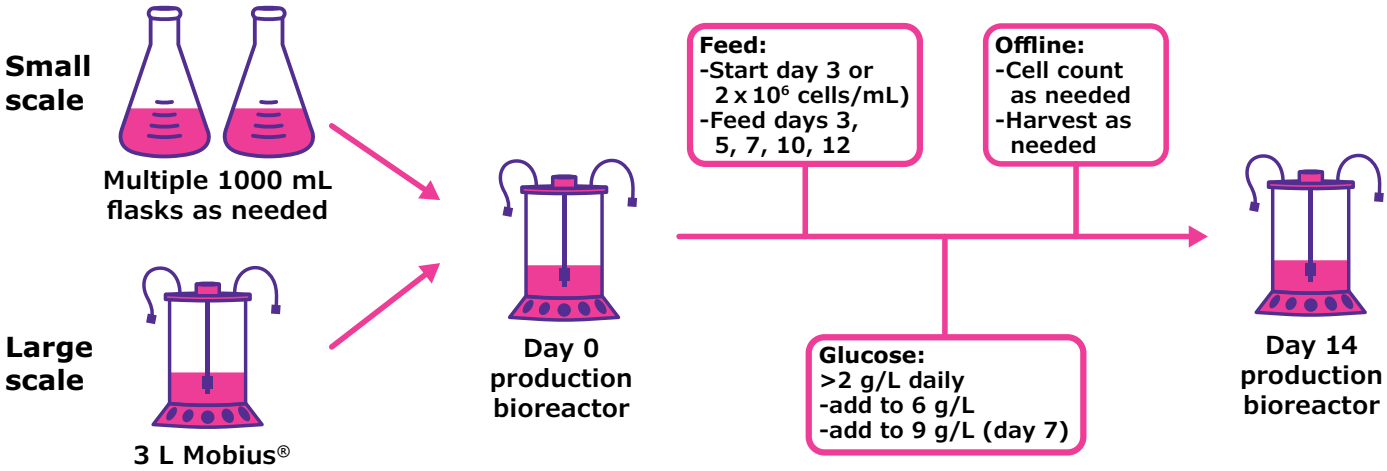

Figure 19: 14-day fed batch bioreactor

Reagents and Equipment

- EX-CELL® Advanced CHO Fed Batch Medium (SAFC 14366C).
- Cellvento® ModiFeed Prime COMP (104132).
- D-(+)-Glucose (45% solution) (G8769).
- Emprove® alternative: HTST Treated Glucose (50% w/v) SAFC 58955C
- Bioreactor for animal cell culture (Mobius® 3L single use benchtop bioreactor CR0003L200 or similar).

Procedure

1. Inoculate the bioreactor by seeding cells in EX-CELL® Advanced CHO Fed Batch medium at the desired inoculation density. Inoculation volume should not exceed more than 20% of the initial working volume. Initial working volume should be set between 60% to 80% of final working volume.
2. Bioreactor parameters such as pH, temperature and dissolved oxygen (DO) may need to be optimized for individual clones. Mobius® bioreactor documentation can be found at https://www.emdmillipore.com/, including:
   a. Scalability of the Mobius® Single-use Bioreactors
   b. Mobius® Single-use Bioreactors: Platform Scalability
   c. Accelerating Cell Line and Process Development

   NOTE Cells expanded in the EX-CELL® CD CHO Fusion Medium can be inoculated directly in the production medium EX-CELL® Advanced CHO Fed Batch Medium without any adaptation.
3. Maintain daily glucose levels above 2 g/L by adding glucose up to 6 g/L. On day 7, glucose addition is adjusted to 9 g/L for no glucose addition over the weekend. A glucose addition schedule is shown in Table 12.
4. Begin feeding Cellvento® ModiFeed Prime COMP starting at $2x10^6$ cells/mL or on day 3, whichever comes first. A feed schedule is shown in Table 12. Choose either a low or high feed schedule for each bioreactor. Continue adding feed every other day except on days 8 and 9, which are the weekend days, until the end of the run. The feed addition and schedule should be optimized for individual clones.
5. Collect samples as appropriate for metabolite, titer and product quality analysis.

