ADDENDUM: PROTOCOLS

Protocol #1: Restriction Enzyme Digestion Protocol (New England BioLabs)

A "Typical" Restriction Digest

Restriction Enzyme	10 unites is sufficient, generally 1 µl is used
DNA	1 µg
10X NEBuffer	5 µl (1X)
Total Reaction Volume	50 µl
Incubation Time	1 hour*
Incubation Temperature	Enzyme dependent

* Can be decreased to 5-15 minutes by using a Time-Saver™ Qualified enzyme.

Enzyme

For additional information, please visit Restriction Enzyme Tips

- Keep on ice when not in the freezer
- Should be the last component added to reaction
- Mix components by pipetting the reaction mixture up and down, or by "flicking" the reaction tube. Follow with a quick ("touch") spin-down in a microcentrifuge. Do not vortex the reaction.
- In general, we recommend 5–10 units of enzyme per µg DNA, and 10–20 units for genomic DNA in a 1 hour digest.
- NEB has introduced a line of High-Fidelity (HF®) enzymes that provide added flexibility to reaction setup.
- Some restriction enzymes require more than one recognition site to cleave efficiently. These are designated with the "multi-site" icon 2*site. Please review recommendations on working with these enzymes.

DNA

- Should be free of contaminants such as phenol, chloroform, alcohol, EDTA, detergents or excessive salts. Extra wash steps during purification are recommended.
- Methylation of DNA can inhibit digestion with certain enzymes. For more information about methylation, Effect of CpG Methylation on Restriction Enzyme Cleavage and Dam and Dcm Methylases of *E.coli*

Buffer

- Use at a 1X concentration
- Supplement with SAM (S-Adenosyl methionine) to the recommended concentration if required.

Reaction Volume

- A 50 µl reaction volume is recommended for digestion of 1 µg of substrate
- Enzyme volume should not exceed 10% of the total reaction volume to prevent star activity due to excess glycerol
- Additives in the restriction enzyme storage buffer (e.g., glycerol, salt) as well as contaminants found in the substrate solution (e.g., salt, EDTA, or alcohol) can be problematic in smaller reaction volumes. The following guidelines can be used for techniques that require smaller reaction volumes.

	Restriction Enzyme*	DNA	10X NEBuffer
10 µl rxn**	1 unit	0.1 µg	1 µl
25 µl rxn	5 units	0.5 µg	2.5 µl
50 µl rxn	10 units	1 µg	5 µl

* Restriction Enzymes can be diluted using the recommended diluent buffer when smaller amounts are needed.
** 10 µl rxns should not be incubated for longer than 1 hour to avoid evaporation.

Incubation Time

- Incubation time is typically 1 hour
- Can often be decreased by using an excess of enzyme, or by using one of our Time-Saver Qualified enzymes.
- It is possible, with many enzymes, to use fewer units and digest for up to 16 hours. For more information, visit Extended Digests with Restriction Endonucleases.

Stopping a Reaction

If no further manipulation of DNA is required:

- Terminate with a stop solution (10 µl per 50 µl rxn) [1x: 2.5% Ficoll®-400, 10mM EDTA, 3.3mM Tris-Hcl, 0.08% SDS, 0.02% Dye 1, 0.001% Dye 2, pH 8.0@25°C] (e.g., NEB #B7024)

When further manipulation of DNA is required:

- Heat inactivation can be used
- Remove enzyme by using a spin column (NEB #T1030) or phenol/chloroform extraction

Storage

- Storage at -20°C is recommended for most restriction enzymes. For a few enzymes, storage at -80°C is recommended for periods longer than 30 days. Please refer to the enzyme's technical data sheet or catalog entry for storage information.
- 10X NEBuffers should also be stored at -20°C

Stability

- All enzymes are assayed for activity every 4 months. The expiration date is found on the label.
- Exposure to temperatures above -20°C should be minimized whenever possible

Control Reactions

If you are having difficulty cleaving your DNA substrate, we recommend the following control reactions:

- Control DNA (DNA with multiple known sites for the enzyme, e.g. lambda or adenovirus-2 DNA) with restriction enzyme to test enzyme viability
- If the control DNA is cleaved and the experimental DNA resists cleavage, the two DNAs can be mixed to determine if an inhibitor is present in the experimental sample. If an inhibitor (often salt, EDTA or phenol) is present, the control DNA will not cut after mixing.

