XtenCHO™

# Mammalian Protein Expression

## Starter Kit Technical Guide and Protocol

Catalog number **PX-XTE-001**

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# Part I
# Introduction

*The XtenCHO™ Starter Kit (Cat. no. PX-XTE-001) is a complete solution for high-yield transient expression based on suspension-adapted XtenCHO™ Cells in a defined, serum-free medium. The XtenCHO™ Starter Kit provides cells, expression medium and reagents to transfect 1 liter of cell culture.*

## The XtenCHO™ Cell Line

The XtenCHO™ cell line is derived from the Chinese Hamster Ovary CHO-K1 cell line, which has been genetically modified. Combined with the optimized expression vector pXten1 and transfected with the optimized protocol and reagents, XtenCHO™ Cells sustain extended transient gene expression, by enhancing plasmid-driven expression and allowing better plasmid maintenance.

The XtenCHO™ Cells are adapted to high-density suspension culture in XtenCHO™ Expression Medium. Frozen cells are supplied, and may be thawed directly into XtenCHO™ Expression Medium.

The XtenCHO™ cell line exhibits the following characteristics:

- Derived from the CHO-K1 cell line
- Adapted to high density, serum-free, suspension growth in XtenCHO™ Expression Medium
- Doubling time of approximately **23 hours**
- High protein expression

The XtenCHO™ Cells are also available separately; see **Page 28** for ordering information.

## XtenCHO™ Expression Medium

The XtenCHO™ Expression Medium is serum-free, animal-component free, and chemically-defined. The XtenCHO™ Expression Medium optimizes productivity with sustained balanced growth and viability post-transfection. The XtenCHO™ Expression Medium supports small- and large-scale transient transfection in XtenCHO™ Cells, enabling researchers to use the same medium for regular subculturing and for transient transfection. XtenCHO™ Expression Medium requires additional supplementation of L-Glutamine and Anticlumping agent.

The XtenCHO™ Expression Medium is also available separately; see **Page 28** for ordering information.

## XtenCHO™ Transfection Reagent

The XtenFect Reagent is optimized for the transfection of nucleic acids into high-density XtenCHO™ cell cultures grown in XtenCHO™ Expression Medium. The XtenFect Reagent is provided as a 10 X concentrated stock solution, and the XtenFect Reagent Working solution has to be reconstituted prior to use on XtenCHO™ Cells. Then the XtenFect Reagent Working solution can be added directly to cells in XtenCHO™ Expression Medium. It is not necessary to pre-form complexes with plasmid DNA before addition to the cell cultures.

Note: Use of transfection reagents other than the XtenFect Reagent to transfect high density XtenCHO™ cultures can lead to substantially reduced performance.

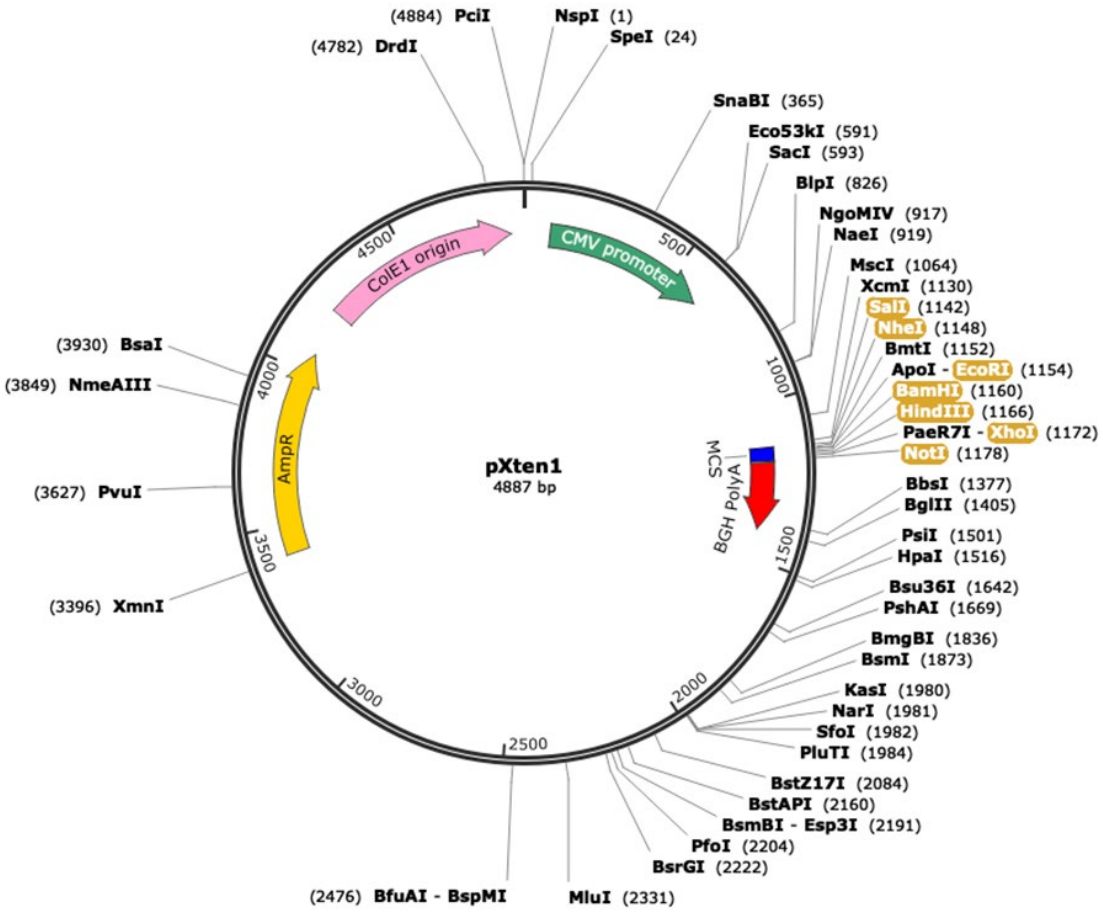
The XtenCHO™ Enhancer is designed to maintain high viability in high density transfected XtenCHO™ Cells, resulting in extended transient gene expression and enhancement of protein production.

## Expression Vector

The pXten1 Expression Vector is an empty vector optimized for the expression of recombinant proteins or antibodies in XtenCHO™ Cells. The plasmid is designed to be used in XtenCHO™ Cells permitting enhanced plasmid-driven expression and better plasmid maintenance, resulting in increased levels of protein expression.

