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Freedom ExpiCHO-S Kit FAQs

An e-book to help you succeed in developing stable cell lines for protein production

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Welcome

The Gibco™ Freedom™ ExpiCHO-S™ Kit is an easy-to-use solution for cloning and expressing recombinant proteins in suspension cultures derived from Chinese hamster ovary (CHO) cells. Developed in collaboration with ProBioGen AG, the Freedom ExpiCHO-S Kit allows you to develop stable clones and quickly move your workflow to process development. The kit includes components and a workflow for vector construction, transfection, expression, and stable cell line selection. The Freedom ExpiCHO-S Kit is also offered with flexible commercial licensing options.

The content in this document is not meant to replace the full User Guide. Please refer to the User Guide for detailed information.

Contents

FAQs about the Freedom ExpiCHO-S Kit and workflow

Vector design and preparation

1. What are your recommendations for cloning inserts into Gibco™ Freedom™ pCHO 3.1 and 3.2 vectors? Should I include Kozak and signal peptide sequences?

   – Use cDNA for your gene(s) of interest.

   – Untranslated 5´ and 3´ regions are not required since the vectors include these functionalities.

   – Insert a Kozak sequence, such as GCCACC, immediately before the start codon.

   – Engineer a signal peptide on the N-terminus of your gene(s) of interest to enable or enhance secretion and facilitate purification of the desired protein(s).

2. NruI is the restriction enzyme suggested for vector linearization before stable pool generation. NruI is sensitive to methylation, so is it compatible with *dam*-positive Invitrogen™ Top10 *E. coli*?

   – We have had no problems digesting Freedom pCHO plasmids with NruI.

3. What is the risk of contamination by bacteria, such as mycoplasmas, or viruses in Freedom pCHO 3.1 and 3.2 vector stocks?

   – The risk of stock vector contamination is low as long as proper cell culture practices are followed.

   – The LB medium used to grow *E. coli* contains Gibco™ SELECT Peptone 140. It is a pancreatic digest of casein and the only component with an animal origin in the vector construction workflow. SELECT Peptone 140 is sourced from New Zealand, Australia, and the United States. The occurrence of transmissible spongiform encephalopathy (TSE) and bovine spongiform encephalopathy (BSE) is very low in these countries, so the risk of TSE or BSE contamination from the medium is minimal.

   – Use an Invitrogen™ PureLink™ HiPure Plasmid Prep kit to prepare your DNA. These kits include a patented anion exchange resin for DNA purification that is equivalent to two passes through a CsCl gradient. PureLink HiPure Plasmid Prep kits efficiently remove proteins, non-plasmid DNA, RNA, and any intact microorganisms that may still be present following the lysis step.

   – The final steps in the workflow require 100% isopropanol and 80% ethanol, which should help remove any living microorganisms remaining after the preceding purification steps.

   – DNA should be prepared using sterile disposable or autoclaved containers and sterile DNA resuspension buffer.

4. Do complete pCHO 3.1 and 3.2 vector inserts have a size limit? Would a large 3.0 kb insert work?

   – We have not fully explored insert size limits for this kit. We have cloned up to 6.0 kb into the pCHO 1.0 vector for the Gibco™ Freedom™ CHO-S™ Kit without any issues. However, increasing vector size generally compromises transfection efficiency. It is thus possible that a large insert could make transfection less efficient.

5. Is it necessary to include untranslated 5´ and 3´ regions, or do the vectors already have them?

   – It is not necessary to include untranslated regions, as the vectors already contain regulatory elements and downstream poly(A) sequences.

6. What should I include in the insert to express my protein of interest?

   – The insert should have two blunt ends. It will be necessary to check the orientation of your insert as described on the next page.

   – Also be sure to include:

     - A Kozak sequence (GCCACC) immediately before the start codon
     - An ATG start codon
     - A signal peptide
     - A cDNA sequence for the gene of interest
     - A stop codon

7. What sequencing primers can I use to verify my construct?

   – We strongly recommend verifying your sequence and its readability before transfecting Gibco™ ExpiCHO-S™ Cells by sequencing the region between the promoter and junction in the recombinant expression plasmid. Examples of primer sequences for this step are shown below. The sequencing primers are not included with the Freedom ExpiCHO-S Kit, so you will need to order them separately for custom synthesis.