Protocol #2: DNA Gel Electrophoresis (University of Virginia)

Preparation of Agarose Gel

1. Prepare 1X TAE buffer by adding 20 mL of 50X TAE Buffer to 980 mL water.
2. Chose % agarose for gel. A 0.9 or 1% agarose gel will work for most applications.

Range of separation	% agarose	Amount of agarose for 50 mL gel
5 kb – 60 kb	0.3	0.15 g
1 kb – 20 kb	0.6	0.30 g
500 bp – 7 kb	0.9	0.45 g
400 bp – 6 kb	1.2	0.60 g
200 bp – 3 kb	1.5	0.75 g

3. Add desired amount of ultra-pure agarose to 1X TAE buffer in a flask. For a standard gel, use 50 mL of 1X TAE Buffer. Swirl the flask to mix.
4. Microwave solution for 1 min. Remove with gloves and swirl. Microwave for longer if there is undissolved agarose. Allow the solution to cool for 1 min.
    - Note: Add a folded paper towel to the opening of the flask when microwaving to prevent steam from escaping.
5. Add 1 µL of ethidium bromide to solution and swirl.
    - Note: Ethidium bromide is a carcinogen and must be handled with care. Dispose of ethidium bromide tips in the designated biohazard bin.
6. Place the gel tray into the cassette and pour the solution into the tray. Insert the comb into the top of the gel and allow the gel to solidify for 30 min. Avoid bubbles in the gel.
    - Choose either an 8- or 16-well gel depending on application. If performing gel extractions, use the 8- well comb to accommodate a larger mass of DNA.
7. Rinse with water and dry the flask to prevent residual gel from solidifying in the flask.

Running Gel Electrophoresis

1. Once the gel has solidified, carefully remove the comb by pulling straight up.
2. Ensure the gel is in the correct orientation, with the negative/black electrode above the wells so that the DNA runs toward the positive/red electrode.
3. Prepare the samples by adding 6X loading buffer to each. For a 16-well gel, combine 5 µL of DNA with 1 µL of 6X loading buffer in order to load 5 µL. [For most applications, load 20-100 ng of DNA/lane.] If doing a gel extraction in an 8-well gel, combine 30 µL DNA with 6 µL 6X loading buffer to load 36 µL.
    - Note: These samples can be prepared on the reverse side of paraffin paper since the volume is so small.
4. Load 5 µL of DNA ladder into one lane of your gel.
    - Note: Choose a ladder that contains the weights of your sample.
5. Load samples into wells. Avoid bubbles.
6. Place lid on cassette and ensure the red and black wires are connected to the matching red and black electrodes on the cassette.
7. Gel can be run at a variety of time and voltage settings depending on the size of samples and desired separation. For most samples, 90V for 30-50 min will work.
8. Remove the tray with the gel and image with UV. Exercise caution when imaging with UV, especially if doing a gel extraction over the UV box.
9. If doing gel extraction, proceed with gel. If not, dispose of the gel in the gel biohazard bin.

Protocol #3: Polymerase Chain Reaction (New England BioLabs)

1. Reaction Setup: We recommend assembling all reaction components on ice and quickly transferring the reactions to a thermocycler preheated to the denaturation temperature (98°C). All components should be mixed and centrifuged prior to use. It is important to add Phusion DNA Polymerase last in order to prevent any primer degradation caused by the 3´→ 5´ exonuclease activity. Phusion DNA Polymerase may be diluted in 1X HF or GC Buffer just prior to use in order to reduce pipetting errors. Please note that protocols with Phusion DNA Polymerase may differ from protocols with other standard polymerases. As such, conditions recommended below should be used for optimal performance.