Table 12: Bioreactor feed schedule

	Total Feed%	Day										
		1	2	3	4	5	6	7	10	11	12	13
Cellvento® ModiFeed Prime COMP (V/V) -Pick one feed % (low or high)	Low (17.5%)	-	-	3%	-	3%	-	5.5%	3%	-	3%	-
	High (22.5%)	-	-	4%	-	4%	-	6.5%	4%	-	4%	-
Glucose (up to) (g/L)	-	6	6	6	6	6	6	9	6	6	6	6

Appendix 1: Service Offerings

BioReliance offers comprehensive services to support biotherapeutics from development and manufacturing. A selection of relevant services are listed below. A full list of services can be found at https://www.bioreliance.com/.

1. Cell Line Development: https://www.sigmaaldrich.com/US/en/services/contract-manufacturing/cell-line-development
2. Process Development
3. Cell Line Sterility
4. Master Cell Bank Manufacturing
5. Monoclonal Antibody Manufacturing

Appendix 2: Abbreviations

Abbreviation	Meaning
bp	Base pair
CD	Chemically Defined
CHO	Chinese Hamster Ovary
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
GS	Glutamine Synthetase
MSX	L-Methionine Sulphoximine
PDL	Population Doubling Level
r-protein	Recombinant protein
SCC	Single Cell Cloning
VCD	Viable Cell Density
ZFN	Zinc Finger Nuclease

Appendix 3: Development of the CHOZN® GS-/- Cell Line

The CHOZN® GS-/- cell line is a suspension-adapted CHO-K1 (Chinese Hamster Ovary) mammalian cell line that has glutamine synthase (GS) activity removed by Zinc Finger Nuclease (ZFN). 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 supplementation requirement (i.e. dihydrofolate reductase, DHFR) or inhibition of key metabolic enzyme (i.e. 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 (or via transgene-mediated GS expression). These unique cells were generated using our CompoZr™ ZFN technology to modify the CHO-K1 cell line (Sigma 85051005).

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 targets in the biopharmaceutical industry. Cells that lack a functional GS enzyme or have this 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 co-transfected with an exogenous GS gene. However, MSX may pose certain hazards and its avoidance of use in a biopharmaceutical cell culture process has many advantages (i.e. less regulatory scrutiny, lower production costs, less variability, etc). Use of MSX is also discouraged as it increases toxic metabolic wastes and reduces stability (Jun et al., 2006). 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. These cells are cGMP cryopreserved 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.

ZFNs are a class of engineered DNA-binding proteins, which facilitate targeted genome editing by binding to a user-specified locus to induce 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.

The suspension adapted CHO-K1 cell line was transfected with the CompoZr™ GS ZFN pair (ZFNGSA9075/ZFNGSB9372, Sigma catalog number ZFNGS), which targets 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 (Figure 20).

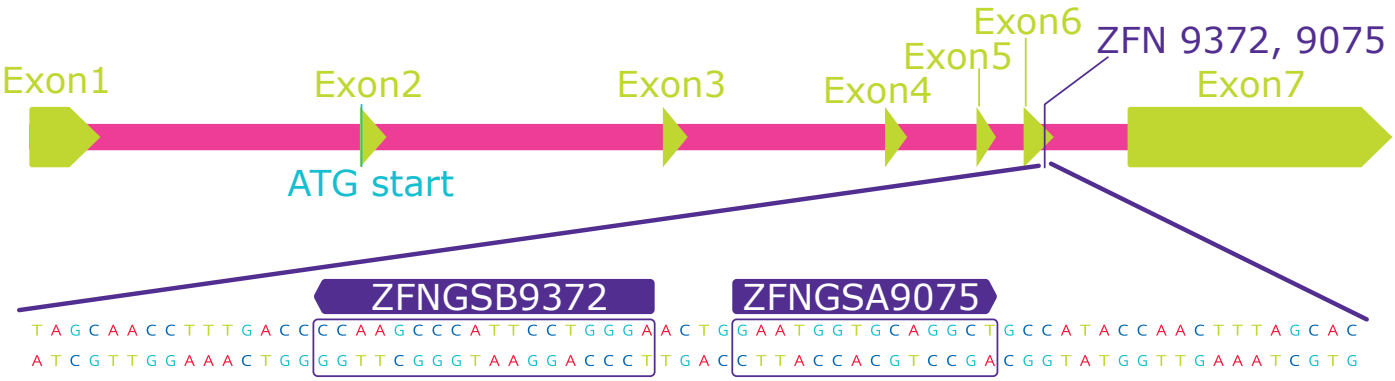

Figure 20: This is a schematic representation of the CHO GS gene and targeted sequence modification sites. The ZFN target site is indicated with the ZFN binding sites identified in the boxes. The ZFN9075/9372 pair target site is located within exon 6 of the GS gene, which codes for the substrate-binding domain of the protein.