Primer	Sequence	Location
Forward primer for CMV/EF1 hybrid promoter	GGTGTCGTGAGGAATTTCAG	Begins ~271 bp upstream of the EcoRV insertion site
Reverse primer for CMV/EF1 hybrid promoter	ACCCACAATTCTTTGACATA	Begins ~249 bp downstream of the EcoRV insertion site

8. How do I check the orientation of my gene of interest after cloning a blunt-end fragment?

   – The simplest way to check the orientation of a fragment cloned by blunt-end ligation is to generate an asymmetric cut in the inserted fragment and a single or double cut in the vector. The asymmetric cut in the gene of interest should be close to either the 5′ or 3′ end of the gene. The resulting digestion fragment can then be easily resolved on an agarose gel to establish the orientation of your gene. Prepare a control by digesting the empty vector with the same restriction enzyme(s).

9. Do you have any more information about the bacterial origin of replication (*ori*) in pCHO plasmids?

   – The pCHO *ori* is derived from the modified pMB1 *ori* from pUC19, and it enables sufficient replication for high copy numbers.

10. Is there a preferred signal peptide to use with Freedom pCHO vectors, and should I optimize my sequence?

   – Successful expression with another cell line or vector does not guarantee expression or secretion when a sequence is placed in a Freedom pCHO vector. We highly recommend that Freedom pCHO constructs be tested via transient transfection before proceeding with selection.

   – We do not have any specific recommendations for particular signal peptides. However, we highly recommend that any sequence, including the signal peptide sequence, be evaluated for optimization to improve CHO-specific codon usage. Invitrogen™ GeneArt™ gene optimization services can do this for you, identify other issues that could affect expression of your gene, and provide you with a report. After reviewing the report, you can then decide whether to have the optimized gene synthesized.

Incubation parameters

1. What are the recommended incubation parameters for mammalian cell culture?

   – The incubation parameters Thermo Fisher Scientific recommends for a shaker with a 19 mm throw (radius) are shown below. If your shaking incubator has a different throw, we recommend optimizing CHO-S cell growth by adjusting the shaker speed prior to initiating cell line development. Calculate the relative centrifugal force (RCF) with the following equation:

   $RCF = 1.12 \times radius \times (RPM/1{,}000)^2$

Incubation parameter	Value
Temperature	37°C
CO_2 concentration	8%
Relative humidity	80%
Agitation	125 RPM
Throw	19 mm

Background on ExpiCHO-S Cells

1. What is the origin of ExpiCHO-S Cells?

   – Gibco™ ExpiCHO-S™ Cells are derived from the CHO cell line established by Robert Tobey at Los Alamos National Laboratory. ExpiCHO-S Cells are distinguished from the commonly used CHO-K1 cell line by karyotyping [1] and more recently by sequence analysis [2].

Cell clumping during the ExpiCHO-S workflow

1. How do I deal with ExpiCHO-S Cells if they clump?

   – Clumping is to be expected with ExpiCHO-S Cells. There are three ways to minimize cell clumping:

   • Remove clumps with a cell strainer at the beginning of the first passage after thawing.

   • Avoid clumps during sampling and cell passage.

   • Do not allow the cell density to exceed 6 x 10^6 viable cells/mL prior to transfection.

   – Do not use an anti-clumping agent prior to transfection. However, an anti-clumping agent will be critical after transfection to enable successful selection.

2. How do you strain the cells?

   – Remove the flask from the incubator and inspect the culture to identify any clumps that would mandate straining.

   – Take the cap off a sterile 125 or 250 mL shake flask while maintaining sterility and replace it with a sterile cell strainer.

   – Pipet or pour the culture through the strainer.

   – Discard the cell strainer, replace the cap, and proceed with cell counting and passage.

Transfection with ExpiFectamine CHO reagent

1. How much monoclonal antibody is typically expressed after transfection with Gibco™ ExpiFectamine™ CHO reagent?

   – There is typically 3–9 µg/mL of IgG in the transfection supernatant on day 2 following transfection.

