Creation and Scale up of a Stable Cell Line using ExpiCHO™ Products

Stable Clone Protocol

Pub. No. MAN0017764 **Rev.** 3.0

WARNING! Read the Safety Data Sheets (SDSs) and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves. Safety Data Sheets (SDSs) are available from **thermofisher.com/support**.

Introduction

This protocol describes the procedure for creating a stable cell line based on the ExpiCHO™ Expression System for use in commercial bioproduction. ExpiCHO-S™ cells are transfected, cloned, and selected in ExpiCHO™ Expression Medium followed by the scale-up in the bioproduction amenable ExpiCHO™ Stable Production Medium.

Required materials not supplied

Unless otherwise indicated, all materials are available through **thermofisher.com**.

Item	Cat. No.
ExpiCHO™ Stable Production Medium	A3711001
ExpiCHO™ Expression Medium	A2910001
GlutaMAX™ I Supplement	35050061
PureLink™ PCR Purification Kit	K310001
ExpiCHO-S™ Cells	A29127
ExpiCHO-S™ Cells (cGMP Banked)	A37785
ExpiCHO™ Expression System Kit	A29133
ExpiFectamine™ CHO Transfection Kit	A29129
L-Glutamine (200 mM)	25030149
EfficientFeed™C+ AGT™ Supplement	A25031
OptiPRO™ SFM	12309

Procedural guidelines

- Do not use ExpiCHO™ Stable Production Medium for transfection.
- Supplement ExpiCHO™ Stable Production Medium with GlutaMAX™ I Supplement at 20 mL/L.
- Supplement ExpiCHO™ Expression Medium with 6mM L-glutamine for limiting dilution cloning. (L-glutamine supplementation at other stages is not necessary as ExpiCHO™ Expression Medium contains GlutaMAX™ I Supplement)
- The shake speed of all orbital shakers is 125 ±5 rpm for shakers with a 19-mm shaking diameter or the adequate converted shaker speed.
- Once stable expressing clones are selected, cGMP cell banks may be produced in ExpiCHO™ Stable Production Medium.
- After the cell line development phase, the banked cells can be thawed directly into ExpiCHO™ Stable Production Medium.
- For Fed-batch culture, we recommend using EfficientFeed™C+ AGT™ Supplement with the ExpiCHO-S™ clones and the ExpiCHO™ Stable Production Medium.

Thaw the cell line to prepare for transfection

1. Remove the vial of ExpiCHO-S™ cells from liquid nitrogen and thaw in a 37°C water bath for 1 to 2 minutes.
2. Just before the cells are completely thawed, decontaminate the vial by wiping it with 70% ethanol before opening it in a biosafety cabinet.
3. Transfer the entire contents of the cryovial into a 125-mL polycarbonate, disposable, sterile, vent-cap Erlenmeyer shaker flask containing 30 mL of pre-warmed ExpiCHO™ Expression Medium.

   Cell viability should be ≥90%.
4. Incubate the cells in a 37°C incubator with ≥80% relative humidity and 8% CO_2 on an orbital shaker platform.
5. Passage cells at 3×10^5 or 2×10^5 seeding density every 3–4 days, respectively, for 3–5 passages before transfection.

   Cell viability should be ≥90% and cell density between $4 \times 10^6 – 6 \times 10^6$ viable cells/mL before each passage.

Create a Kill Curve

This protocol is based on a standard suspension cell culture in a shaker flask. It can be scaled down appropriately to be performed in a 6-well, 12-well, or 24-well plate.

1. Seed at 1×10^6 ExpiCHO-S™ cells/mL five to ten 125-mL shake flasks in 30 mL of ExpiCHO™ Expression Medium.
2. Add selective pressure to each flask in increasing concentrations. See "Reference ranges for selection markers". Also, include a flask with no selective pressure as the negative control.
3. Assess the flasks daily for viability and viable cell density for up to 7 days.

   Note: The ideal concentration of the selective pressure will kill about 50% of cells and/or inhibit growth around days 3–5.

Reference ranges for selection markers

Unless otherwise indicated, all materials are available through **thermofisher.com**. MLS: Fisher Scientific (**fisherscientific.com**) or other major laboratory supplier.

