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User Guide

CHOZN® Elite Cell Line

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Contents

Overview

The CHOZN® Platform is a CHO (Chinese Hamster Ovary) mammalian cell expression system designed for the selection and scale-up of stable clones that produce recombinant proteins (r-proteins). The core of the CHOZN® Platform is the CHOZN® Elite CHO cell line, which requires glutamine supplemented in the medium due to disruption of the endogenous glutamine synthetase (GS) gene using CompoZr™ zinc finger nuclease (ZFN) technology. Refer to Development of the CHOZN® Elite Cell Line for more information regarding the development of the CHOZN® Elite CHO cell line.

Transfection with a plasmid containing a GS transgene enables metabolic selection in glutamine-free medium during r-protein production. The CHOZN® Platform contains chemically defined (CD) media for cell expansion and cloning, as well as a media and feed designed to support cell growth and r-protein production.

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

Precautions and Disclaimer

The CHOZN® Elite 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.

Development of the CHOZN® Elite Cell Line

CHOZN® Elite was derived from the CHOZN® GS$^{-/-}$ (CHOGS-1VL) host and selected for its improved bioproduction phenotype. These phenotypes included predictable growth, high r-protein productivity, and stability. The derivation of the cell line consisted of three distinct development phases.

During the first phase, the CHOZN® GS$^{-/-}$ host was transfected with a plasmid with a benchmark IgG transgene flanked by wild type LoxP sites. After mini-pool selection, clones were obtained by dilution cloning from the top-expressing mini-pools. Clones containing only one stable integration of the benchmark transgene underwent fed batch assays to determine growth and productivity. Clones displaying enhanced growth and productivity compared to controls proceeded to phase two. In phase two, the stably integrated transgene was removed. This was done by introducing Cre mRNA to cells via electroporation. Post-recovery, transfected pools were cloned by limiting dilution. To identify suitable hosts, these clones were screened by ddPCR, surface staining, NGS, and selection to demonstrate that the transgene was fully removed and that no r-protein or glutamine synthetase expression was detected. In addition, a single LoxP sequence was to be left at the integration site intact, which signified correct operation of the Cre protein.

In the final phase, host candidates were tested in CLD with a new IgG and Fc-fusion protein. CHOZN® Elite was identified as the clone with phenotypes most advantageous for bioproduction.

Media, Feeds, Supplements and Reagents

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® Elite Cells	BioReliance	ELITE-1VL
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
pCGS3.2 standard CHOZN® GS plasmid	Aldevron	PCGS32-1VL

Plasticware and Equipment

Table 1: Equipment descriptions and recommendations

Equipment	Description/Recommendations
25 cm^2 and T-75 cm^2 suspension culture flasks	Greiner Bio-one 690195 (C6731) and 658195 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 plates for cloning	Corning® CellBIND® CLS3300 or similar
24 well suspension plates	Greiner Bio-one 662102 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
Electroporation instrument	Bio-Rad Genepulser® or similar
Controlled rate freezing vessel (1°C/minute cooling)	Nalgene Mr Frosty™ or similar

CHOZN® Elite Cell Line Handling Protocols

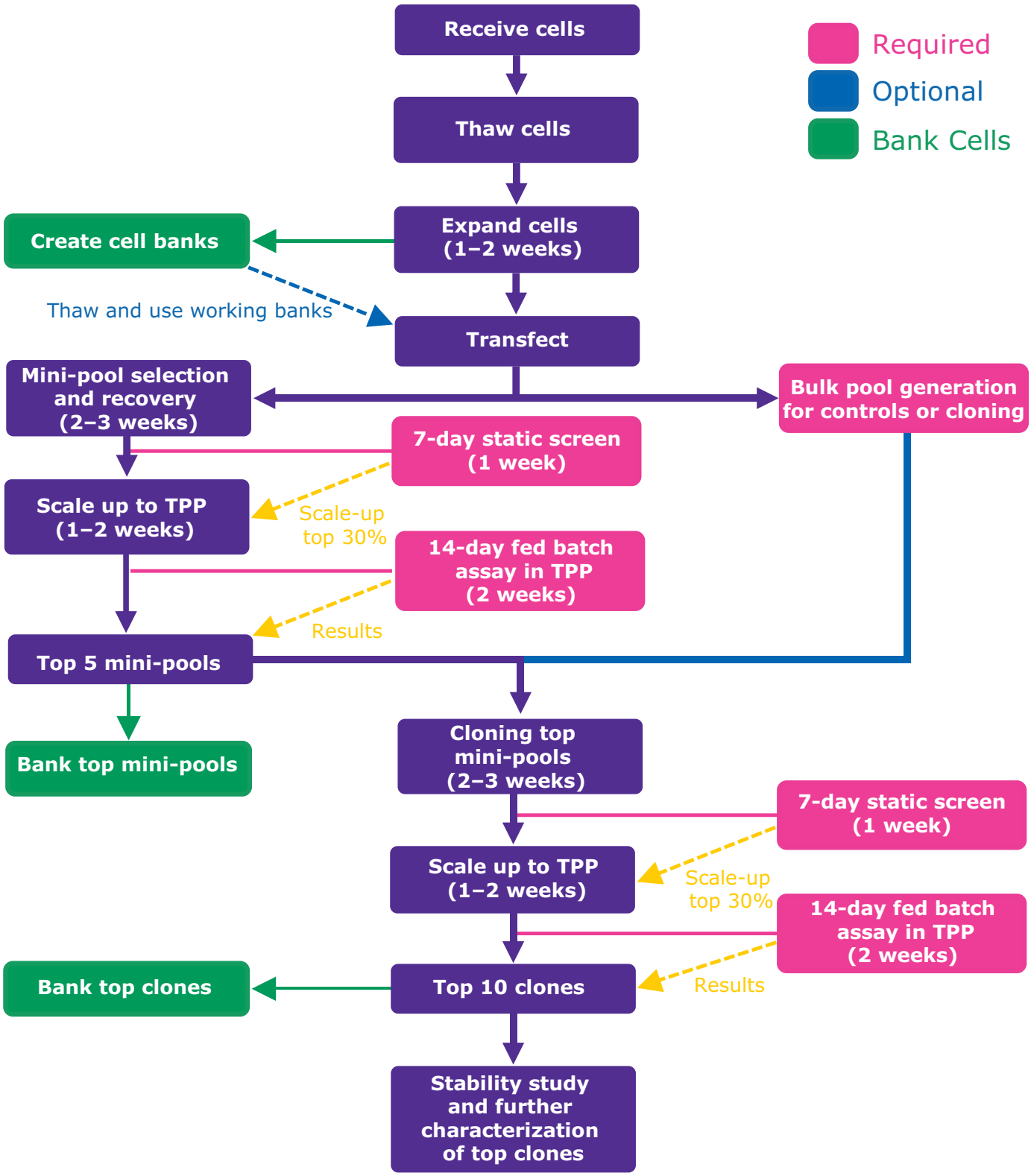
NOTES The following procedures should only be performed by personnel trained to:

- 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.