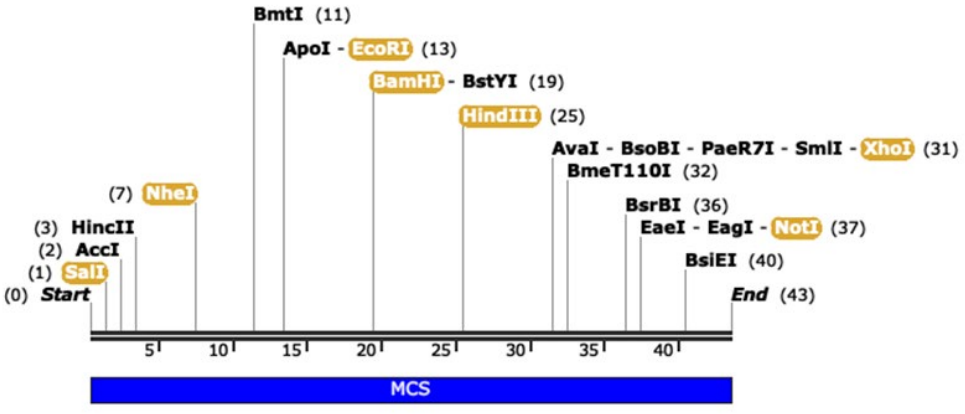
The Antibody Expression Positive Control Vector is provided as a control for transfection and expression in XtenCHO™ Cells. The control consists of pXten1 plasmids expressing the heavy and light chains of a human IgG1 antibody. The control is a transfection-grade plasmid provided at a concentration of 1 µg/µL with a 1:1 light chain / heavy chain ratio and is enough to transfect up to 150 mL of XtenCHO™ Cells.

## pXten1 Vector Information




Sequencing **primers** (after subcloning into the Multiple Cloning Site, **MCS**):

| | |
|---|---|
| **Forward primer (5' - 3')** | TCCACAGGTGTCCACTC |
| **Reverse primer (5' - 3')** | AACAGATGGCTGGCAACTA |




**pXten1 MCS**
43 bp

# Part II
# Product Components

*Each XtenCHO™ Starter Kit includes the following components:*

| Component | Quantity/Size | Storage |
|---|---|---|
| XtenCHO™ **Cells** (1 x $10^7$ cells/mL) | 2 x 1 mL | Liquid nitrogen[1] |
| XtenCHO™ Expression **Medium** | 1 L | 4°C, protect from light |
| XtenCHO™ Transfection **Kit** 1 L, contains: | 1 kit | |
| XtenFect Reagent, **Stock solution** *(dilute prior to use)* | 1 x 0.51 mL | -80°C, until dilution |
| XtenFect Reagent, **Working solution** *(reconstitute prior to use)* | 3 x 1.44 mL | -20°C, until reconstitution |
| XtenCHO™ **Enhancer** | 3 x 1.2 mL | -20°C |
| pXten1 Expression **Vector** (1 µg/µL in T10E1 buffer, pH8) | 1 x 10 µL | -20°C |
| Antibody Expression Positive **Control** Vector (1 µg/µL in T10E1 buffer, pH8) | 1 x 150 µL | -20°C |

[1]Store the frozen cells in liquid nitrogen, in the vapor phase until thawing. Do not store the cells at -80°C.

*Reagents to be supplied by the user:*

- **L-Glutamine** solution, culture grade, 200 mM
- **Anticlumping agent**, culture grade
- **Endotoxin-free,** transfection-grade **plasmids** expressing your protein of interest
- **DMSO**

# Part III
# Product Details

| | |
|---|---|
| **Kit Size** | The XtenCHO™ Starter Kit provides cells, culture medium, and reagents to transfect **1 liter** of cell culture. |
| **Shipping Conditions** | Dry ice for cells and reagents and blue ice for the medium. |
| **Storage Conditions** | Stable for **12 months** from the date of manufacture, if cells, reagents and medium are conserved at their optimal storage temperature. Diluted XtenFect Reagent, Working solution, and XtenCHO™ Enhancer should be used within **4 weeks** after dilution and thawing, respectively. |
| **Validation data** | Tested with Antibody Expression Positive Control Vector. |
| **Expected Control Results** | Productivity **> 250 mg/L** (before purification). |

# Part IV
# XtenCHO™ Protocol

## Preparation of Working Solutions and Complete Media

### A. Preparation of Complete XtenCHO™ Expression Medium

The XtenCHO™ Expression Medium is formulated without **L-Glutamine** and **Anticlumping agent**. L-Glutamine is quickly hydrolysed in the medium, decreasing its actual concentration and generating by-products of hydrolysis which can be toxic to the cells.

To ensure optimal L-Glutamine concentration in medium, end users should supplement the XtenCHO™ Expression Medium with the recommended concentration of fresh L-Glutamine (8 mM). An Anticlumping agent (such as 0010057AE from Gibco) should also be added to the cultures at a final concentration of 0.5% for cell subculturing prior to transfection and post transfection.

However, the Anticlumping agent should not be added to the cultures during the transfection or it will result in decreased protein yields. It is highly recommended to remove the Anticlumping agent during the last passage before transfection, by replacing completely the culture medium with XtenCHO™ Expression Medium supplemented with 8 mM L-Glutamine and without Anticlumping agent.

To prepare complete XtenCHO™ Expression Medium:

- Supplement XtenCHO™ Expression Medium with **8 mM L-Glutamine**
- Add **0.5% Anticlumping agent** to the culture when required (cell subcultures and maintenance, and cultures post-transfection)

### B. Preparation of XtenFect Reagent, Working solution

To ensure optimal and prolonged performance of the transfection reagent, the XtenFect Reagent is supplied as a 10 X concentrated stock solution and the XtenFect Reagent Working solution has to be reconstituted prior to use on XtenCHO™ Cells. XtenFect Reagent Stock solution should not be used non-diluted on cells or it will dramatically affect cell viability.

To reconstitute the XtenFect Reagent Working solution:

1. Thaw one tube labeled "**XtenFect Reagent, Working solution**" and let it reach room temperature. The tube contains 1440 µL of XtenFect Dilution Buffer.

2. Thaw the tube of labeled "XtenFect Reagent, Stock solution". The tube contains 510 µL of 10 X concentrated XtenFect Reagent.

3. Pipet 160 µL of "XtenFect Reagent, Stock solution" and add them to the XtenFect Dilution Buffer into the tube labeled "XtenFect Reagent, Working solution".

4. Mix by inversion.

5. The reconstituted XtenFect Reagent Working solution is now ready to use.

6. Concentrated XtenFect Reagent Stock solution should be frozen again and stored at -80°C. Avoid multiple freeze-thaw cycles as it can lead to reduced transfection performances. If needed, the XtenFect Reagent Stock solution can be aliquoted.

7. The diluted XtenFect Reagent Working solution could be stored at 4°C and should be used within 4 weeks for optimal performance.

# Culture of XtenCHO™ Cells

## A. Recommendations for XtenCHO™ cell culture

Follow the general guidelines below to thaw, subculture and expand XtenCHO™ Cells.

