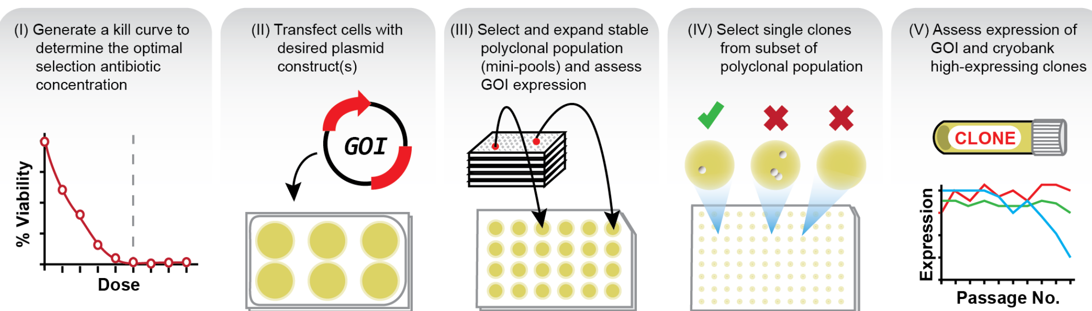
Generation of Stable CHO Cell Lines for Protein Production

Instructions for use with MIR 6260 and 6270

Using the CHOgro® High Yield Expression System, exceptional yields of recombinant protein can be obtained by transient transfection of suspension CHO cells. Though transgene expression ceases for the vast majority of cells within one to two weeks post-transfection, a small fraction of transfected CHO cells continues to stably express the recombinant protein through random chromosomal integration of the transfected gene. Stable cell lines can be established through selection and expansion of single cells or a pool of cells that continually produce high levels of recombinant protein. To facilitate the selection process, transfection of auxotrophic-marker or antibiotic-resistance genes in *cis* or *trans* to the plasmid containing the gene-of-interest is performed. Additional selection pressure, e.g. sublethal doses of MSX on CHO-GS$^{-/-}$ cells, can then be applied to amplify gene copy number and identify high-expressing clones. Generating stable cell lines is a lengthy process (requiring 9-12 weeks), but ultimately has the potential to yield gram per liter quantities of protein with high batch-to-batch consistency for extended periods of time.

The figure below represents a protocol for generating stable cell lines in suspension CHO cells using antibiotic selection.



Note: Not all serum-free complete media formulations can support transient transfection. Thus, CHOgro® Expression Medium is recommended for use with this protocol. However, it is not compatible with DHFR-based methods of selection because it contains hypoxanthine and thymidine.

I. Generate a kill curve to determine the optimal selection antibiotic concentration

The first critical step for stable cell line generation is determining the optimal antibiotic concentration for selecting stable cell colonies. A kill curve is a dose-response experiment where cells are subjected to increasing amounts of antibiotic to determine the minimum antibiotic concentration that is needed to kill all the cells over the course of one week. Performing a kill curve is recommended with each new cell type or when a new selection antibiotic is used.

1. Seed suspension CHO cells at 5×10^5 cells/ml in 2 ml of complete growth medium per well in multiple 6-well non-tissue culture treated plates (e.g. Falcon Polystyrene Microplates, VWR #15705-056). Suspension CHO cells do not grow optimally in smaller plate formats.
2. Add increasing amounts of antibiotic to each well, in duplicate. Include a 0 µg/ml condition as a negative control for cell health.
3. Incubate cells at 37°C in 8% CO_2 with shaking.
4. Every 2-3 days for up to a week, pellet cells to remove media. Replace with fresh media containing antibiotic. Examine the cells daily for signs of visual toxicity. Determine the lowest concentration of antibiotic at which all cells are dead after one week of antibiotic selection.

II. Transfect cells with desired plasmid construct(s)

Optional: Linearize your target plasmids before transfection. When generating a stable cell line, the transfected plasmid undergoes recombination during chromosomal integration. The recombination event can occur within any region of the plasmid, including the gene expression or selectable marker cassettes that might disrupt their function. To increase the likelihood that recombination will occur in non-essential plasmid regions, such as the bacterial replicon or bacterial marker gene, linearize the plasmid with restriction enzyme(s) that cut within these non-essential regions. Prior to transfection, purify the linearized DNA by ethanol precipitation or column purification.