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.

Interactive CHOZN® Elite Cell Line Flow Chart and Timeline for Mini-Pool Process

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



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Protocol 1: Thawing CHOZN® Elite Cells

Purpose

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

NOTE Parental CHOZN® Elite cells require EX-CELL® CD CHO Fusion Medium *with* 6 mM L-glutamine.

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

Reagents and Equipment

- T-75 cm^2 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® Elite cells (SAFC® ELITE-1VL).

  NOTE CHOZN® Elite cells are cryopreserved at approximately 10 x 10^6 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 for parental CHOZN® Elite cells.
- EX-CELL® CD CHO Fusion Medium *without* L-glutamine (SAFC® 14365C) for pools and clones.

Table 2: Recommended vessel and volume for initial thaw

Vessel	Cells	Volume (mL)	Orbital Shaker#
T-75 cm^2 suspension flask	<5x10^6 cells	10 to 15	None
TPP® (TPP50) TubeSpin tube	>5x10^6 cells	20	• 50 mm throw, 200 RPM • 25 mm throw, 285 RPM
125 mL shake flask	>5x10^6 cells and not sensitive	22 to 50	• 50mm throw, 90 RPM • 25mm throw, 125 RPM

#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.
    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® Elite cells (1 mL), 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. Invert vial three times then carefully transfer cells 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 warm EX-CELL® CD CHO Fusion Medium. Slowly pipette up and down to break up the cell clumps.
    f. Transfer the 10 mL cell suspension to T-75 cm^2 suspension flask or TPP50 guided by Table 2.

3. Initial cell culture:

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

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.

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.

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

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 2: Culturing CHOZN® Elite Cells.

b. TPP® (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.

iii. Proceed to Protocol 2: Culturing CHOZN® Elite Cells.

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Protocol 2: Culturing CHOZN® Elite Cells

Purpose

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

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® Elite 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.
2. Determine viable cell density and viability. Prepare to passage into new TPP50 at a density of $0.3x10^6$ cells/mL in no more than 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.
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 no more than 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	≤ 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 3: Cryopreservation of CHOZN® Elite Cells

Purpose

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

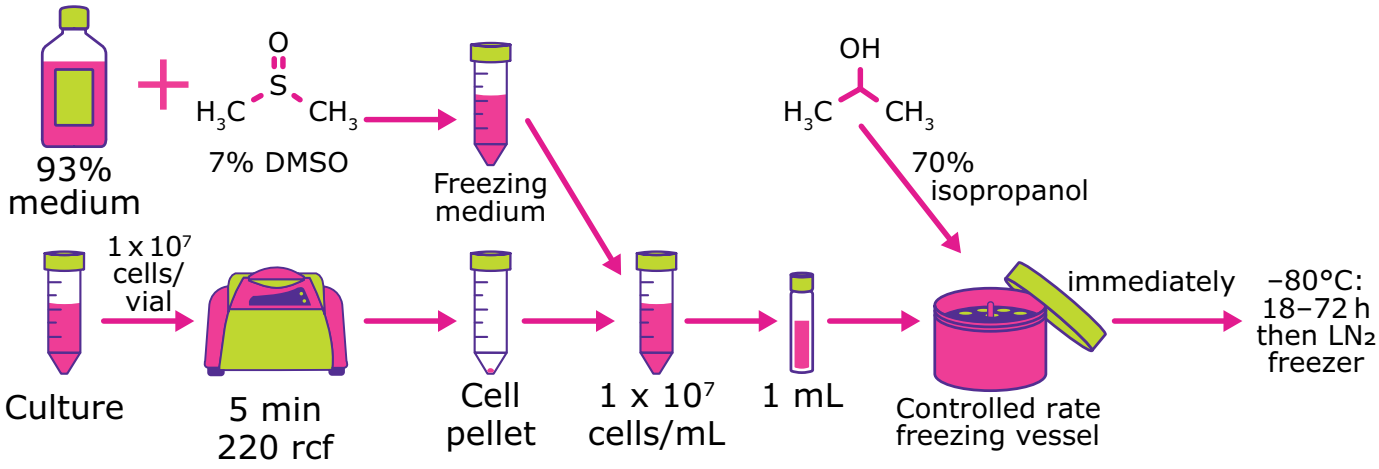

Figure 1: Cryopreservation of CHOZN® Elite cells

Reagents and Equipment

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

  NOTE Use fresh DMSO from an unopened bottle to ensure sterility.
- 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 1.2 times the freezing medium needed by mixing 93% EX-CELL® CD CHO Fusion Medium (with L-glutamine, if necessary) and 7% DMSO.
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 1 and Protocol 2 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 appropriate vessels 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 4: Transfection of CHOZN® Elite Cells

Purpose

This protocol explains how to transfect CHOZN® Elite cells using electroporation with the pCGS3.2 plasmid, achieving 60 to 80% efficiency. Figure 2 shows a fluorescent reporter expressed in transfected cells.

The number of mini-pools or bulk pools to be created will determine how many cells and transfections are needed. The amount of transfections also depends on the equipment used. This protocol is optimized for the Bio-Rad Gene Pulser® with 4 mm cuvettes.

To ensure enough cells for mini-pool plating, transfect twice the required number. If multiple transfections use the same plasmid, they can be combined into one pool before plating.

The pCGS3.2 plasmid is recommended for the CHOZN® Elite GS platform and is designed to support high expression of recombinant proteins, such as monoclonal antibodies. It includes a glutamine synthetase (GS) cassette, allowing cells to grow without glutamine after plasmid integration and selection.

NOTE Chemical based transfection may be used with the CHOZN® Elite cells. Mirus Bio CHOgro® High Yield Expression System (MIR 6200) is recommended for transient transfection. 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.