All cell cultures must be performed in **microbiological safety cabinet** using aseptic technique to ensure sterility. All media and reagents that come in contact with the cells must be sterile.

Store the frozen cells in liquid nitrogen in the vapor phase until thawing. **Do not store the cells at -80°C.** For all cell manipulations, mix the cells by gentle **swirling; avoid extensive shaking/pipetting.** Allow freshly thawed cells to recover in culture for **3 passages** before transfecting.

For maintenance of cells, passage XtenCHO™ Cells when they reach a density of approximately **$1.5 \times 10^6$ - $2.5 \times 10^6$ viable cells/mL** (i.e., early log-phase growth), typically every **2-3 days.** Please refer to **Table 1** on **Page 13** to consult recommended cell seeding densities.

Note: Cells that are subcultured at densities outside of this early log-phase growth window may show longer doubling times and lower titers over time. Modify the initial seeding density to reach the target cell density of $1.5 \times 10^6$ - $2.5 \times 10^6$ viable cells/mL at the time of subculturing.

XtenCHO™ Cells should be cultured between **2 and 20 passages.** Optimal performances are obtained in these conditions. Use a **hemocytometer** with the trypan blue exclusion method or an **automated cell counter** to determine cell viability. Log-phase cultures should be >95% viable. When thawing cells, **transfer cells into pre-warmed medium.**

## B. Thaw and establish XtenCHO™ Cells

### Introduction

The protocol below describes steps to thaw the XtenCHO™ Cells and initiate cell culture. The XtenCHO™ Cells are supplied in a vial containing 1 mL of cells at 1 × $10^7$ viable cells/mL in 90% XtenCHO™ Expression Medium and 10% DMSO. Thaw the cells directly into **XtenCHO™ Expression Medium supplemented with L-Glutamine 8 mM, pre-warmed at 37°C.**

### Required materials

- XtenCHO™ Cells, frozen cryovial
- XtenCHO™ Expression Medium, pre-warmed to 37°C
- L-Glutamine, 200 mM
- Anticlumping agent
- 125-mL disposable, sterile, vented, baffled Erlenmeyer shake flask
- Reagents and equipment to determine viable cell density and viability (e.g., hemocytometer or an automated cell counter, trypan blue)
- Incubator shaker set at 37°C with ≥80% relative humidity and 5% $CO_2$

NOTE: Store the frozen cells in liquid nitrogen in vapor phase, until thawing. Do not store the cells at -80°C. For all cell manipulations, mix the cells by gentle swirling; avoid extensive shaking/pipetting.

### Thawing of XtenCHO™ Cells

1. Remove the vial of cells from liquid nitrogen and immediately swirl in a **37°C** water bath for 90 seconds maximum to thaw the cells rapidly until only a small amount of ice remains. Do not submerge the vial in water.

2. Just before the cells are completely thawed, decontaminate the vial by wiping it with 70% ethanol before opening it in a laminar flow hood.

3. Quickly transfer the entire contents of the cryovial into **8 mL** of pre-warmed XtenCHO™ Expression Medium. In order to recover all the XtenCHO™ Cells, we advise rinsing the cryovial with XtenCHO™ Expression Medium. Mix by inversion.

4. Centrifuge the cellular suspension for **5 minutes at 300 x *g***. Discard the supernatant. This step is intended to remove the freezing medium.

5. Resuspend the cells in a small volume of XtenCHO™ Expression Medium supplemented with 8 mM L-Glutamine **(2 mL)**.

6. Determine viable cell number and viability.

7. Transfer the total amount of cells to pre-warmed XtenCHO™ Expression Medium

supplemented with 8 mM L-Glutamine in a baffled shake flask at a final seeding density of **0.3 × $10^6$ cells/mL**. Add Anticlumping agent at a final concentration of **0.5%**.

8. Incubate the flask in a **37°C** incubator with **≥80%** relative humidity and **5%** $CO_2$ on an orbital shaker platform until cultures reach a density of **1.5 - 2.5 × $10^6$ viable cells/mL**.

   Note: Set the shake speed to 140-150 rpm for shakers with a 19 mm shaking diameter, 125-130 rpm for shakers with a 25 mm shaking diameter and 95-100 rpm for shakers with a 50 mm shaking diameter. Please refer to **Table 2** on **Page 13** for shaking speed recommendations.

9. On **Day 2 or 3** post-thaw, determine viable cell density and viability percentage. Cell viability should be ≥90% by 3 days post-thaw.

10. Continue to monitor cell density and viability and subculture the cells once the culture has reached **1.5 - 2.5 × $10^6$ viable cells/mL** (typically 2-3 days post-thaw) using the procedure described below.

## C. Subculture XtenCHO™ Cells

### Introduction

XtenCHO™ Cells are capable of achieving high cell densities; therefore, we recommend that the cells reach a density of **1.5 - 2.5 × $10^6$ viable cells/mL** at the time of subculturing.

NOTE: Do not allow the cells to reach a density of 3.5 - 4 x $10^6$ and higher during cell subculture.

### Required materials

- XtenCHO™ cell cultures at 1.5 - 2.5 × $10^6$ viable cells/mL
- XtenCHO™ Expression Medium supplemented with 8 mM L-Glutamine
- Anticlumping agent
- Disposable, sterile, vented, baffled Erlenmeyer shake flask
- Reagents and equipment to determine viable cell density and viability (hemocytometer or an automated cell counter, trypan blue)
- Orbital shaker in a 37°C incubator with ≥80% relative humidity and 5% $CO_2$
- DMSO

### Passage of XtenCHO™ Cells

1. Determine the viable cell density of the culture and calculate the volume of cell suspension required to seed a new shake flask according to the recommended seeding densities in **Table 1** and the recommended culture volumes in **Table 2**.

**Table 1.** Recommended seeding densities for routine subculturing.

| Subculture timing | Recommended seeding density |
|---|---|
| For cells ready 2 days post-subculture | 0.3 x $10^6$ viable cells/mL |
| For cells ready 3 days post-subculture | 0.2 - 0.3 x $10^6$ viable cells/mL |

**Table 2.** Recommended volumes and shaking speeds for routine cell culture maintenance.

| Flask size | 125 mL | 250 mL | 500 mL | 1000 mL | 2000 mL | 3000 mL |
|---|---|---|---|---|---|---|
| **Culture volume (mL)** | 30-40 | 60-100 | 125-200 | 250-400 | 500-800 | 750-1200 |
| **Shaking speed** | 140 ± 10 rpm (19-mm shaking diameter) | | | | | 95 ± 5 rpm |
| | 130 ± 5 rpm (25-mm shaking diameter) | | | | | 90 ± 5 rpm |
| | 95 ± 5 rpm (50-mm shaking diameter) | | | | | 85 ± 5 rpm |
| **Flask type** | Vented, baffled | | | | | |

2. Transfer the calculated volume of cells to a new tube and centrifuge for **5 min at 300 x *g***. Discard the supernatant. Resuspend the pellet in fresh XtenCHO™ Expression Medium supplemented with **8 mM L-Glutamine** and transfer the cellular suspension to a baffled shake flask. Add Anticlumping agent to the cultures at a final concentration of **0.5%**.