Item	Source	Concentration	Method of action
Geneticin™ Selective Antibiotic (G418 Sulfate)	10131027	200–500 µg/mL	Interferes with the function of 80S ribosomes and protein synthesis in eukaryotic cells
Puromycin	A1113802	10–50 µg/mL	Inhibits protein synthesis by disrupting peptide transfer on ribosomes causing premature chain termination during translation.
MTX	MLS	150–1000 nM	Inhibits dihydrofolate reductase, an enzyme that participates in the tetrahydrofolate synthesis.
Zeocin™ Selection Reagent	R25005	75–500 µg/mL	Causes cell death by intercalating into and cleaving DNA.
Hygromycin-B	10687010	100–1000 µg/mL	Inhibits protein synthesis by disrupting translocation and promoting mistranslation at the 80S ribosome.
Blasticidin S HCl	A1113902	2–20 µg/mL	Nucleoside antibiotic, a potent translational inhibitor

Prepare vector

1. Linearize plasmid using restriction enzyme of choice and confirm linearization by running a gel.
2. Isolate linearized plasmid DNA using method of choice.

   We recommend PureLink™ PCR Purification Kit or do a Phenol-Chloroform Isoamyl alcohol / Chloroform-Isoamyl alcohol extractions followed by DNA precipitation for larger amount of DNA.

Transfect cells

1. On the day prior to transfection (Day -1), split the ExpiCHO-S™ culture of $4 \times 10^6–6 \times 10^6$ viable cells/mL to a final density of $1 \times 10^6–2 \times 10^6$ viable cells/mL and allow the cells to grow overnight.
2. On the day of transfection (Day 0), determine viable cell density and viability.

   The cells should have reached a density of approximately $2 \times 10^6–6 \times 10^6$ viable cells/mL. Viability should be 95–99% to proceed with transfection.

3. Dilute the cells to a final density of 1×10^6 viable cells/mL (in total 25 mL) with fresh ExpiCHO™ Expression Medium, pre-warmed to 37°C.

   Swirl the flasks gently to mix the cells. Do not re-use high density cells (>6×10^6) for routine sub culturing.

4. Prepare ExpiFectamine™ CHO/linearized plasmid DNA complexes using cold reagents (4°C), as described.

   Total plasmid DNA in the range of 0.5–1.0 µg per mL of culture volume to be transfected is appropriate.

   a. Gently invert the ExpiFectamine™ CHO Reagent bottle 4–5 times to mix thoroughly.

   b. Dilute plasmid DNA with cold OptiPRO™ SFM to a total volume of 2 mL. Mix by inversion.

   c. Dilute 40 µL ExpiFectamine™ CHO Reagent with 1.96 mL OptiPRO™ SFM (included in the ExpiCHO™ Expression System). Mix by inversion. Do not exceed 5 minutes before complexation with diluted DNA.

   d. Add the diluted ExpiFectamine™ CHO Reagent to diluted DNA. Mix by swirling the tube or by inversion.

5. Incubate ExpiFectamine™ CHO/plasmid DNA complexes (from Step 4d) at room temperature for 1–5 minutes as after such time the efficacy decreases, and then slowly transfer the solution to the shaker flask from Step 3, swirling the flask gently during addition.

6. Incubate the cells in a 37° C incubator with a humidified atmosphere of 8% CO_2 on an orbital shaker.

7. On the day 2 after transfection check flasks for viability, viable cell counts, and titer (above 4 mg/L ideally).

8. Passage flasks into selection.

Selection Phase 1

1. Passage cells into a 125-mL shake flask with 30 mL ExpiCHO™ Expression Medium at a density of 5×10^5 viable cells/mL.

2. Add adequate selective pressure to the flasks depending on the results of the kill-curve done during the vector preparation step.

3. Incubate the cells on a shaking platform at a 37°C, 70–80% relative humidity, and 8% CO_2.

4. Sample flasks on day 7 post-selection for a viable cell count only.

5. Thereafter, passage the cells in shake flasks every 3–4 days, seeding them at 3×10^5 viable cells/mL at each passage.

   Maintain selective pressure appropriate for the volume of fresh medium added.

   Centrifugation for full medium exchange is only required when the dilution factor is <2. Use a cell strainer whenever clumping is observed.

6. Selection Phase 1 is complete when viability exceeds 85% and the viable cell density exceeds 1×10^6 viable cells/mL.

7. Cryopreserve at least 3 vials of cells from each Selection Phase 1 pool as a back-up and proceed directly to Selection Phase 2. See "Cryopreserve cells" on page 6.