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 at the GS locus. After extensive characterization of the clones, one clone was identified as having more robust characteristics than the others (Table 13). The clone ID for this CHOZN® GS-/- cell line is 2E3. Cells from clone 2E3 were subsequently cryopreserved under cGMP conditions in EX-CELL® CD CHO Fusion Medium with L-glutamine and were thoroughly characterized according to FDA and EMEA testing requirements for master cell banks.

Table 13: The sequence of the GS mutations within Clone 2E3 (CHOZN® GS-/- cell line) caused by ZFN transfection of the CHO-K1 cell line

Allele	Sequence	Modification
WT Reference	CCAAGCCCATTCCTGGGAACTGGAATGGTGCAGGCT	Reference
Clone 2E3 Allele 1	CCAAGCCCATTCCTGGGAACT---------CA-GCT	10bp deletion
Clone 2E3 Allele 2	CCAAGCCCATT----------------TGCAGGCT	17bp deletion

The biallelic disruption of both copies of the GS gene is noted. Allele 1 has a 10 base deletion and allele 2 has a 17 base deletion.

Appendix 4: EX-CELL® Advanced CHO Feed 1 Feed Schedule for 14-Day Fed Batch Assay

Purpose

This is the feed schedule for use of EX-CELL® Advanced CHO Feed 1 as the feed for 14-day fed batch. EX-CELL® Advanced CHO Feed 1 was the previously recommended feed for CHOZN® GS-/- cells but since has been replaced with Cellvento® ModiFeed Prime.

Reagents and Equipment

- EX-CELL® Advanced CHO Feed 1 (24367C with glucose /24368C without glucose)

Procedure

1. To use EX-CELL® Advanced CHO Feed 1 instead of Cellvento® ModiFeed Prime COMP, refer to Table 14 replacing the feed from Table 8.
   a. In Protocol 8c, replace step 3F.
   b. On day 3, 5, 10, and 12, add 5% (v/v, 1.25 mL) of EX-CELL® Advanced CHO Feed 1.
   c. On day 7, add 7.5% (v/v, 2.25 mL) of EX-CELL® Advanced CHO Feed 1.

   NOTE If using EX-CELL® Advanced CHO Feed 1 with glucose (24367C, 30 g/L of glucose), subtract the glucose to be added by the amount in the feed.

   a. Example part 1: If 5% feed is to be added into 25 mL *Assay TPP50*, then 1.25 mL of 24367C feed is to be added, which will contain 0.0375 g of glucose (similar to 1.5 g/L glucose).
   b. Example part 2: If 6 g/L glucose is to be added into 25 mL *Assay TPP50*, then 0.15 g of glucose is required and subtracting the 0.0375 g of glucose from the feed, then only 0.1125 g of glucose is required to be added from D-(+)-Glucose (45% solution) Sigma-G8769.

Table 14: Feed addition schedule for EX-CELL® Advanced CHO Feed 1

	Day					
	3	5	7	10	12	14
Cell counting	Y	Y	Y	Y	Y	Y
Store supernatant for titer analysis	-	-	Y	Y	Y	Y
Measure Glucose	Y	Y	Y	Y	Y	Y
Glucose	to 6 g/L	to 6 g/L	to 9 g/L	to 6 g/L	to 6 g/L	-
EX-CELL® Advanced CHO Feed 1	5%	5%	7.5%	7.5%	5%	-

References

- Jun, S.C., Kim, M.S., Hong, H.J., and Lee, G.M. (2006). Limitations to the Development of Humanized Antibody Producing Chinese Hamster Ovary Cells Using Glutamine Synthetase-Mediated Gene Amplification. Biotechnol. Prog. *22*, 770–780.
- Puck, T.T., Cieciura, S.J., and Robinson, A. (1958). Genetics of somatic mammalian cells. III. Long-term cultivation of euploid cells from human and animal subjects. J. Exp. Med. *108*, 945–956.
- WHO (2020). Laboratory biosafety manual Fourth edition.

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