Stable pool selection

1. What titers can I expect after phase I and phase II of selection with puromycin and methotrexate?

   – Titers will vary depending on the monoclonal antibody being expressed. Typical phase I pool titers are in the range of 400–1,200 mg/L by day 14. Phase II pool titers are in the 600–1,600 mg/L range in a shake flask batch fed with glucose and supplemented with Gibco™ EfficientFeed™ C+ 2X Supplement.

2. How long does it take to select a stable pool, and how much does viability drop?

   – Selection phases I and II for antibody expression typically take 18–25 days and 10–14 days, respectively. The entire selection process will thus take a total of 28–39 days. Viability can drop below 15% in phase I, but it usually does not fall below 50% in phase II.

   – In Figure 1, selection took a total of 33 days. The viability of cells transfected with the control vector dropped below 15% during the first 8 days of phase I, which made it possible to distinguish true recovery during phase II of selection. The control vector did not need to be maintained beyond phase I of selection.

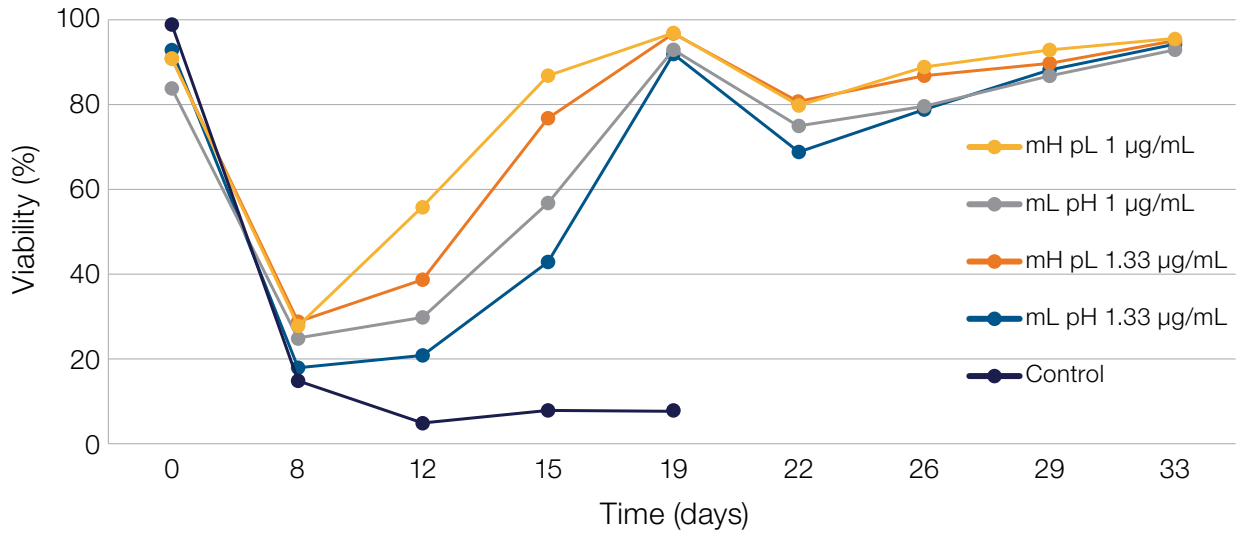

Figure 1. Viability of ExpiCHO-S Cells in phase I and phase II of stable pool selection. The vector DNA concentration (µg/mL) is shown on the right. m = pCHOm; p = pCHOp; H = heavy chain; L = light chain.

3. How should I handle stable pools once they are recovered?

   – A frozen stock should be prepared for each stable pool as soon as the pools recover after phase II of selection.

   – Stable pools should not be passaged without selection pressure.

   – Pool drift is possible, so it is best to seed stable pools for limited dilution cloning as soon as possible after stable pool recovery.

   – If you cannot clone from the stable cell pools after recovery, thaw the frozen stable pools for cloning in Gibco™ ExpiCHO™ Stable Production Medium supplemented with glutamine, and seed for limited dilution cloning 2 or 3 days later without additional pool passage. Applying selection pressure is not recommended. The cell pools should be ≥95% viable before you proceed with limited dilution cloning.