Component	20 µl Reaction	50 µl Reaction	Final Concentration
Nuclease-free water	To 20 µl	To 50 µl	
5x Phusion HF or GC Buffer	4 µl	10 µl	1x
10 mM dNTPs	0.4 µl	1 µl	200 µM
10 Forward Primer	1 µl	2.5 µl	0.5 µM
10 Reverse Primer	1 µl	2.5 µl	0.5 µM
Template DNA	variable	variable	<250 ng

Notes: Gently mix the reaction. Collect all liquid to the bottom of the tube by a quick spin if necessary. Overlay the sample with mineral oil if using a PCR machine without a heated lid.

Transfer PCR tubes from ice to a PCR machine with the block preheated to 98°C and begin thermocycling:

Thermocycling conditions for a routine PCR:

STEP	TEMP	TIME
Initial Denaturation	98 °C	30 seconds
25-35 cycles	98 °C 45-72 °C 72 °C	5-10 seconds 10-30 seconds 15-30 seconds per kb
Final Extension	72 °C	5-10 minutes
Hold	4-10 °C	

General Guidelines:

Template:
Use of high quality, purified DNA templates greatly enhances the success of PCR. Recommended amounts of DNA template for a 50 µl reaction are as follows:

DNA	Amount
Genomic	50ng – 250 ng
Plasmid or viral	1pg – 10 ng

If the template DNA is obtained from a cDNA synthesis reaction, the volume added should be less than 10% of the total reaction volume.

2. Primers:
Oligonucleotide primers are generally 20–40 nucleotides in length and ideally have a GC content of 40–60%. Computer programs such as Primer3 can be used to design or analyze primers. The final concentration of each primer in a reaction using Phusion DNA Polymerase may be 0.2–1 µM, while 0.5 µM is recommended.

1. Mg^{++} and additives:
   Mg^{++} is critical to achieve optimal activity with Phusion DNA Polymerase. The final Mg^{++} concentration in 1X Phusion HF and GC Buffer is 1.5 mM. Excessive Mg^{++} can prevent full denaturation of DNA as well as cause non-specific binding of primers. The optimal Mg^{++} concentration is affected by dNTP concentration, the template being used and supplements that are added to the reaction. This can also be affected by the presence of chelators (e.g. EDTA). Mg^{++} can be optimized in 0.5 mM increments using the $MgCl_2$ provided.
   Amplification of difficult targets, such as those with GC-rich sequences or secondary structure, may be improved by the presence of additives such as DMSO (included). A final concentration of 3% DMSO is recommended, although concentration can be optimized in 2% increments. It is important to note that if a high concentration of DMSO is used, the annealing temperature must be lowered as it decreases the primer T_m (2). Phusion DNA polymerase is also compatible with other additives such as formamide or glycerol.

2. Deoxynucleotides:
   The final concentration of dNTPs is typically 200 µM of each deoxynucleotide. Phusion cannot incorporate dUTP.

3. Phusion DNA Polymerase Concentration:
   We generally recommend using Phusion DNA Polymerase at a concentration of 20 units/ml (1.0 units/50 µl reaction). However, the optimal concentration of Phusion DNA Polymerase may vary from 10–40 units/ml (0.5–2 units/50 µl reaction) depending on amplicon length and difficulty. Do not exceed 2 units/50 µl reaction, especially for amplicons longer than 5 kb.

4. Buffers:
   5X Phusion HF Buffer and 5X Phusion GC Buffer are provided with the enzyme. HF buffer is recommended as the default buffer for high-fidelity amplification. For difficult templates, such as GC-rich templates or those with secondary structure, GC buffer can improve reaction performance. GC buffer should be used in experiments where HF buffer does not work. Detergent-free reaction buffers are also available for applications that do not tolerate detergents (e.g. microarray, DHPLC).

5. Denaturation:
   An initial denaturation of 30 seconds at 98°C is sufficient for most amplicons from pure DNA templates. Longer denaturation times can be used (up to 3 minutes) for templates that require it.

   During thermocycling, the denaturation step should be kept to a minimum. Typically, a 5–10 second denaturation at 98°C is recommended for most templates.