3. Incubate flasks in a **37°C** incubator with **≥80%** relative humidity and **5%** $CO_2$ on an orbital shaker platform until cultures reach a density of **1.5 - 2.5 × $10^6$ viable cells/mL**.

4. Repeat Steps 1-3 to amplify the cells for transfection.

## Cryopreservation

1. Cultivate cells during **3 passages** before freezing, let the cells reach a viable cell density of **1.5 - 2.5 × $10^6$ cells/mL** and >95% viability before freezing. Do not freeze cells at <90% viability.

2. Determine the viable cell density of the culture and calculate the volume of cell suspension required to freeze aliquots of XtenCHO™ Cells, at a density of **1 × $10^7$ viable cells/mL**.

3. Calculate the required volume of freezing medium, prepare it and maintain it at 4°C. Freezing medium should be composed of **90 % XtenCHO™ Expression Medium** (supplemented with L-Glutamine and Anticlumping agent) and **10% DMSO**.

4. Centrifuge the cells at **300 × *g* for 5 minutes**, discard the medium, and replace it with chilled freezing medium. Gently resuspend the cell pellet by pipetting.

5. Aliquot **1 mL** of the cell suspension into each cryovial.

6. Place cryovials quickly in a freezing container designed to achieve a cooling rate of **-1°C/minute**, the optimal rate for cell preservation, then place the freezing container in a -80°C freezer.

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

NOTE: Cells should not be in contact with the freezing medium more than 10 minutes before putting them in the -80°C freezer. Moreover, it is important to achieve a cooling rate of -1°C/minute. When not followed, this may increase cell death during freezing.

# Transfection of XtenCHO™ Cells

## A. Recommendations for transfection of XtenCHO™ Cells

### Introduction

Use freshly thawed cells and allow them to recover in culture for **3 or more passages** before transfecting. We recommend not using XtenCHO™ Cells aged more than 20 passages for transfection to keep optimal transfection efficiency and yields.

During all cell manipulations, mix the cells by **gentle swirling; avoid vigorous mixing/ pipetting.** Cell health is critical to maximal performance.

Make sure you have **replaced** the culture medium by **medium without Anticlumping agent** the day before the transfection.

For optimal transfection of high-density suspension XtenCHO™ cultures, use the XtenFect Reagent included in the transfection kit. Make sure to dilute the **"XtenFect Reagent, Stock solution"** into the **"XtenFect Reagent, Working solution"** before use (see protocol for preparation of XtenFect Reagent Working solution on Page 9). The use of transfection reagents other than the XtenFect Reagents to transfect high density XtenCHO™ cultures can lead to substantially reduced performance.

The **plasmid DNA of interest** or **Antibody Expression Positive Control Vector** should be added directly on the cells and cultures, and mixed by gentle swirling.

For expression of recombinant antibodies, we observed that a **1:1 mass ratio of heavy and light chain encoding plasmids can be used with the XtenCHO™ system**. Most of the cells will be co-transfected with the two plasmids.

The expression of the recombinant antibody will then be dependent on the expression rate

of each chain and can be further **optimized**, if necessary, by **modifying heavy to light chain plasmid ratio**. We recommend cloning the heavy and light-chain subunits separately into the **pXten1 expression plasmid**, and then optimizing the ratios of the two plasmids.

It is important to add XtenFect Reagent Working solution **slowly, drop-by-drop** to the cells while swirling the flask. The cell cultures have to be placed in a **37°C** incubator with an orbital shaker, **≥80%** relative humidity, and **5%** $CO_2$.

The XtenCHO™ Enhancer should be added **2 hours post-transfection** and the cell cultures placed back in a **37°C** incubator, under the same conditions.

Unlike some other serum-free media formulations, XtenCHO™ Expression Medium **does not inhibit transfection**. XtenCHO™ Expression Medium is specifically formulated to enable transfection without the need to add feed.

**The harvesting time point** depends on the nature of the protein expressed and should be adjusted accordingly. Expression levels can vary greatly from protein to protein, and the yield will also be impacted by the stability of the protein.

Transfected cells can be kept until viability drops under **50%** or till **day 15** post-transfection. Maintaining high cell viability at the time of harvest facilitates downstream processes, such as protein purification.

For stable proteins, as recombinant antibodies, time of harvest occurs generally on **day 13 to day 15**. For intracellular or unstable proteins, it might be necessary to harvest the cultures at an earlier time point, to avoid toxicity or degradation. Optimal time of harvest should be determined empirically.

### Required materials

- XtenCHO™ Cells cultured in XtenCHO™ Expression Medium + 8 mM L-Glutamine
- High quality and purity; endotoxin-free plasmid DNA preparations of expression vectors
- Antibody Expression Positive Control Vector
- XtenFect Reagent Working solution, reconstituted from Stock solution, pre-warmed to room temperature (see protocol for Preparation of XtenFect Reagent Working solution on Page 9)
- XtenCHO™ Expression Medium, pre-warmed to room temperature, supplemented with 8 mM L-Glutamine
- Anticlumping agent
- XtenCHO™ Enhancer, pre-warmed to room temperature
- Disposable, sterile Erlenmeyer baffled flasks
- Orbital shaker in a 37°C incubator with ≥80% relative humidity and 5% $CO_2$
- Reagents and equipment to determine viable cell density and percent viability

### Scale-up of transfections

The XtenCHO™ Starter Kit allows scaling up and down transfection volumes, from 30 mL to 1000 mL. Follow the guidelines indicated in **Table 3** for cell number, transfection and reagent volumes. For large flasks (Fernbach), the shaking speed of the cultures must be adjusted, please see **Table 2** on **Page 13** for guidelines.

**Table 3.** Guidelines for cell number and volumes for transfection at different scales.

| Cell culture vessel | Cell number (x$10^6$) | DNA quantity (µg) | XtenFect Reagent, Working Solution (µL) | XtenCHO™ Enhancer (µL) | Transfection volume (mL) | Final transfection volume (mL) |
|---|---|---|---|---|---|---|
| 125 mL flask | 75 | 48 | 144 | 96 | 15 | 30 |
| 250 mL flask | 200 | 128 | 384 | 256 | 40 | 80 |
| 500 mL flask | 500 | 320 | 960 | 640 | 100 | 200 |
| 1000 mL flask | 625 | 400 | 1200 | 800 | 125 | 250 |
| 2000 mL flask | 1250 | 800 | 2400 | 1600 | 250 | 500 |
| 3000 mL flask | 2500 | 1600 | 4800 | 3200 | 500 | 1000 |

## B. Transfection of XtenCHO™ Cells

Determine first the volume of cells you wish to transfect and refer to Table 3 above, for cell number and corresponding volumes for plasmids and transfection reagents.