Selection Phase 2

1. For each Selection Phase 1 pool, determine the viable and total cell counts.

2. Seed a new 125-mL shake flask per Selection Phase 1 pool at 5×10^5 viable cells/mL in 30 mL of ExpiCHO™ Stable Production Medium.

3. To the shake flask, add selective pressure to a 2–5 times higher concentration depending on the vector's selection marker.

4. Incubate the cells on a shaking platform at 37°C, 80% relative humidity, and 8% CO_2. Sample the flasks every 3–4 days; if cells do not show signs of recovery (i.e., cell densities above the last measured value), leave as-is and perform a complete medium exchange once a week.

   Once cells show signs of recovery, proceed.

5. Passage the cells in shake flasks every 3–4 days, seeding them at 3×10^5 viable cells/mL at each passage. Maintain selective pressure appropriate for the volume of fresh media added.

   Cell pelleting and full media exchange is only required when the dilution factor is <2.

6. Selection is complete when viability meets or exceeds 90%.

7. Cryopreserve at least 5 vials of cells from each stable pool as a back-up. See "Cryopreserve cells" on page 6.

Assess productivity of the pools

1. Seed fully recovered cell pools (viability >90%) at 3×10^5 viable cells/mL using 30 mL ExpiCHO™ Expression Medium without selective pressure in 125-mL shake flasks.

2. Incubate the cells on a shaking platform at 37°C, 80% relative humidity, 8% CO_2.

3. Sample cultures on days 0, 3, 5, 7, 10, 12, and 14 (to fit the normal workweek assuming a day 0 on a Friday) to determine the cell density, viability, and productivity until culture viability drops below 50% or day 14 of culture is reached.

4. After sampling, feed the cultures with glucose as follows:
   - **Day 3**: add 4 g/L of glucose
   - **Day 5**: add 4 g/L of glucose
   - **Day 7**: add 6 g/L of glucose

Perform limiting dilution cloning

Limiting dilution cloning is used to separate single clones in the pool and scale up such clones. For this section we recommend 6 mM of L-glutamine supplementation to the ExpiCHO™ Expression Medium.

1. Create a cloning medium by adding L-glutamine to at 6 mM and pre-warm it to 37°C.

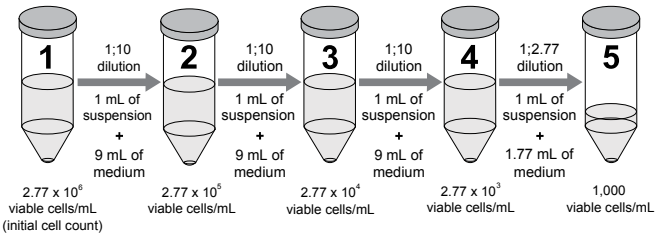
2. Thaw frozen stable pool(s) for limiting dilution cloning 2–5 days in advance lacking selective pressure; no more than one passage should be needed to reach >90% viability before seeding limiting dilution cloning.

3. For each pool, label five 50-mL conical tubes "1" through "5".

4. Use a cell strainer such as Fisherbrand™ 40-µm nylon mesh (if cell clumping is observed) cell strainer to obtain a uniform single-cell suspension into the 50-mL tube labeled "1".

5. Accurately determine the viable cells/mL of the strained pool.

6. Serially dilute the cells to a final concentration of 1,000 viable cells/mL using cloning medium:



7. From tube 5, pipette an 8 µL droplet of cell suspension each in 12 wells of a 96-well plate.

8. Observe the 96-well plate under a microscope and count the number of cells in each of the droplets.

   To achieve 1000 cells/mL, the droplets should average 8 cells/droplet. If the cell numbers are inconsistent with this target, dilute or concentrate the cells in tube 5 accordingly.

9. Pipette 0.1 mL of the cell suspension from "Tube 5" (1,000 cells/mL) into tubes containing 39.9 mL of ExpiCHO™ Expression Medium with 6 mM L-glutamine. This brings the final volume in each tube to 40 mL with a cell density of 2.5 cells per mL, allowing a seeding density of 0.5 cell per well when 200 µL of diluted cells is added into each well.

10. Mix the cell suspension gently by inverting the tube 5 or 6 times and transfer it into a sterile reagent reservoir or trough.

11. Use a multi-channel pipettor to aseptically dispense 200 µL of the diluted cells into each of the wells of the 96-well plate.

    Note: To make it easier to later see and focus the cells under the microscope, add 20 µL (about 20 cells) of the 1000 cells/mL (tube 5) into the first well (A1) of each 96-well plate.