6. Annealing:
   Annealing temperatures required for use with Phusion tend to be higher than with other

PCR polymerases. **The NEB Tm calculator should be used to determine the annealing temperature when using Phusion.** Typically, primers greater than 20 nucleotides in length anneal for 10–30 seconds at 3°C above the T_m of the lower T_m primer. If the primer length is less than 20 nucleotides, an annealing temperature equivalent to the T_m of the lower primer should be used. A temperature gradient can also be used to optimize the annealing temperature for each primer pair. For two-step cycling, the gradient can be set as high as the extension temperature.

For high T_m primer pairs, two-step cycling without a separate annealing step can be used.

7. Extension:
   The recommended extension temperature is 72°C. Extension times are dependent on amplicon length and complexity. Generally, an extension time of 15 seconds per kb can be used. For complex amplicons, such as genomic DNA, an extension time of 30 seconds per kb is recommended. Extension time can be increased to 40 seconds per kb for cDNA templates, if necessary.

8. Cycle number:
   Generally, 25–35 cycles yields sufficient product.

9. 2-step PCR:
   When primers with annealing temperatures ≥ 72°C are used, a 2-step thermocycling protocol is recommended.

Thermocycling conditions for a routine 2-step PCR:

STEP	TEMP	TIME
Initial Denaturation	98 °C	30 seconds
25-35 cycles	98 °C 72 °C	5-10 seconds 15-30 seconds per kb
Final Extension	72 °C	5-10 minutes
Hold	4-10 °C	

10. PCR product:
    The PCR products generated using Phusion DNA Polymerase have blunt ends; if cloning is the next step, then blunt-end cloning is recommended. If TA-cloning is preferred, then DNA should be purified prior to A-addition, as Phusion DNA Polymerase will degrade any overhangs generated.

    Addition of an untemplated -dA can be done with *Taq* DNA Polymerase (NEB #M0267) or Klenow exo– (NEB #M0212).

Protocol #4: Ligation Protocol with T4 DNA Ligase (M0202) (New England BioLabs)

1. Set up the following reaction in a microcentrifuge tube on ice.
(T4 DNA Ligase should be added last. Note that the table shows a ligation using a molar ratio of 1:3 vector to insert for the indicated DNA sizes.) Use NEBioCalculator to calculate molar ratios.

COMPONENT	20 µl REACTION
T4 DNA Ligase Buffer (10X)*	2 µl
Vector DNA (4 kb)	50 ng (0.020 pmol)
Insert DNA (1 kb)	37.5 ng (0.060 pmol)
Nuclease-free water	To 20 µl
T4 DNA Ligase	1 µl

- ** The T4 DNA Ligase Buffer should be thawed and resuspended at room temperature.*
- Gently mix the reaction by pipetting up and down and microfuge briefly.
- For cohesive (sticky) ends, incubate at 16°C overnight or room temperature for 10 minutes.
- For blunt ends or single base overhangs, incubate at 16°C overnight or room temperature for 2 hours *(alternatively, high concentration T4 DNA Ligase can be used in a 10 minute ligation)*.
- Heat inactivate at 65°C for 10 minutes.
- Chill on ice and transform 1-5 µl of the reaction into 50 µl competent cells.

Protocol #5: Creation and Scale up of a Stable Cell Line using ExpiCHO™ Products (ThermoFisher Scientific)

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 shakerspeed.
- 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 × 10^6 – 6 × 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
2. choice and confirm linearization by running a gel.

   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
2. ExpiCHOS™ culture of 4×10^6–6×10^6 viable cells/mL to a final density of 1×10^6–2×10^6 viable cells/mL and allow the cells to grow overnight.

   On the day of transfection (Day 0), determine viable cell density and viability.

   The cells should have reached a density of approximately 2×10^6–6×10^6 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, prewarmed 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

Creation and Scale up of a Stable Cell Line using ExpiCHO™ Products User Bulletin ™6 mM of L-glutamine supplementation to the ExpiCHO Expression Medium.