Limited dilution cloning

1. How do I know if I have cells in the final dilution for limited dilution cloning?

   – Assuming you want 0.5 cells per well in a 96-well plate, aim for a cell density of 1,000 cells/mL in your second-to-last dilution. Add 100 µL of this dilution to 39.9 mL of the cloning medium to achieve a final cell density of 2.5 cells/mL. Plating 200 µL per well will then give the desired 0.5 cells per well. Perform the final dilution and calculations as described below.

   - Seed 12 wells in a flat-bottom 96-well plate with 8 µL of the 1,000 cell/mL dilution. If the initial cell count and subsequent dilution are accurate, you should have ~8 cells per well on average.
   - Count the total number of cells in all 12 wells and divide by 96 µL. Multiply by 1,000 to calculate the concentration of the cell suspension, and use this corrected value to achieve your desired final dilution. For example, if you count 65 cells in 96 µL, you will have 65/96 x 1,000 = 677 cells/mL. The following calculation gives the volume needed to achieve 2.5 cells/mL in 40 mL of cloning medium:

     2.5 cells/mL x [40 mL/(677 cells/mL)] x 1,000 = 148 µL

     If 677 cells/mL is an accurate cell count, diluting 148 µL with 39.85 mL of the cloning medium will give 2.5 cells/mL: (0.148 mL x 677 cells/mL)/39.85 mL.

2. How should I scale up the clones from 96-well plates to shake flasks?

   – Figure 2 is a suggested workflow that includes the main steps of clone scale-up and screening.

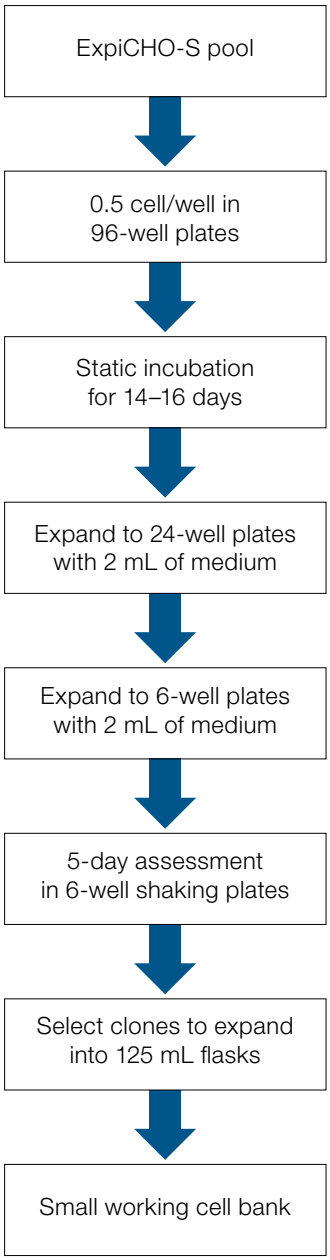

Figure 2. Suggested workflow for clone scale-up and screening.

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References

1. Deaven LL and Petersen DF (1973) The chromosomes of CHO, an aneuploid Chinese hamster cell line: G-band, C-band, and autoradiographic analyses. *Chromosoma* 41(2):129-144.
2. Lewis NE, Liu X, Li Y et al. (2013) Genomic landscapes of Chinese hamster ovary cell lines as revealed by the *Cricetulus griseus* draft genome. *Nature Biotechnol* 31(8):759-765.

Ordering information

Description	Quantity	Cat. No.
Freedom ExpiCHO-S Kit	**1 kit**	**A46847**
ExpiCHO-S Cells (cGMP Banked)	1 vial (~1 x 10^7 cells)	A37785
ExpiCHO Expression Medium	1 L	A29100
ExpiCHO Stable Production Medium	1,000 mL	A3711001
Freedom pCHOm 3.1 and pCHOp 3.2 vectors*	–	
Freedom ExpiCHO Expression System		
ExpiFectamine CHO Transfection Kit	For 1 L of culture	A29129
	For 10 L of culture	A29130
	For 50 L of culture	A29131

* Only available with Freedom ExpiCHO-S Kit purchase. Not sold separately.

Learn more at **thermofisher.com/freedom**