1. Subculture and expand XtenCHO™ Cells until reaching a density of approximately **1.5 - 2.5 × $10^6$ viable cells/mL**.

### Day -1: Passage of XtenCHO™ Cells

2. On the day prior to transfection (Day -1), passage the XtenCHO™ culture to a final density of 1.2 × $10^6$ viable cells/mL in XtenCHO™ Expression medium supplemented with 8 mM L-Glutamine. **Do not add the Anticlumping agent to the culture.** Allow the cells to grow for 24 hours.

## Day 0: Transfection of XtenCHO™ Cells

3. On the next day (**Day 0**), determine viable cell density and viability. The cells should have attained a density of approximately **2 - 3 × $10^6$ viable cells/mL**. Viability should be **>90%** to proceed with transfection. Centrifuge, then resuspend XtenCHO™ Cells at a final density of **5 × $10^6$ viable cells/mL** in half of the final volume wanted with fresh XtenCHO™ Expression Medium supplemented with 8 mM L-Glutamine, following the guidelines indicated in **Table 3** on **Page 16**. **Do not add the Anticlumping agent**, as it will dramatically impact your transfection efficiency. Swirl the flasks gently to mix the cells.

   *For example, for a final 30 mL transfection volume, prepare a cell culture with 5 x $10^6$ cells/mL in 15 mL XtenCHO™ Expression Medium with 8 mM L-Glutamine.*

   NOTE: We recommend discarding the remaining cells and not using high-density cells for routine subculturing.

4. Prepare the XtenFect Reagent Working solution as indicated on **Page 9** and allow it to reach room temperature. Mix the XtenFect Reagent Working solution by inversion.

5. Thaw the expression plasmids and mix them by inverting the tubes.

6. Add the expression plasmids directly on the cells in a row. Refer to **Table 3** on **Page 16** for DNA quantity. Gently swirl the flasks to mix.

   *For example, for a final 30 mL transfection volume, add 48 µg of expression plasmids to the cells.*

7. Add the XtenFect Reagent Working solution slowly, drop-by-drop on the cells, while swirling the flask during addition. Refer to **Table 3** on **Page 16** for XtenFect Reagent Working solution volume.

   *For example, for a final 30 mL transfection volume, add 144 µl of XtenFect Reagent Working solution to the cells.*

8. Incubate the cells in a **37°C** incubator with a humidified atmosphere containing **5%** $CO_2$ in air on an orbital shaker.

## Day 0, 2 hours post-transfection: Add Expression Medium and XtenCHO™ Enhancer

9. Allow XtenCHO™ Expression Medium supplemented with 8 mM L-Glutamine and XtenCHO™ Enhancer to reach room temperature. **2 hours post-transfection**, add the other half volume of XtenCHO™ Expression Medium supplemented with 8 mM L-Glutamine to the culture. Add the required volume of XtenCHO™ Enhancer directly on the cells. Refer to **Table 3** on **Page 16** for XtenCHO™ Enhancer volume. Gently mix by swirling the flask. Viable cell density and viability can be checked at this point.

   *For example, for a final 30 mL transfection volume, add 15 mL of XtenCHO™ Expression Medium (supplemented with 8 mM L-Glutamine) and add 96 µL of XtenCHO™ Enhancer to the transfected cells.*

NOTE: It is normal to observe a drop in cell viability. However, cell viability should not be <80% on Day 0.

10. Return the flasks to the **37° C** incubator with a humidified atmosphere containing **5%** $CO_2$ on an orbital shaker.

### Day 1: Add Anticlumping agent and shift cultures to 33°C

11. **24 hours** post-transfection, add Anticlumping agent to the flask at a final concentration of **0.5%**, gently swirling the flask during the process. Shift XtenCHO™ Cells to a **33°C** incubator with a humidified atmosphere containing **5%** $CO_2$ with shaking. Viable cell density, viability and transfection efficiency can be checked 24 hours and 48 hours after transfection.

    NOTE: It is normal to observe a drop in cell viability. However, cell viability should not be <60% on Day 1 and Day 2.

### Days 10 to 15: Monitor culture viability and harvest when necessary

12. Monitor cell density and viability regularly in the cultures and maintain transfected cells culture until viability drops under **50%** or till **Day 15** post-transfection. Note that if protein is not stable or toxic, the time of harvest has to be adapted. When expressing a protein for the first time, you could harvest cells or media at several time points post-transfection to optimize the length of the expression run.

Harvest by centrifugation cultures for **5 min at 300 x *g*** to pellet cells. For recombinant proteins expected to be expressed intracellularly, keep and freeze cell pellets at **-80°C** for further analysis. Culture supernatants can be further clarified by centrifugation for **30 min at 5000 x *g*** and/or filtration using a **0.22 µm membrane**. It is recommended to proceed quickly with the purification of the protein of interest, or to freeze supernatants at **-80°C** for later use.

### Transfection and expression control

The Antibody Expression Positive Control Vector is provided as a positive control for transfection and expression in XtenCHO™ Cells. The control contains **pXten1 plasmids** expressing the heavy and light chains of a **human IgG1 antibody**. The control is a transfection-grade plasmid provided at a concentration of **1 µg/µL** with a **1:1 light chain / heavy chain ratio** and is sufficient to transfect up to **150 mL** of XtenCHO™ Cells.

The antibody will be expressed and secreted into XtenCHO™ Expression Medium, with optimal yields obtained between **Days 13-15** post-transfection. Typically, yields in crude cell culture supernatants reach concentrations of **> 250 mg/L**, as determined with a Pall Life Sciences FortéBio™ Octet™ instrument with a protein A biosensor. We recommend to transfect **30 mL of XtenCHO™ Cells using 48 µL of the Antibody Expression Positive Control Vector** with the optimal transfection protocol and reagents as described above.