1. Immediately before transfection, seed suspension CHO cells at 5×10^5 cells/ml in 2 ml of complete growth medium per well of a 6-well non-tissue culture treated plate.
2. Transfect cells as directed in Table 1. Leave one of the wells untransfected. This control is important as a reference during the selection process (Step III). Do not add selection antibiotic until 24 hours post-transfection.
3. Incubate cells at 37°C in 8% CO_2 with shaking.

Table 1. Recommended Transfection Conditions per Well of a 6-well Plate

CHOgro® Complex Formation Solution	200 µl
*Trans*IT-PRO® Transfection Reagent	2 µl
Total Plasmid DNA (1 µg/µl)*	2 µl
Incubate transfection complexes for no more than 5 min before adding to cultures.	

** If the antibiotic selection marker is in* trans *of the gene of interest, then maintain a 10:1 ratio of gene-of-interest plasmid over antibiotic-selection plasmid.*

III. Select and expand stable polyclonal population (mini-pools) and assess GOI expression

1. At 24 hours post-transfection, centrifuge the transfected cells and resuspend to 500 cells/ml in complete CHOgro® Expression Medium containing the selection antibiotic at the optimal dose identified in Step I.
2. Transfer 100 µl of this diluted cell suspension to each well of multiple 96-well plates, e.g. 20 plates.
3. To control for antibiotic efficacy, the untransfected cells should also be diluted to 500 cells/ml in complete CHOgro® Expression Medium containing the selection antibiotic. They can be plated in a new well of a 6-well plate. All untransfected cells should be dead after one week.
4. Incubate cells at 37°C in 8% CO_2 with shaking.
5. Examine the cells for antibiotic resistance daily. Wells with surviving cells after nine days post-transfection represent cell populations stably expressing the transgene.
6. Upon reaching 80% confluency, transfer the wells with surviving cells to 24-well plates.
7. At this point, each well is considered a polyclonal mini-pool of stable CHO cells and should be assessed for expression level of the target protein. Mini-pools with the highest productivity can be expanded and frozen and/or re-selected for a monoclonal population (Step IV).

Note: The wells containing antibiotic-resistant cells can easily be identified by holding the 96-well plate up to a light. Mark these wells with a permanent marker.

IV. Select single clones from subset of polyclonal population

The polyclonal cell culture can be further processed to isolate clones using various techniques such as:

- **Limiting dilution:** In this method, individual cells are isolated by plating at very low cell densities (< 1 well per well in 96 well plates).
- **Fluorescence activated cell sorting (FACS):** Using a FACS machine, single cells can be isolated. Depending on the gene of interest, the expressed protein may also be detected using direct or indirect fluorescence. Single cells exhibiting fluorescence can then be enriched and expanded to generate a monoclonal lineage.
- **Automated clone picking:** Sophisticated instrumentation-based methods, e.g. ClonePix™ technology (Molecular Devices), allow a completely automated process of high producer clone identification and expansion.

Use of conditioned media or culturing cells in semi-solid media such as soft-agar can increase cell survival. Of the above-mentioned methods, limiting dilution is the most cost-effective and frequently adopted technique; a detailed protocol using limiting dilution to generate monoclonal cell lines is as follows:

1. Dilute the polyclonal CHO cells to a density of 10 cells/ml. Transfer 100 µl of the cell suspension to each well of a 96-well tissue culture plate (i.e. 1 cell per well).
2. Allow the cells to recover in the incubator for 2-4 hours (37°C in 8% CO_2 with shaking).
3. Use a microscope to determine which wells contain only one cell. Note these wells.
4. After four days, visually assess the wells to determine if the single cells have divided. These wells can be considered a monoclonal population.
5. Allow the cells to expand to over 80% confluency in each well of the 96-well plate before proceeding to Step V.

Note: To increase the likelihood of obtaining monoclones, limited dilution can be repeated several times.

V. Assess expression of GOI and cryobank high-expressing clones

1. When the monoclonal cells reach high density (4 - 6 x 10^6 cells/ml), transfer them to sequentially larger vessels, e.g. 24-well plate to 6-well plate to shake flasks, to expand the culture for cryobanking. After each expansion, incubate cells at 37°C in 8% CO_2 with shaking.
2. Upon reaching high density in 24-well plates (4 - 6 x 10^6 cells/ml), a portion of cells should be assessed for target gene expression, and high -expressing clones should be further expanded and cryobanked.
3. To determine the long-term stability of target gene expression, a portion of cells should also be assessed over 50+ doublings. It is advisable to freeze multiple vials of the same pool or clone at an early passage number to prolong their use after thaw as loss of transgene expression is known to occur spontaneously over time.

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