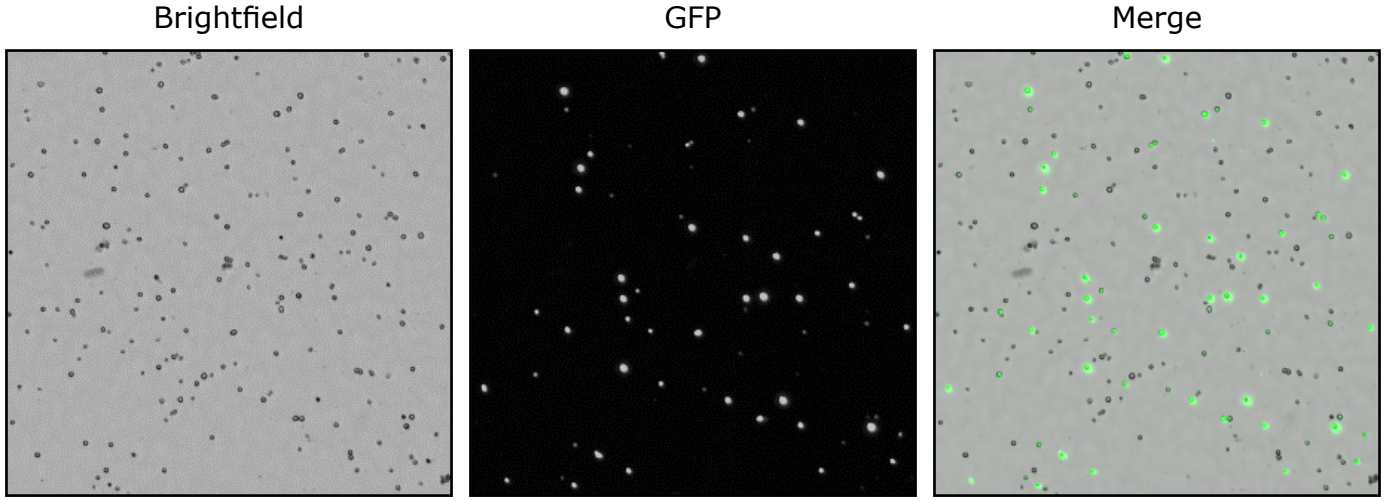

Figure 2: pCGS3.2-GFP transfected CHOZN® Elite cells

Images of pCGS3.2-GFP transfected CHOZN® Elite 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 5: Transfection

Purpose

Transfection of plasmid DNA into CHOZN® Elite cells.

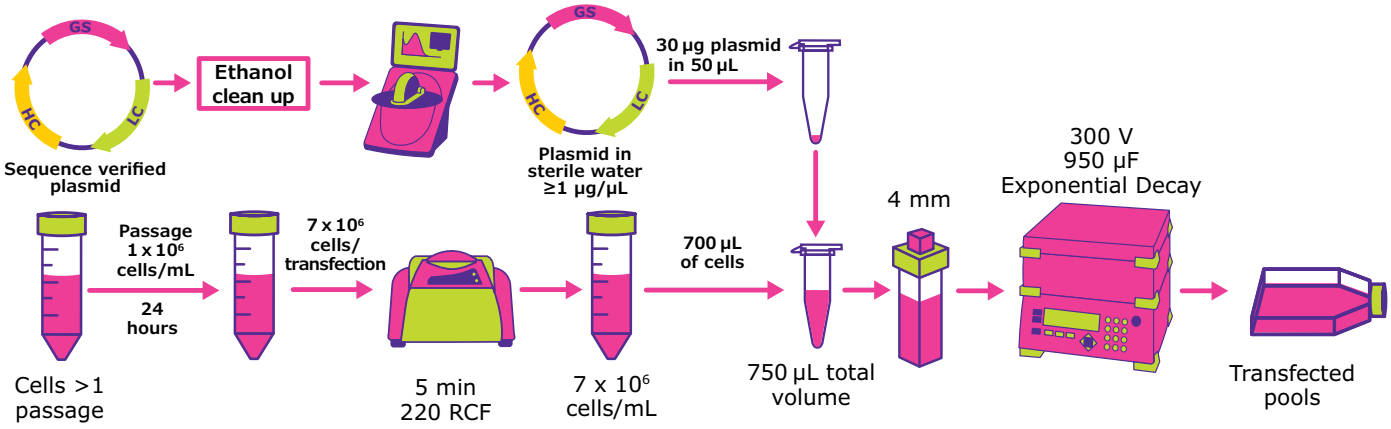

Figure 3: Transfection workflow

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.
- 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 are specific to the Bio-Rad Gene Pulser®.

Procedure

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.*
        iii. Calculate the amount of DNA required for the transfection protocol to ensure enough has been purified.
            1. For Bio-Rad Gene Pulser®, use 30 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 culture vessel with 1.2x the required number of CHOZN® Elite cells at $1x10^6$ cells/mL in EX-CELL® CD CHO Fusion Medium *with* L-glutamine.
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*.
    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 until minimum viability is met.
   b. Use $7x10^6$ cells for each transfection and transfer to 15 mL or 50 mL conical tube.
   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 30 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.
   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. If a fluorescent protein positive control was included, analyze by flow cytometry analysis or fluorescent imaging 24 hours post-transfection, then discard.
      i. If the cells are <30% fluorescently expressing, consider repeating the transfection.

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Protocol 6: Selection and Recovery

Purpose

Selection may proceed either by mini-pools or bulk pools. Although the generation of mini-pools lengthens the cell line development timeline, they increase the likelihood of isolating higher producing clones, because a limited number of transfected cells are included in each mini-pool. Clones are 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 9 to 12 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 (Figure 4) 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. Proceed to Protocol 6a for instructions on the mini-pool selection method.

Rather than serving as only a control, clones may be derived directly from bulk pools instead of mini-pools. Bulk pool selection dramatically decreases the cell line development timeline and R&D banks may be generated between 80 and 90 days after transfection, however, this shorter timeline may result in lower productivity top clones. Bulk selection pools are generated by transferring the entire transfection culture to glutamine-free media. They recover in 9 to 12 days, after which productivity will be assessed to determine the pools from which to clone. Proceed to Protocol 6b for bulk pool selection method.