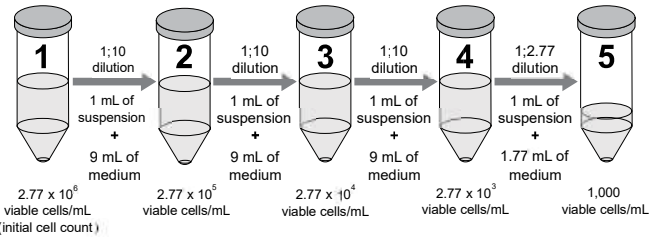
1. Create a cloning medium by adding L-glutamine to at 6 mM and pre-warm it to 37°C.

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.

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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 prewarmed 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.

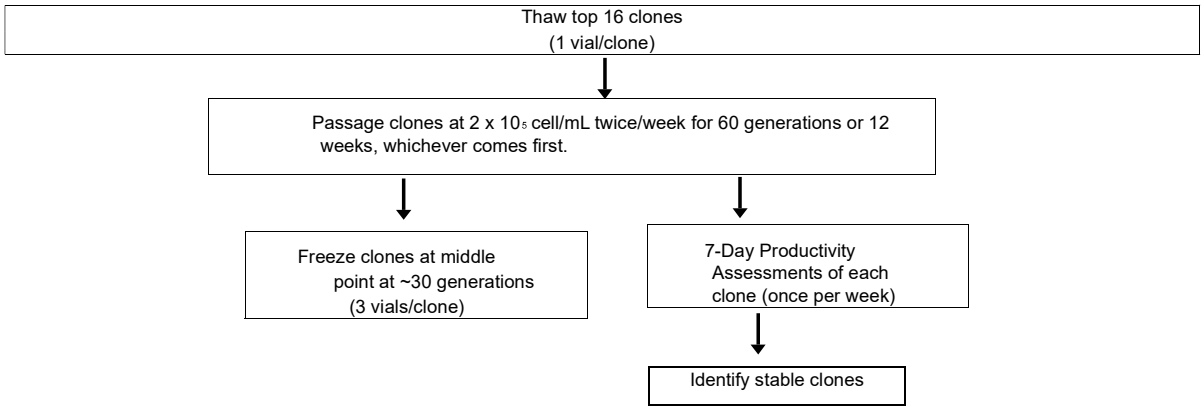
5. At generation 30, we recommend to do a safety freeze of 3 vials of each clone to mitigate loss. See “Cryopreserve cells“ on page 6.

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.

Protocol #6: Jeff's E. coli Transformation Protocol (The Blumberg Combined Protocols)

1. Thaw the desired competent E. coli cells on ice for 5 min
    - (DH5 alpha, STBL3, MACHT1, BL21DE3)
2. Add 1-10ng of DNA to 10-25µl cells
    - Can use more DNA when transforming a ligation
3. Incubate on ice for 10 min
4. Heat shock at 42°C for 30 sec
5. Incubate on ice for 2 min
6. Add 200-400ul SOC media
7. Incubate for 0.5-1 hour on 37°C heat block
8. Add E. coli to 4mL LB broth with ampicillin (100ug/mL) or kanamycin (25ug/mL) in 50mL centrifuge tube or plate on appropriate LB agar plate with antibiotic
9. Shake overnight at 37°C and 225-250rpm
10. Miniprep to extract DNA and measure concentration on nanodrop

Protocol #7: Plasmid Prep Protocol, Alkaline Lysis Method, LiCl & PEG Precipitations (Cold Spring Harbor Laboratory)

Alkaline Lysis Miniprep Procedure (3 mL culture):

1. Transfer 1.5 mL of culture to 1.5 mL eppendorf tube and centrifuge culture @ 14k rpm for 5 minutes. Decant and pipette off residual supernatant. Repeat until entire culture is consolidated in one pellet in one tube.

2. Resuspend pellet in 100 µL ice cold Solution I by vortexing and pipetting until no clumps remain.

3. Add 200 µL room temperature Solution II. Gently invert 5-10x to lyse cells then place on ice. Contents will become transluscent and viscous.

4. Add 150 µL Solution III. Vortex in inverted position for 10 seconds or until precipitate fullly develops and contents are no longer viscous.