12. Incubate the plates undisturbed for 12–14 days at 37°C and 5–8% CO_2 in humidified air in a static (non-shaking) incubator. Stack no more than 5 plates together.

> **CAUTION!** If the incubator is not sufficiently humidified, you may want to take steps to prevent evaporation from the plates.

13. After day 12 of incubation, examine the wells visually using a microscope for growth of monoclonal colonies.

    Note: If note of step 11 was followed, A1 wells can be used to focus the microscope as they will have a lot of cells. If not many colonies are observed, conditioned media can be used to perform the limited dilution cloning.

    Optional: On day 13–14, feed wells with 25–50 µL of ExpiCHO™ Expression Medium supplemented with 6 mM L-glutamine.

14. Calculate the percent cloning efficiency:

$$\text{Cloning efficiency} = \frac{\text{Number of wells showing growth}}{\text{Number of wells seeded x cells per well seeded}}$$

    For example, the cloning efficiency with 120 colonies growing out of total 600 seeded wells (10 plates with 60 seeded wells/plate) at seeding density of 0.5 cell per well is 40%.

    The example assumes that peripheral wells of the 96-well plate will be filled with water to prevent loss of medium in the test wells.

Scale up clone

1. When individual clones from limiting dilution cloning are > 60% confluent (day 17 or 18 post-seeding) in 96-well plates, aseptically harvest the desired clones by pipetting the cells up and down gently and transferring the entire content of each well into a separate well of 24-well tissue culture plates containing 0.3–0.5 mL of fresh ExpiCHO™ Expression Medium.

2. After 2–5 days, transfer the desired clones into 6-well plates (non-shaking) using the same procedure.

   The final culture volume in a 6-well plate is 2–3 mL.

3. Perform a passage in a 6-well plate shaking at 125 rpm. Ensure that the vast majority of the clones are >90% viable before performing the primary screen.

Primary Screen: 5-Day Productivity Assessment

4. Set up the primary screen in shaking 6-well plates, consistent with the conditions used in the preceding step.

5. Seed the cells at 3×10^5 viable cells/mL in 3 mL of ExpiCHO™ Expression Medium plus 3g/L glucose.

6. Incubate for 5 days before sampling for productivity.

7. Expand the top 15–40 producing clones into 125-mL shaker flasks in 30-mL of ExpiCHO™ Expression Medium.

8. Incubate the cells at 37°C and 8% CO_2, with shaking at 125 rpm.

9. Passage clones two times in shaker flasks.

10. Cryopreserve 3–5 vials of cells from each clone as a back-up and proceed to the tertiary screening. See "Cryopreserve cells" on page 6.

    This cell bank should be used for further expansion depending upon the clone selected in the subsequent steps.

Secondary Screen: 14-Day Simple Fed-Batch Assessment

Note: The purpose of this screen is to further select from the clones chosen from the primary screen. This screen may be skipped if all clones selected in the primary screen can be tested in the tertiary screen.

11. Seed the expanded clones from step 9 at 3×10^5 cell/mL in 125-mL shaker flasks in 30 mL of ExpiCHO™ Expression Medium.

    Alternatively thaw a vial from step 10 and perform at least two passages before starting the study. See "Thaw the cell line to prepare for transfection" on page 2.

12. Sample cultures on days 0, 3, 5, 7, 10, 12, and 14 to determine the cell density, viability, and productivity until culture viability drops below 50% or day 14 of culture is reached.

13. After sampling, feed the cultures with glucose as follows:
    - **Day 3**: add 4 g/L of glucose
    - **Day 5**: add 4 g/L of glucose
    - **Day 7**: add 6 g/L of glucose – Discard cultures at the end of the run.
    - Discard cultures at the end of the run.

Tertiary Screen: 14-Day Fed-Batch Assessment

14. Thaw one vial of each clone selected from the primary or secondary screen into 30 mL ExpiCHO™ Expression Medium into a 125 mL shake flask. Incubate in a shaker at 125 rpm, 37°C, 70–80% relative humidity, and 5–8% CO_2.

15. Passage cells 2–5 times every 3–4 days prior to commencing the productivity screen by seeding at 2×10^5 cells/mL (4 day passage) or 3×10^5 cells/mL (3 day passage). Incubate in a shaker at 125 rpm, 37°C, 70–80% relative humidity, and 5–8% CO_2.

    Note: Anti-clumping agent may also be added if cell clumping is observed.