# Part V
# Troubleshooting Guide

| Problem | Solution |
|---|---|
| **Cells show poor growth and viability post thaw. What can I do?** | The viability of XtenCHO™ Cells can be monitored on the day of thawing and should be >90%. The viability should rise quickly after one or two passages post-thaw and reach >95%.<br><br>**We advise being very quick when thawing:** do not let the cells thaw at room temperature and do not let them stay in the freezing medium for too long time before diluting them with XtenCHO™ Expression Medium.<br><br>**We advise using freshly supplemented XtenCHO™ Expression Medium:** an old preparation is not optimal for cells after thawing as significant degradation of L-Glutamine can occur. Avoid using medium supplemented with L-Glutamine more than 7 days ago. It is also suggested to use pre-warmed complete XtenCHO™ Expression Medium for thawing.<br><br>During all cell manipulations, **we recommend avoiding extensive mixing and pipetting of the cells.** It is highly recommended to keep a separate flask for cell culture maintenance and never use cells that have reached high density for routine culture.<br><br>For optimal performance, it is critical that the shaking diameter, shaking speed, flask size/type and volume of culture to be transfected **correspond to the recommended conditions** described in the present guide, for both regular subculture and expression experiments.<br><br>We advise ensuring the equipment is calibrated for $CO_2$, temperature and humidity. **$CO_2$ levels should not exceed 6%.**<br><br>If poor viability and growth persist, we recommend checking the temperature, $CO_2$, humidity and shaking of your incubator. Discard cells if you notice morphological changes in the culture. It is highly recommended to thaw another vial of cells rather than trying to keep culturing these cells. |
| **What can I do to avoid clumping of my XtenCHO™ Cells?** | First, make sure that the expression medium is supplemented with the **Anticlumping agent.** Then transfer the cells into a new vented and baffled flask if you notice the occurrence of clumps around the neck during subculturing. Clumps may result in the underestimation of the cell density when seeding, leading to overgrown cultures. Such clumps can be also observed after transfection but will not dramatically affect the culture.<br><br>**Do not try to pick up cells attached around the neck of the flask.**<br><br>During subculturing, if cell clumps are noticed, resuspend cells carefully and at each passage, allow cell clumps to settle down in the vessel for a few seconds and harvest the suspended cells for subculturing.<br><br>Clumping should not affect cell viability, but it can affect growth as cell density cannot be measured accurately if clumps are present in the suspension. We recommend verifying that the cells are cultured according to the recommendations in the present guide in the presence of 0.5% of Anticlumping agent, below passage 20, and free of contamination. |

| Problem | Solution |
|---|---|
| **What can I do if I observe a premature drop in viability post transfection?** | Depending on the nature of the protein to be expressed and the expression strategy (**intracellular or secreted expression**), the proper time of harvest should be adjusted. Unstable proteins or toxic proteins can lead to a premature drop in viability.<br><br>A drop in viability could also result from suboptimal culture conditions. The shaking speed of the incubator, as well as temperature, $CO_2$ and humidity levels should be verified. Volume and size of the flask should be appropriate. Depending on the flask brand and the model of the incubator, shaking speed might have to be adjusted. |
| **The expression of my recombinant protein is lower than expected with the XtenCHO™ Expression system. What should I do to improve my results?** | Combined with the optimized expression vector, **pXten1**, and transfected with the optimized protocol and reagents, XtenCHO™ Cells sustain extended transient gene expression, by enhancing plasmid-driven expression and allowing better plasmid maintenance.<br><br>Therefore, **we recommend using the pXten1 Expression Vector** to express your protein of interest. XtenCHO™ Cells can sustain high expression levels from various expression plasmids but should show optimal performance when using pXten1 as the expression vector.<br><br>It is recommended to use **high quality endotoxin-free plasmid** preparations for transfection, as low quality and low purity plasmids will result in low transfection efficiency and increased cell toxicity, dramatically affecting bioproduction yield.<br><br>Expression levels can vary greatly from protein to protein; and yield will also be impacted by protein stability. We strongly recommend expressing the positive antibody control to determine if the low yield is rather due to low expression of the protein of interest, suboptimal culture conditions or suboptimal transfection conditions. If you are not achieving the expected yield with the antibody positive control, we recommend checking the following:<br><br>• Monitor cell viability and verify that XtenCHO™ Cell's viability is > 95% during subculture and at time of transfection<br><br>• Verify that the doubling time of XtenCHO™ Cells is close to 24 hours<br><br>• Verify that your shake speed is about 120-130 rpm for 25 mm orbit shakers and about 140-150 rpm for 19 mm orbit shakers<br><br>Further culture optimization may be required. |

# Part VI
# Frequently Asked Questions

## 1. How does the XtenCHO™ Expression system work?

Combined with the optimized pXten1 Expression Vector and transfected with the optimized protocol and reagents, XtenCHO™ Cells sustain extended transient gene expression, by enhancing plasmid-driven expression and allowing better plasmid maintenance.

## 2. How should the XtenCHO™ Cells be stored upon receipt?

Upon receipt of the cells on dry ice, place the vials immediately into liquid nitrogen until thawing; do not store cells at -80°C. Once thawed and transferred into medium in a vented, baffled shake flask, cells should be cultured at 37°C with 5% $CO_2$ on a shaker platform set to 125-130 rpm for a shaker with a 25 mm orbital diameter or 140-150 rpm for a 19 mm orbital diameter. Cells should show a high viability upon thawing and should recover quickly post-thaw, reaching their normal 23-hour doubling time within 1-2 passages.

If viability or growth after 1-2 passages are low, cells may have been compromised during either storage, or thawing, or shipment, and should not be used.

## 3. How should reagents of the XtenCHO™ kits be stored upon receipt?

XtenCHO™ Cells should be placed immediately in liquid nitrogen, in the vapor phase. The XtenCHO™ Expression Medium (1L) should be kept at 4°C. The "XtenFect Reagent, Stock solution" should be stored at -80°C upon receipt. The "XtenCHO™ Enhancer" and the non-reconstituted "XtenFect Reagent, Working solution" can be stored at -20°C. Plasmids (pXten1 Expression Vector and Antibody Expression Positive Control Vector) can be stored at -20°C.

## 4. How should reagents of the XtenCHO™ kits be handled, prepared and stored?

All reagents need to be brought to room temperature prior to use for transfection. Allow the XtenCHO™ Enhancer to thaw and equilibrate at room temperature prior to use. The XtenCHO™ Enhancer is provided as a ready to use solution in 3 aliquots. The XtenCHO™ Enhancer should not be frozen again. It can be stored at 4°C and should be used within 4 weeks.

To ensure long term stability of the transfection reagent, the "XtenFect Reagent, Stock solution" is provided as a 10 X concentrated stock solution. It must be diluted 10 times prior to use and should not be used undiluted on cells.

On the day of transfection, allow the "XtenFect Reagent, Stock solution" and the non-reconstituted "XtenFect Reagent, Working solution" to thaw and equilibrate at room temperature. Then pipet 160 µL of "XtenFect Reagent, Stock solution" into the tube "XtenFect Reagent, Working solution", which contains 1.44 mL of XtenFect Dilution Buffer, and mix by inversion.

Concentrated "XtenFect Reagent, Stock solution" should be frozen again and stored at -80°C. Avoid multiple freeze-thaw cycles for "XtenFect Reagent, Stock solution" as it can lead to reduced transfection performances. If needed, the "XtenFect Reagent, Stock solution" can be aliquoted. The diluted "XtenFect Reagent, Working solution" could be stored at 4°C and should be used within 4 weeks for optimal performance.