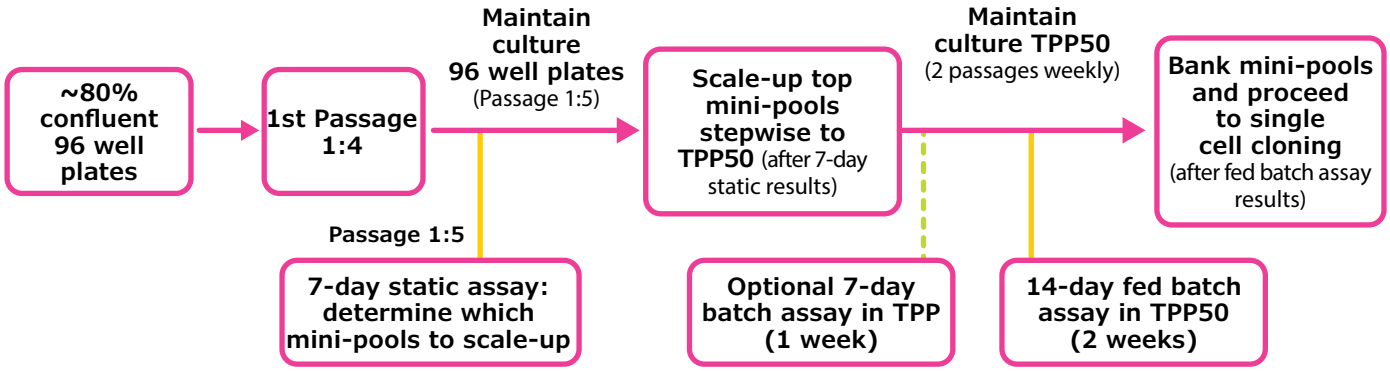

Figure 4: Mini-pool selection and recovery workflow

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Protocol 6a: Plating Mini-Pools

Purpose

Mini-pool plating about 24 hours post-transfection.

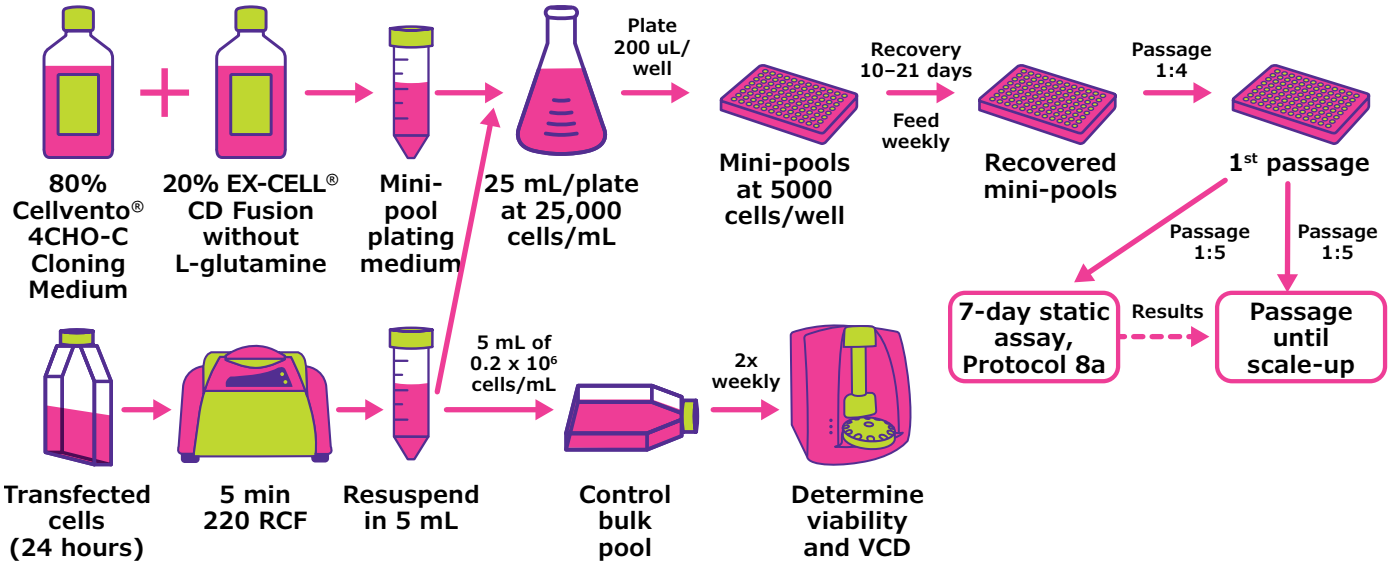

Figure 5: Mini-pool plating, selection, recovery, assay workflow

Reagents and Equipment

- Transfected cell culture (approximately 24 hours post-transfection, from Protocol 5).
- 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).
- Multi-channel 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. 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 to 25 cm^2 flask and add EX-CELL® CD CHO Fusion Medium *without* L-glutamine to 5 mL total volume (5 mL of $0.2x10^6$ cells/mL).

    - 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, evaluate confluency on day 14. If wells are approximately 80% confluent, proceed to step 3a. If not, add 40 µL of EX-CELL® CD CHO Fusion Medium *without* L-glutamine to each well to replace evaporated media.

c. If colonies begin to form bi-layers (typically between days 11 and 14), cells may be dispersed by gently triturating by pipette to enhance outgrowth.

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 selection recovery.

   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 culture + 160 µL medium) 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 at least 80 mini-pools.

e. Perform 7-day batch (optional) 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 3.

Return to Flow Chart

Protocol 6b: Bulk Pool Selection

Purpose

Bulk pool selection to be initiated about 24 hours post-transfection.

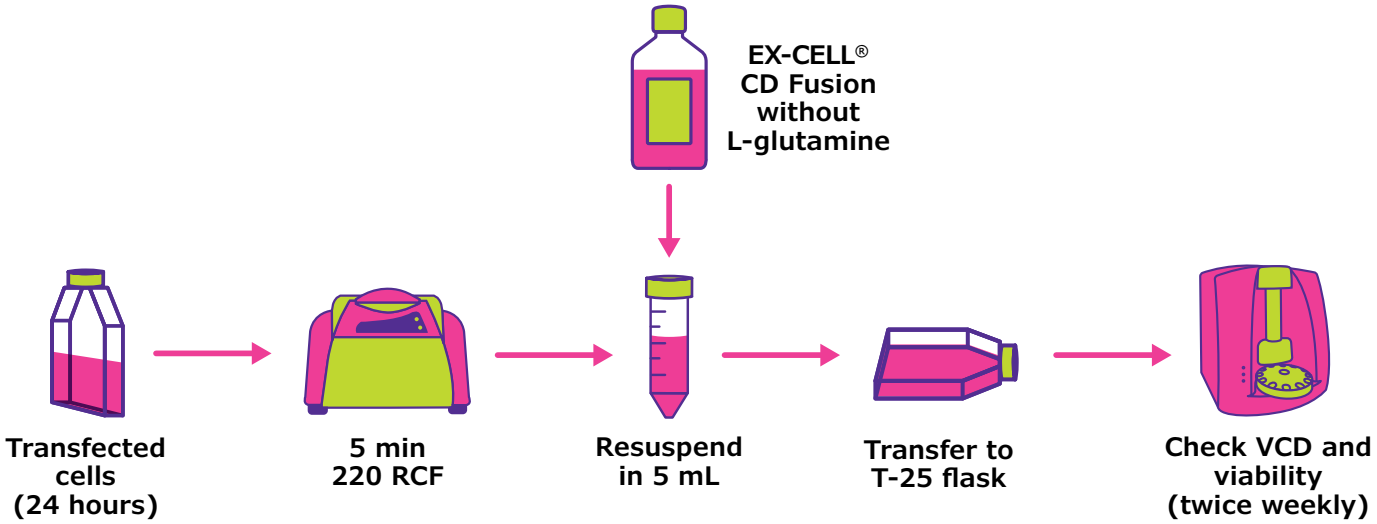

Figure 6: Bulk pool selection, recovery, assay workflow

Reagents and Equipment

- Transfected cell culture (approximately 24 hours post-transfection, from Protocol 5).
- EX-CELL® CD CHO Fusion Medium *without* L-glutamine (SAFC® 14365C/24365C).
- T-25 cm^2 suspension cell culture flasks (C6731 or similar).