16. As needed for biological replication, seed 1–3 shake flasks per clone at 3×10^5 cells/mL in ExpiCHO™ Expression Medium. Incubate in a shaker at 125 rpm, 37°C, 70–80% relative humidity, and 5–8% CO_2.

17. Sample cultures on Day 0 and Days 3–14 to determine cell density, viability, glucose, and productivity (other metabolites may also be measured if desired). After sampling, feed the cultures with glucose up to 6 g/L based upon glucose measurements. Also, add 2% EfficientFeed™C+ AGT™ Supplement (2X) daily from Day 3 to 13.

18. Discard cultures at the end of the run.

Assess stability

Stability is determined by how long the generated clones can keep producing the protein of choice at a constant level (without losing too much productivity with time). It can be done after the tertiary assessment or concurrently.

Select the top 16 identified clones during Primary Screening to sub-culture them for up to 60 generations or 12 weeks, whichever comes first with a single lot of medium. At this stage, the ExpiCHO™ Stable Production Medium can be used. For subsequent cell banking, scale-up and process development ExpiCHO™ Stable Production Medium should be used.

Note: Determine the optimal generation number for your experiment.

1. Thaw each clone in a 125-ml shake flask with 30 mL pre-warmed ExpiCHO™ Expression Medium.

2. Passage clones at a seeding density of 2×10^5 cells/mL twice a week (3–4 day passages) for up to 60 generations or twelve weeks, whichever comes first.

3. Calculate generations by the following simple formula:

$$\text{Previous generation} + \frac{\ln(\text{VCD/seeding density})}{\ln(2)}$$

4. Set up a 7-Day productivity assessment for each clone one week after thaw in a different shake flask.

   These assessments will be seeded at 2×10^5 cells/mL once a week, fed 5 g/L glucose on day 3 or 4, and measure viability, VCD and productivity on day 7. This will be done throughout the entire study once a week.

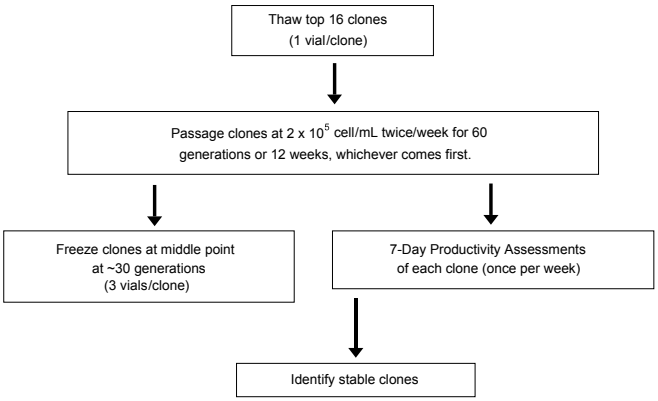
5. At generation 30, we recommend to do a safety freeze of 3 vials of each clone to mitigate loss. See "Cryopreserve cells" on page 6.

6. Use the data from the productivity assessments, plot a regression line and establish a slope.

7. Calculate the changes in titer (or growth or both) over the 60 generations to determine clone stability.

8. Establish a criteria for clone stability.

   We have used in the past a decrease in titer of 30% or less to indicate that the specific clone is stable.



Cryopreserve cells

1. Allow cells to attain a viable cell density of 4×10^6–6×10^6 cells/mL and > 95% viability before harvest.

2. Centrifuge the cells at 200 × *g* for 5 minutes to pellet, discard the spent medium, and replace it with cold ExpiCHO™ Expression Medium (Selection I, Selection II, Primary Screen) with 10% DMSO.

3. Gently resuspend the cell pellet by pipetting.

4. Dilute the cells to a final density of 1×10^7 viable cells/mL and aliquot 1 mL per cryovial.

   For five vials:

   Cells: $5 \times 10^6 = 50 \times 10^6$ needed /VCD flask = mL required to be centrifuged (200 g for 5 min)

   DMSO medium: 5 mL × 0.1 = 0.5 mL DMSO + 4.5 mL medium

   After decanting supernatant from the centrifuged tubes, resuspend in the DMSO medium and aliquot into 1 mL cryovial.

5. Freeze the cells in an automated or manual controlled-rate freezing apparatus following standard procedures.

   For ideal cryopreservation, the freezing rate should be a decrease of 1°C per minute.

6. Transfer frozen vials to liquid nitrogen for long-term storage.

Limited product warranty

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