## 5. How to prepare ready-to-use XtenCHO™ Expression Medium?

The XtenCHO™ Expression Medium is serum-free, animal-component-free, and chemically-defined. The XtenCHO™ Expression Medium optimizes productivity with sustained balanced growth and viability post-transfection. The XtenCHO™ Expression Medium supports small- and large-scale transient transfection in XtenCHO™ Cells, enabling researchers to use the same medium for regular subculturing and for transient transfection.

The XtenCHO™ Expression Medium is formulated without L-Glutamine and Anticlumping agent. L-Glutamine is quickly hydrolyzed in medium, decreasing its actual concentration and generating byproducts of hydrolysis which can be toxic to the cells. To ensure optimal L-Glutamine concentration in medium, end users should supplement the XtenCHO™ Expression Medium with the recommended concentration of fresh L-Glutamine (8 mM). The XtenCHO™ Expression Medium should also supplemented with an Anticlumping agent (such as 0010057AE from Gibco) for subculturing prior transfection and post transfection.

However, the Anticlumping agent should not be added in the XtenCHO™ Expression Medium during the transfection or it will result in decreased protein yields. It is highly recommended to remove the Anticlumping agent during the last passage before the transfection, by replacing complete XtenCHO™ Expression Medium (XtenCHO™ Expression Medium + 8 mM L-Glutamine + 0.5% Anticlumping agent) with XtenCHO™ Expression Medium only supplemented with 8 mM L-Glutamine.

## 6. How many passages are required before using XtenCHO™ Cells and how long can they be maintained in culture?

XtenCHO™ Cells should be passaged at least three times after thawing and should be cultured within the ranges specified in the XtenCHO™ Starter Kit User Guide, prior to

transfection. Cells can be maintained up to 20 passages without alterations in performance if maintained in accordance with the cell culture maintenance guidelines in the guide.

## 7. What is the expected doubling time for XtenCHO™ Cells?

The doubling time for XtenCHO™ Cells is about 23 hours.

## 8. From which cell line are XtenCHO™ Cells derived?

XtenCHO™ cell line is derived from a CHO-K1 cell line.

## 9. What are the normal growth characteristics of XtenCHO™ Cells?

Within 2-3 passages after thawing, the doubling time of XtenCHO™ Cells should be of approximately 23 hours. When cells are seeded at 0.2 - 0.3 x $10^6$ cells/mL, viable cell density should be approximately 1 - 3 x $10^6$ cells/mL within 2 or 3 days. Typically, cells should be subcultured each 2 -3 days, when they reach a density of 2-3 x $10^6$ cells/mL. If cells are not growing within these approximate ranges, cell culture conditions will require further optimization. In such cases, different shaking speeds could be tested to determine which conditions allow optimal cell growth and high viability.

## 10. What is the expected viability for XtenCHO™ Cells and how can I analyse cell viability of XtenCHO™ Cells?

For both regular passaging and for transfection of XtenCHO™ Cells, viability should be greater than 95% to achieve best results with the XtenCHO™ Expression System. We recommend analysing cell viability with trypan blue exclusion method.

## 11. For how many passages would you recommend using XtenCHO™ Cells before replacing them with new cells that were frozen at earlier passages?

Optimal and consistent expression results will be obtained when cells are cultured for less than 20 passages.

## 12. What are the recommended culture conditions for XtenCHO™ Cells?

For cell line maintenance, we recommend passaging the cells every 2-3 days, at a cell density of 0.2 - 0.3 x $10^6$ cell/mL. Cells should be passaged the day before transfection and seeded at a higher density (1.2 x $10^6$ cells/mL). On the day of transfection, cells are seeded at a density of 5 x $10^6$ cells/mL. In case of cell clumping, let cell clumps settle in the vessel and only use the cells in suspension for subculturing. Indeed, the presence of clumps could alter significantly the accuracy of counting data. It is recommended to discard remaining cells as high-density cell cultures should not be used for seeding of maintenance flasks.

## 13. Can the XtenCHO™ Expression System be scaled down to smaller volumes?

Yes. Small scale transfections can be performed in vented 50 ml Falcon or in deep well plates with shaking. Reagents, plasmids and cells should be scaled down accordingly. Contact us for advices for small volume transfections.

## 14. When subculturing XtenCHO™ Cells the day before a transfection, would you recommend using fresh medium to resuspend cells or just diluting cells to the desired cell density?

We recommend to change out the medium for XtenCHO™ Cells the day before a transfection. This is particularly important as the medium should not contain Anticlumping agent for transfection. On day of transfection, it is also advised to use fresh medium, without Anticlumping agent to resuspend cells. During the subculturing and amplification of the cells, it is also preferable to use fresh medium at each passage to reach optimal performance. However, to amplify large volume of cultures, half of the medium volume could be replaced for passages before the transfection, provided that Anticlumping agent is totally removed for the transfection.

## 15. What is the recommended density for the XtenCHO™ Cells at the time of transfection?

XtenCHO™ Cells should be subcultured at a density of 1.2 x $10^6$ cells/mL one day prior to transfection to obtain a cell density of approximately 2-3 x $10^6$ cells/mL on the day of transfection. These cells should be diluted to a final density of 5 x $10^6$ cells/mL with fresh media and gently swirled to mix prior to transfection. It is recommended to discard remaining cells as high-density cell cultures should not be used for seeding of maintenance flasks.

## 16. Should I change the medium after the transfection?

No. The XtenCHO™ Expression System does not require any medium exchange. There is no need to remove transfection complexes or to change expression medium following transfection. No feed is required. However, the addition of the XtenCHO™ Enhancer, of XtenCHO™ Expression Medium two hours post-transfection, and the temperature shift to 33°C on Day 1 after transfection, are necessary to achieve optimal results.

## 17. When should the XtenCHO™ Enhancer be added to the cultures? When should I add the XtenCHO™ Expression Medium?

The XtenCHO™ Expression Medium and the XtenCHO™ Enhancer should be added two hours post-transfection for best results. The XtenCHO™ Expression Medium and the XtenCHO™ Enhancer may be added to the flasks with pre-warming at room temperature. It

is recommended to add first the XtenCHO™ Expression Medium and then the XtenCHO™ Enhancer in the transfected cultures. It is recommended to add the XtenCHO™ Enhancer closer to the timepoint for best performance.

## 18. Do I need to use two incubators at 37°C and 33°C or can I simply change the temperature of the 37°C incubator down to 33°C?

Optimal expression performances are obtained when using two incubators. However, for more convenience, the temperature can be lowered from 37°C to 33°C on the same incubator on Day 1 post-transfection (It can require some time to reach the desired temperature). The impact on the final expression yield will vary depending on the protein of interest.