Procedure

1. Bulk pool seeding:
    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. For each transfected culture, 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.
    e. Determine viability and viable cell density of the resuspended transfected cultures.
2. Stable bulk pool recovery:
    a. Determine viable cell density and viability twice weekly after day 5. Gently triturate to include attached cells in the count.
    b. Add 1.5 mL of EX-CELL® CD CHO Fusion Medium *without* L-glutamine weekly.
    c. Scale up and cryopreserve cultures when recovered (>90% viability and stable doubling time, Protocol 4).
3. Stable bulk pool assays:
    a. Perform a 7-day batch assay on all bulk pools (Protocol 8b). To shorten the assay, top bulk pools may be determined by analyzing supernatant for titer on day 4.
        i. Optionally, a 14-day fed batch assay (Protocol 8c) may be performed to gather more data on the bulk pools or collect early r-protein for testing.
    b. Proceed to Protocol 9 for limiting dilution cloning.

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 7. 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.

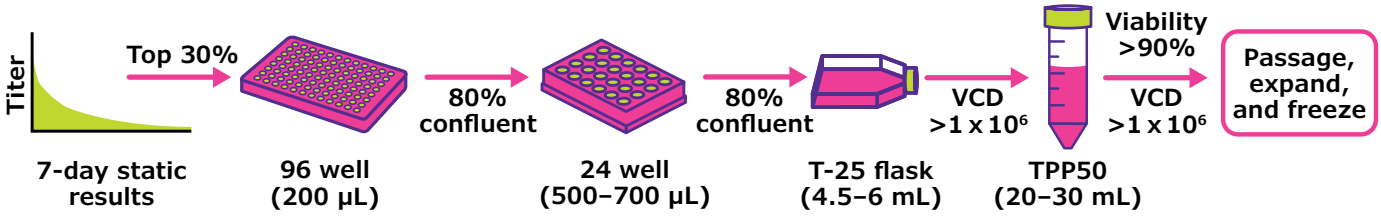

Figure 7: Mini-pool/clone scale-up workflow

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 the strategy outlined in Table 6.
2. 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 2.
    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 3, 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 µL per well	80% confluent	3 to 5
T-25 cm^2 flask	Greiner-one 690195	5 mL	VCD >1x10^6 cells/mL	3 to 5
TPP® (TPP50) TubeSpin tube	MilliporeSigma Z761028	20 to 30 mL	Passage twice weekly to 0.3x10^6 cells/mL	3 to 4

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Protocol 8: Recombinant Protein Assays

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, a relatively high number of 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.

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 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.

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

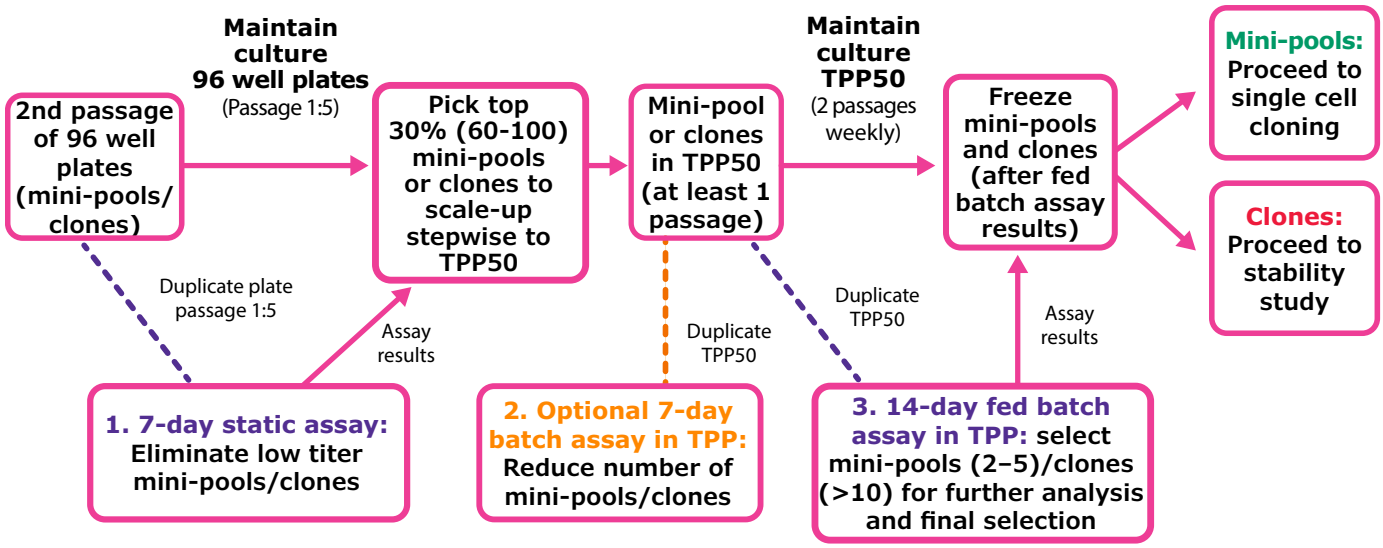

Figure 8: R-protein assays and decisions workflow for mini-pools and clones

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 Bulk pools: 1	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.