5. Centrifuge for 5 minutes at 14k rpm in table top centrifuge.

6. Carefully transfer supernatant to fresh 1.5 mL eppendorf tube. If pellet is loose, you can filter the supernatant through a 0.2-0.45 µm syringe filter into fresh tube.

7. Precipitate DNA by adding 2x volumes of 90-100% EtOH at room temperature. Vortex and let stand for 2-5 minutes to allow precipitate to develop.

8. Pellet DNA by centrifugation for 5 minutes at 14k rpm in table top centrifuge.

9. Remove supernatant with p1000 pipettor.

10. Rinse pellet with 1 mL of 70% EtOH, then remove wash ith p1000 pipettor. Respin for 10 seconds and remove residual supernatant with p200 pipettor.

11. Let tubes dry with lids open.

12. Resuspend pellet in 100 µL TE buffer.

13. Obtain [DNA] with UV scan from 240-340 nM of a 100x dilution of plasmid prep in TE using extinction coefficient at 260 nm = 50 µg/mL/OD. Take note of 260/280 ratio.

14. Store at -20 °C, or proceed with RNAase treatment.

15. Add 1% (v/v = 1 µL) of 1 mg/mL RNAase A to DNA prep and incubate at RT for 60 minutes.

16. Proceed with LiCl and PEG precipitation steps.

LiCl Precipitation:

LiCl precipitation helps remove RNA and trace proteins from the DNA prep.

Materials:

· 7.5 M LiCl, 0.2 µm filtered and stored at -20 C.

LiCl precipitation procedure:

1. Add a volume of ice-cold 7.5 M LiCl equal to 50% of the volume of DNA solution, and mix well by pipetting. Final concentration of LiCl will be 2.5 M.

a. Miniprep (3 mL culture): 100 µL DNA + 50 µL 7.5 M LiCl

b. Maxiprep (50 mL culture): 400 µL DNA + 200 µL 7.5 M LiCl

2. Incubate the mixture on ice or in the -20 C Freezer for at least 15 minutes to overnight.

3. Centrifuge in table top Eppendorf centrifuge for 15 minutes at max speed

4. Decant supernatant which contains the DNA to a clean tube. Discard the pellets.

PEG Precipitation:

A subsequent purification of the plasmid DNA to remove residual RNA and contaminating proteins can be performed by PEG precipitation as follows:

Materials:

· 1.6 M NaCl/13% (w/v) polyethylene glycol 8000 (PEG 8k), 0.2 um filtered and stored at -20 C.

PEG precipitation procedure:

1. To the LiCl supernatant, add equal volume of PEG precipitation solution at RT and incubate for 15 minutes, or overnight at 2-8 C for higher yield.

a. Miniprep (3 mL culture): 150 µL LiCl sup + 150 µL PEG solution

b. Maxiprep (50 mL culture): 600 µL LiCl sup + 600 µL PEG solution

2. Recover precipitated DNA by centrifugation at max rpm for 5 minutes at RT.

3. Remove supernatant with p1000, respin, and remove residual PEG precipitation solution.

4. Rinse pellet with 1 mL 70% EtOH, remove wash and respin to remove residual wash.

6. Resuspend DNA in 400-500 µL TE.

7. Obtain [DNA] with UV scan from 240-340 nm of a 100x dilution of plasmid prep in TE using double stranded DNA extinction coefficient at 260 nm = 50 µg/mL/OD. Take note of 260/280 and 260/230 ratios.

a. 260/280 ≥ 2.0 indicates significant RNA impurity; 260/280 = 1.8 -1.9 indicates clean DNA; Pure RNA has a 260/280 ratio of 2.0; 260/280 < 1.8 indicates contaminating proteins in DNA

b. 260/230 ratios < 2 indicate small molecule impurities in the preparation

8. Store at -20 °C.

Protocol #8: Protein Purification Protocol

Phosphoethanolamine Resin Affinity Chromatography

Purpose: purify histidine tag proteins

1. Prepare buffers

    a. Sanitization buffer (0.1M NaOH)

    b. Equilibration buffer (20mM Tris, 150mM NaCl, 1 mM CaCl2, pH 8)

    c. Wash buffer (20mM Tris, 1 M NaCl, 1 mM CaCl2, pH 8)

    d. Elution buffer (20mM Tris, 150 mM NaCl, 5 mM EDTA, pH 8)