## 19. Can I use a different medium for XtenCHO™ Cells in my transfection?

The XtenCHO™ Starter Kit is optimized for use with the provided medium. This medium is chemically-defined, animal component-free medium and designed to achieve high yields of recombinant protein by transient transfection. Using another medium would not guarantee to achieve high rates of production for recombinant proteins, and could inhibit recombinant protein expression.

## 20. Can the amount of plasmid be changed to transfect XtenCHO™ Cells?

The protocol is optimized for use with the indicated DNA quantity for optimal complexation with the "XtenFect Reagent, Working solution". However, it is possible to modify the quantity of DNA to transfect and conditions should be tested empirically for optimal results with your protein of interest.

## 21. For the XtenCHO™ transfection protocol, what is the optimal time point for harvesting?

Harvesting time point depends on the nature of the protein expressed and the expression strategy (intracellular or secreted). Transfected cells can be kept until viability drops under 50% or till day 15 post transfection. Maintaining high cell viability at the time of harvest facilitates downstream processes, such as protein purification.

For stable secreted proteins, as recombinant antibodies, time of harvest occurs generally on day 13 to day 15. For intracellular or unstable proteins, it might be necessary to harvest culture at an earlier time point, to avoid toxicity or degradation. Optimal time to harvest should be determined empirically.

## 22. How should I process the cell supernatant?

It is recommended to harvest and centrifuge cultures for 5 min at 300 x *g* to pellet cells.

For recombinant protein whose expression is expected to be intracellular, keep and freeze cell pellets at -80°C for further analysis. Culture supernatants can be further clarified by centrifugation for 30 min at 5000-10000 x *g* and/or filtration through a 0.22 µm filter. It is recommended to proceed quickly to purification of the protein of interest, or to freeze crude supernatants for later use.

## 23. How does the protein expression yield of the XtenCHO™ Expression System compare to ExpiCHO™ or CHO-S™ expression systems?

We observe higher yields for the production of various recombinant proteins and therapeutic molecules using the XtenCHO™ Expression System compared to CHO-S™ and ExpiCHO™ expression systems.

## 24. What amount of heavy and light chain encoding plasmids should be used to express recombinant antibody in XtenCHO™ Cells?

We observed that a 1:1 mass ratio of heavy and light chain encoding plasmids can be generally used for expression of recombinant antibody with the XtenCHO™ Cells. Most of the cells will be cotransfected by the two plasmids. However, the expression of the recombinant antibody may then be dependent on the expression rate of each chain and can be further optimized, if necessary, by modifying heavy to light chain plasmid ratio. We recommend cloning the heavy- and light-chain subunits separately into the pXten1 expression plasmid and before optimizing the ratios of the two plasmids.

## 25. Can XtenCHO™ Cells be used to generate stable cell lines?

Our XtenCHO™ Cells are derived from CHO-K1 cells, thus stable CHO-K1 selection methods are applicable to these cells. Note that XtenCHO™ Cells are engineered cells and are resistant to geneticin. It is recommended to use a different selection to establish stable cell lines.

## 26. Can a different transfection reagent be use for the transfection of XtenCHO™ Cells?

XtenFect Reagent, XtenCHO™ Enhancer and XtenCHO™ Expression Medium are optimized to work together to provide optimal protein expression levels. The XtenFect Reagent provides high transfection efficiency of high-density cultures. Combined with pXten1 Expression Vector, and transfected with the optimized protocol and reagents, XtenCHO™ Cells sustain extended transient gene expression, by enhancing plasmid-driven expression and allowing better plasmid maintenance.

## 27. Can the XtenCHO™ Expression Medium be used with other CHO cell lines than the XtenCHO™ cell line?

Yes, you may be able to adapt your CHO cells into XtenCHO™ Expression Medium. This

change may increase productivity of your CHO cells and could allow high-density growth. However, there is no guarantee that CHO lines other than the XtenCHO™ cell line will achieve the same levels of expression as the XtenCHO™ Cells. Indeed, XtenCHO™ Cells have been engineered to provide optimal protein expression. Combined with the pXten1 Expression Vector and transfected with the optimized protocol and reagents, XtenCHO™ Cells sustain extended transient gene expression, by enhancing plasmid-driven expression and allowing better plasmid maintenance.

## 28. How should I add reagents for the transfection?

High quality and purity plasmid DNA could be added all at once to the XtenCHO™ cell culture. Then the prepared "XtenFect Reagent, Working solution" is added drop-by-drop while swirling gently the flask. Two hours post-transfection, the XtenCHO™ Expression Medium, prewarmed to room temperature can be added all at once to the transfected cells. Similarly, the XtenCHO™ Enhancer, prewarmed to room temperature, should be added to the culture. It is recommended to add the XtenCHO™ Expression Medium before adding the XtenCHO™ Enhancer to the transfected cells. Gently swirl the flask to ensure even distribution.

## 29. Can I use minipreps to transfect XtenCHO™ Cells?

We recommend using only high quality, endotoxin-free plasmid preparation for transfecting XtenCHO™ Cells as low quality and low purity plasmids will result in low transfection efficiency, lead to cell toxicity, and will dramatically affect bioproduction yield.

# Ordering Information

Ordering information is provided below. The following reagents supplied in the XtenCHO™ Starter Kit and other products suitable for use with the kit are available separately. For more information, refer to proteogenix.science or contact the **Technical Support**.

| Products | Amount | Reference |
|---|---|---|
| **XtenCHO™ Starter Kit, includes:** | | |
| XtenCHO™ Cells | 2 x 1 mL | **PX-XTE-001** |
| XtenCHO™ Expression Medium | 1 x 1 L | |
| XtenFect Reagent, Stock solution (to dilute prior to use) | 1 x 0.51 mL | |
| XtenFect Reagent, Working solution (to reconstitute prior to use) | 3 x 1.44 mL | |
| XtenCHO™ Enhancer | 3 x 1.2 mL | |
| pXten1 Expression vector | 10 µL | |
| Antibody Expression Positive Control Vector | 150 µL | |
| **XtenCHO™ Expression Medium** | 1 x 1 L | **PX-XTE-002** |
| **XtenCHO™ Transfection Kit, includes:** | | **PX-XTE-003** |
| XtenFect Reagent, Stock solution (to dilute prior to use) | 1 x 0.51 mL | |
| XtenFect Reagent, Working solution (to reconstitute prior to use) | 3 x 1.44 mL | |
| XtenCHO™ Enhancer | 3 x 1.2 mL | |
| **XtenCHO™ Cells** | 1 x 1 mL | **PX-XTE-004** |
| **XtenCHO™ Cell Bank Pack** | 6 x 1 mL | **PX-XTE-005** |