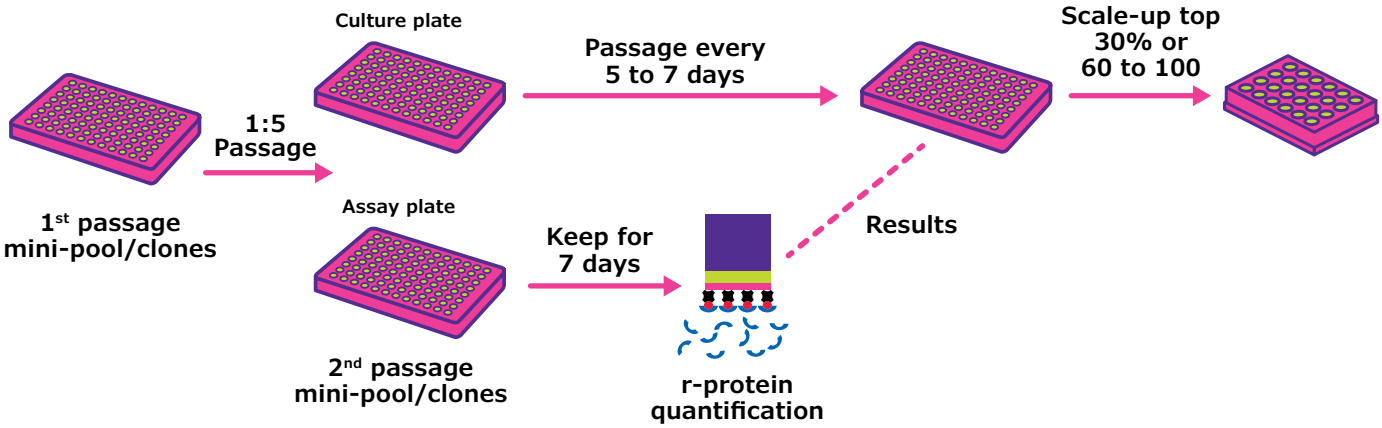

Figure 9: 7-day static assay workflow

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 is 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. It is also the first r-protein assay performed on bulk pools. 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.

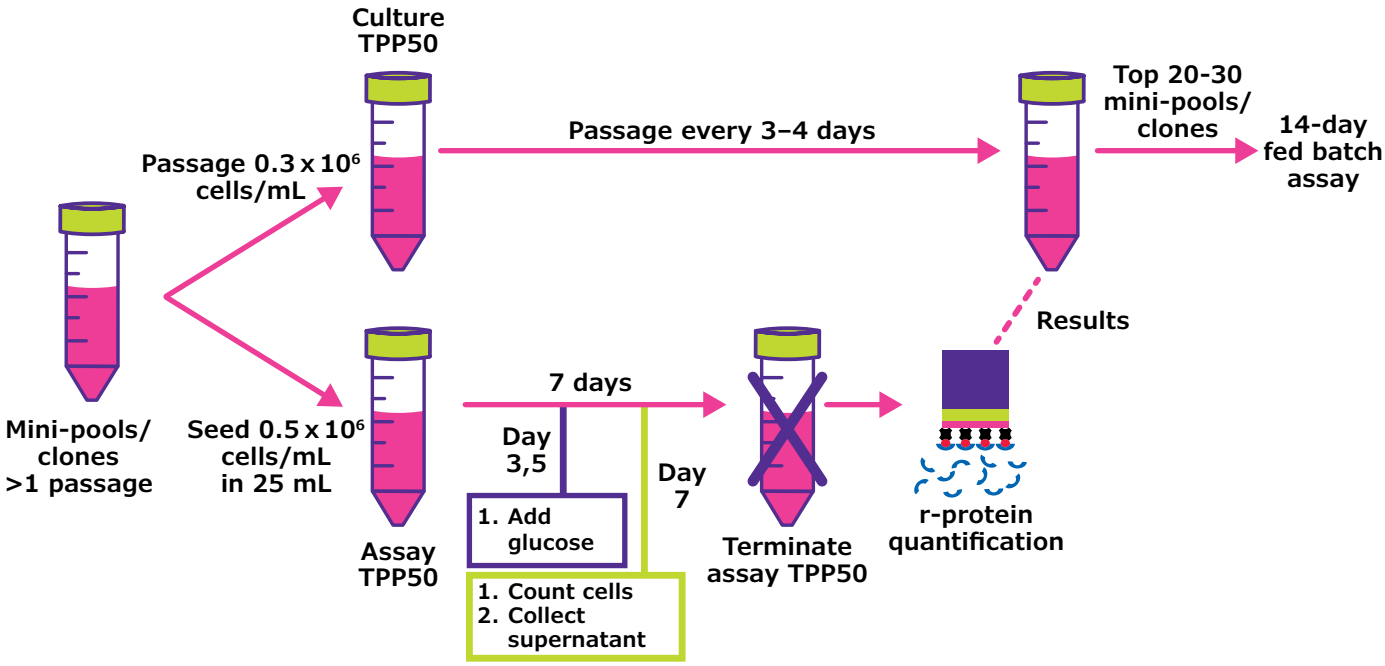

Figure 10: 7-day batch assay workflow

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 (20°C to 22°C) 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 2, 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, which is 6 g/L on top of what is already present. This is a blind feed.

4. On day 7, count cells, then collect and 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.

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Protocol 8c: 14-Day Fed Batch Assay

Purpose

This is the final and most predictive of the r-protein assays. The number of candidate 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.