2. Pack chromatography column

    a. Slurry phosphoethanolamine (PE) resin

    b. Pour slurry into chromatography column to target volume

    c. Attach flow adapters and flow sanitization buffer over column until 5 column volumes have flowed through column

    d. Flow 10 column volumes of equilibration buffer through column and ensure pH in effluent is 8.

    e. Condition load by adding to a final concentration of 10 mM Tris at pH 8 and 5 mM CaCl2.

    f. Load protein sample onto column.

    g. Flow 5 column volumes of equilibration buffer.

    h. Flow 5 column volumes of wash buffer.

    i. Flow 5 column volumes of equilibration buffer.

    j. Flow 10 column volumes of elution buffer.

    k. Collect ½ cv fractions until 10 cv of elution buffer have flown through column.

    l. Flow 5 cv sanitization buffer over resin.

    m. Unpack chromatography column and store in tube.

Protocol #9: ExpiCHO Transient Transfection Protocol

Table 3. Recommended volumes for transfection at various scales

Flask size	125 mL	250 mL	500 mL	1 L	2 L	3 L
Number of cells required	1.5×10^8	3.0×10^8	6.0×10^8	1.2×10^9	2.4×10^9	4.5×10^9
Culture volume to transfect	25 mL	50 mL	100 mL	200 mL	400 mL	750 mL
Shake speed[1]	125 ± 5 rpm (19-mm shaking diameter) 120 ± 5 rpm (25-mm shaking diameter) 95 ± 5 rpm (50-mm shaking diameter)					**75**± 5 **rpm** **80**± 5 **rpm** **80**± 5 **rpm**
Flask type	Vented, non-baffled					
Amount of plasmid DNA	0.5–1.0 µg total plasmid DNA per mL of culture volume to transfect					
Volume of plasmid DNA[2]	20 µL	40 µL	80 µL	160 µL	320 µL	600 µL
OptiPRO™ SFM[3]	1 mL	2 mL	4 mL	8 mL	16 mL	30 mL
ExpiFectamine™ CHO Reagent	80 µL	160 µL	320 µL	640 µL	1280 µL	2400 µL
OptiPRO™ SFM[4]	920 µL	1.84 mL	3.7 mL	7.4 mL	14.8 mL	28 mL
ExpiCHO™ Enhancer	150 µL	300 µL	600 µL	1200 µL	2400 µL	4500 µL
ExpiCHO™ Feed (Standard)[5]	6 mL	12 mL	24 mL	48 mL	96 mL	180 mL
ExpiCHO™ Feed (High Titer)[5]	6 mL	12 mL	24 mL	48 mL	96 mL	180 mL
ExpiCHO™ Feed (Max Titer)[6]	4 mL on Days 1 and 5	8 mL on Days 1 and 5	16 mL on Days 1 and 5	32 mL on Days 1 and 5	64 mL on Days 1 and 5	120 mL on Days 1 and 5
Final culture volume	~35 mL	~70 mL	~140 mL	~280 mL	~560 mL	~1 L

During all cell manipulations, mix the cells by gentle swirling; avoid vigorous mixing/pipetting. Cell health is critical to maximal performance. Refer to Table 3, page 13, for suggested volumes for transfection at various scales.

- Subculture and expand ExpiCHO-S™ cells until the cells reach a density of approximately $4 \times 10^6 – 6 \times 10^6$ viable cells/mL.

Day –1: Split cells

On the day prior to transfection (Day –1), split the ExpiCHO-S™ culture from Step 1 to a final density of $3 \times 10^6 – 4 \times 10^6$ viable cells/mL and allow the cells to grow overnight.