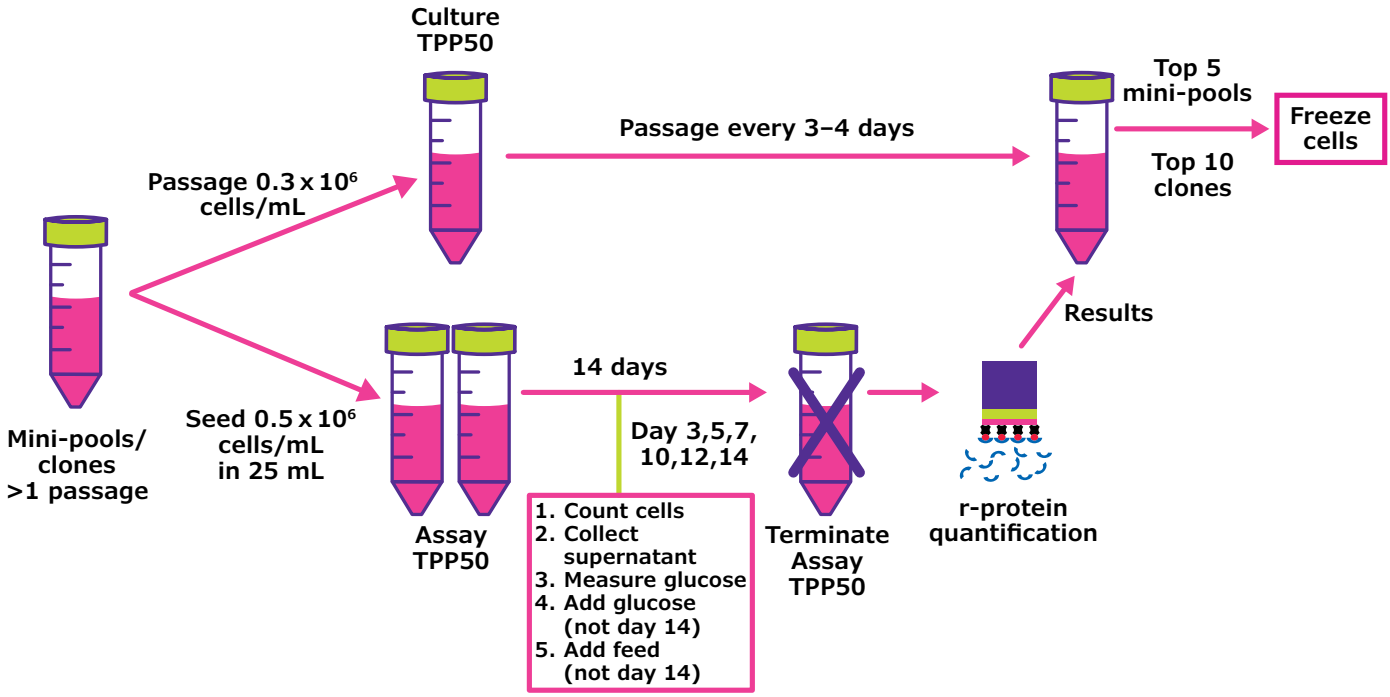

Figure 11: 14-day batch assay workflow

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 (20°C to 22°C).
    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 2, 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 1 mL sample and clarify via centrifugation 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. Assay glucose using half the clarified supernatant.
        ii. 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 enough glucose so the final concentration is 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).
    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.
    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.

g. Evaluate pools to clone from by considering titer, specific productivity, growth characteristics and protein quality attributes, if available. Typically, 2 to 5 mini- pools or 1 to 2 bulk pools are selected. Cloning is guided by Protocol 9. If working with clones, select at least 10 for further analysis, such as stability assessment, guided by Protocol 10.

h. Refer to Protocol 3 for cryopreservation.

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% (0.75 mL)	3% (0.75 mL)	5.5% (1.38 mL)	3% (0.75 mL)	3% (0.75 mL)	-

Return to Flow Chart

Protocol 9: Clone Isolation by Limiting Dilution

Purpose

Single cell clones (SCC) are isolated from mini-pools or bulk pools. Cloning from multiple pools increases the likelihood of finding a clone that meets project criteria. Clones are isolated from each 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. Alternatively, instruments such as flow cytometers or cell printers may be used to vastly increase outgrowth.

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.

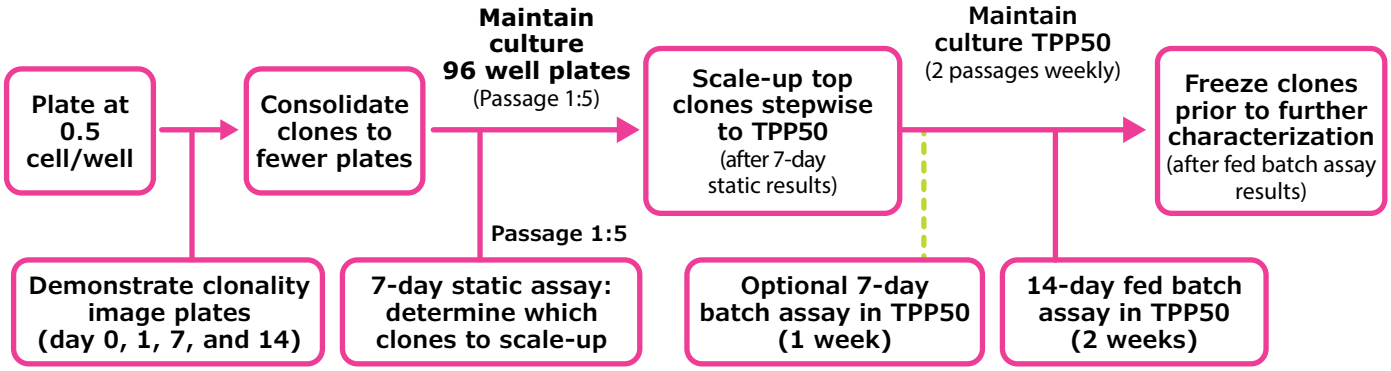

Figure 12: Clone isolation, recovery, and assay workflow

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

Prepare clone plating medium for clone isolation. Cloning medium included 20% of conditioned medium from the established pool. The inclusion of conditioned medium helps to improve clone outgrowth.

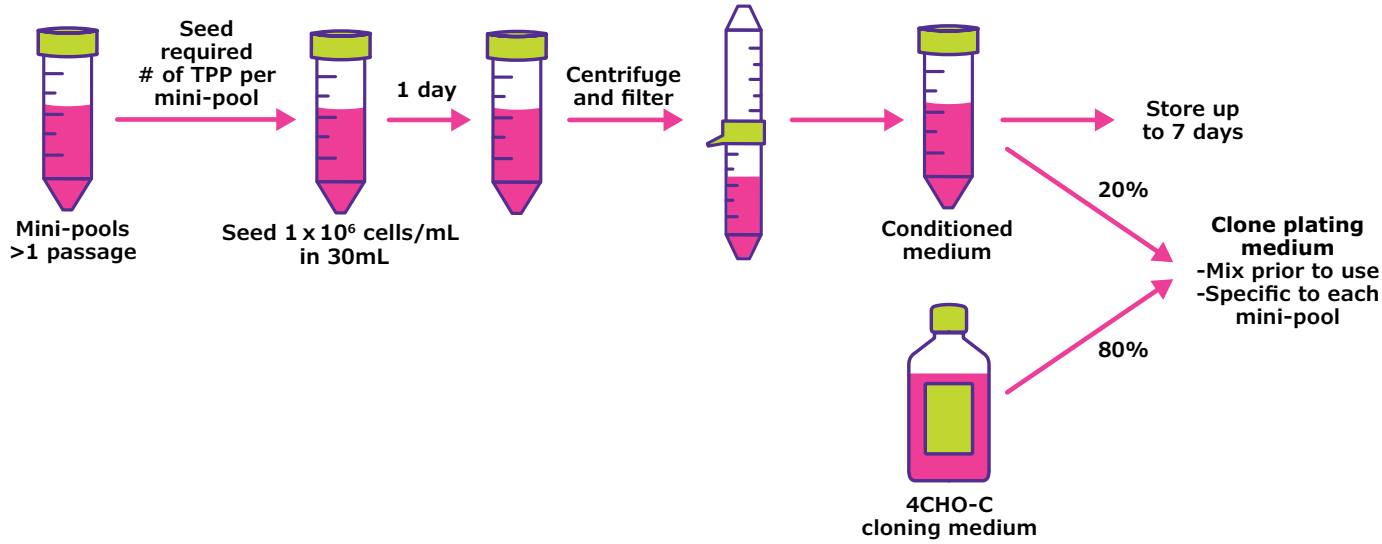

Figure 13: Clone plating medium preparation

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 2.
    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 (20°C to 22°C).
    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 (refer to 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 step 1 above)	20%
Cellvento® 4CHO-C Cloning Medium	14390C	80%

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Protocol 9b: Single-Cell Cloning by Limiting Dilution

Purpose

Clone isolation by limited dilution averaging 0.5 cells/well.

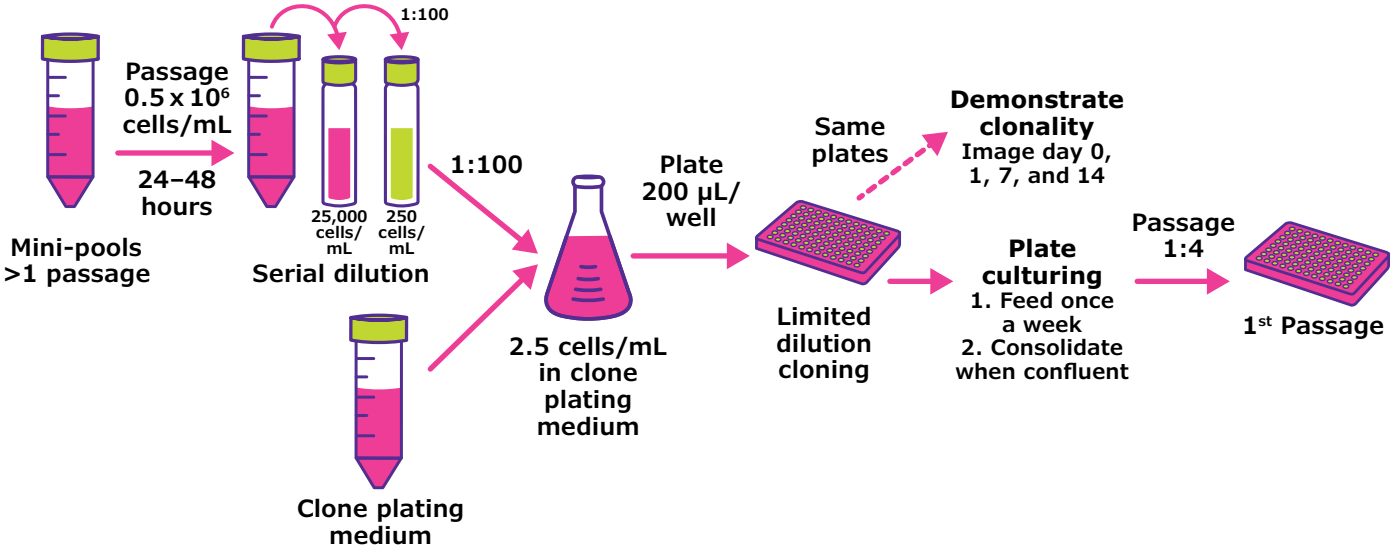

Figure 14: Limited dilution cloning workflow

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. On Day 14, if wells are approximately 70% confluent, proceed to step 6b. Otherwise, feed 40 µL EX-CELL® CD CHO Fusion to replace evaporated media.
        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 to establish Research Cell Banks (RCBs) (refer to Protocol 3). 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.

Return to Flow Chart

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 2) and r-protein productivity is compared in cells before and after continuous passaging (refer to Protocol 8c).

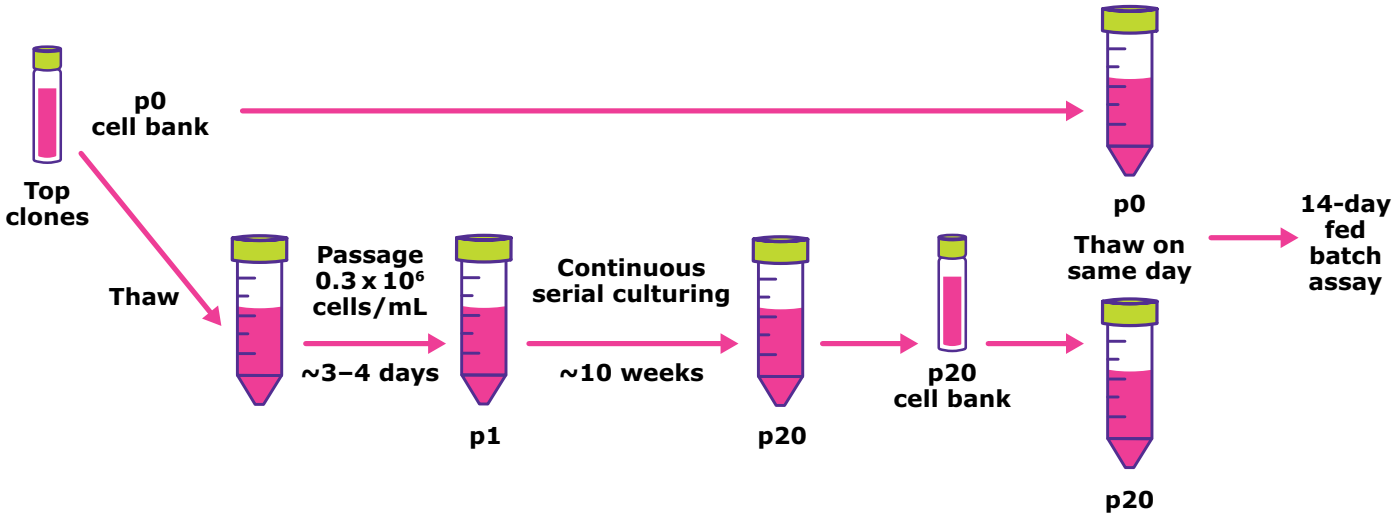

Figure 15: Clone stability assessment

Reagents and Equipment

- EX-CELL® CD CHO Fusion Medium *without* L-glutamine (SAFC® 14365C).
- TPP® (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 1) "Passage 0" cells created from step 8 of Protocol 9b.

   b. Serially passage (refer to Protocol 2) 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 3). 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 1).

   b. Maintain clones from Passage 0 and Passage 20 for at least 2 passages (refer to Protocol 2) 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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