Day 0: Transfect cells

- On the next day (Day 0), determine viable cell density and percent viability. The cells should have reached a density of approximately $7 \times 10^6 – 10 \times 10^6$ viable cells/mL. Viability should be 95–99% to proceed with transfection.

- Dilute the cells from Step 2 to a final density of 6 × 106 viable cells/mL with fresh ExpiCHO™ Expression Medium, pre-warmed to 37°C. Swirl the flasks gently to mix the cells.

Note: Discard the remaining cells; do not re-use high density cells for routine subculturing.

- Prepare ExpiFectamine™ CHO/plasmid DNA complexes using cold reagents (4°C), as described below. It is not necessary to keep reagents on ice during complexation. Simply remove reagents from refrigeration and commence with DNA complexation.

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

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

b) Dilute plasmid DNA with cold OptiPRO™ medium. Mix by swirling the tube and/or by inversion.

c) Dilute ExpiFectamine™ CHO Reagent with OptiPRO™ medium. Mix by swirling the tube and/or by inversion or gentel pipetting 2–3 times.

Note: Dilute the ExpiFectamine™ CHO reagent with cold OptiPRO™medium just prior to addition to the diluted DNA. Holding diluted ExpiFectamine™ CHO reagent for longer than 5 minutes before addition to diluted plasmid DNA can lead to reduced protein titers. See Troubleshooting section for additional information.

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

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

- Incubate the cells in a 37° C incubator with a humidified atmosphere of 8% CO2 in air on an orbital shaker (refer to Table 3, page 13, for suggested shake speeds).

Day 1: Add ExpiFectamine™ CHO Enhancer and ExpiCHO™ Feed

On the day after transfection (Day 1, 18–22 hours post-transfection), perform the following additions depending on the protocol chosen (see Table 3, page 13, for the volumes to add):

Note: It is not necessary to pre-warm the ExpiFectamine™ CHO Enhancer or the ExpiCHO™ Feed prior to addition to flasks.

Note: ExpiFectamine™ CHO Enhancer and ExpiCHO™ Feed may be premixed together immediately prior to adding to flasks.

Standard Protocol: Add ExpiFectamine™ CHO Enhancer and ExpiCHO™ Feed to the flask (according to Table 3, page 13), gently swirling the flask during addition. Return the flask to the 37°C incubator with a humidified atmosphere of 8% CO_2 with shaking.

High Titer Protocol: Add ExpiFectamine™ CHO Enhancer and ExpiCHO™ Feed to the flask (according to Table 3, page 13), gently swirling the flask during addition. Transfer the flask to a 32°C incubator with a humidified atmosphere of 5% CO_2 in air with shaking.

Max Titer Protocol: Add ExpiFectamine™ CHO Enhancer and ExpiCHO™ Feed to the flask (according to Table 3, page 13), gently swirling the flask during addition. Transfer flask to a 32°C incubator with a humidified atmosphere of 5% CO_2 in air with shaking.

Day 5:

For Max Titer Protocol Only: On Day 5 post-transfection, add the second volume of ExpiCHO™ Feed to the flask (according to Table 3, page 13) and immediately return the flask to 32°C incubator with shaking.

Optimal time to harvest protein will depend on the specific properties of the protein being expressed and the protocol chosen. Typical harvest times to reach maximum titers for the various protocols are as follows:

Standard Protocol: 8–10 days post-transfection

High Titer Protocol: 10–12 days post-transfection

Max Titer Protocol: 12–14 days post-transfection

Optimize protein expression

- Expression levels will vary depending on the specific recombinant protein expressed and the vector used; however, the ExpiCHO™ Expression System will exhibit consistent expression level for any particular protein from one transfection to the next.
- When expressing a protein for the first time, you may want to perform a time course (e.g., harvest cells or media at several time points post-transfection) to optimize the length of the expression run.
- The ExpiCHO™ Expression Medium is designed to support transiently transfected cultures for up to 14 days in conjunction with ExpiFectamine™ CHO Enhancer and ExpiCHO™ Feed in the Max Titer protocol, however, it will not always be necessary, or desirable, to take expression runs out to 14 days for a given protein.