Protein expression

Nalgene 5 L Angled-Bottom Shake Flask for scale-up of the ExpiCHO Expression System

Purpose

This application note describes scale-up of the Gibco™ ExpiCHO™ Expression System into the Thermo Scientific™ Nalgene™ 5 L Angled-Bottom Shake Flask. In contrast to other 5 L shake flasks that can impart damaging levels of shear stress on mammalian cells, the unique design of the Nalgene 5 L Angled-Bottom Shake Flask creates a gentle environment allowing for cell growth and expression levels equivalent to those obtained in small-scale shake flasks. The Nalgene 5 L Angled-Bottom Shake Flask was the only 5 L flask tested that enabled such highly efficient scale-up.

Introduction

Thermo Scientific™ Nalgene™ 5 L shake flasks are exceptional choices for the culture of suspension cells used in mammalian, insect, and prokaryotic expression systems for the production of recombinant proteins or viral vectors. Our newest additions to the wide range of Nalgene flask offerings are the Nalgene 5 L Angled-Bottom and 5 L Faceted-Bottom Shake Flasks.

The ExpiCHO Expression System brings together a high-expressing CHO cell line and an optimized medium and transfection kit that together provide titers as much as 160x higher than the Gibco™ FreeStyle™ MAX CHO Expression System and 4x higher than the Gibco™ Expi293™ Expression System. The ultrahigh yields of the ExpiCHO Expression System (up to 1–3 g/L for some proteins) allow you to scale up your expression run and achieve significant cost savings compared to other transient expression technologies.

A known challenge with transient protein expression in CHO cells is suboptimal protein expression levels at the 5 L shake flask scale. This is likely due to the higher levels of shear stress introduced to the cells in current 5 L flask offerings, which lead to decreased cell viability and lower protein titers. The improved design of the Nalgene 5 L Angled-Bottom Shake Flask provides a gentle environment for cells, making this flask an excellent choice for growth and protein expression of Gibco™ ExpiCHO-S™ cells, as well as other cell types.

Note: Due to the enhanced mixing dynamics of the Nalgene 5 L Faceted-Bottom Shake Flasks, they should not be used with the ExpiCHO Expression System; use only the angled-bottom flasks for protein expression in the ExpiCHO system.

In this study, we compared the growth and protein expression of ExpiCHO-S cells in the Nalgene 5 L Angled-Bottom Shake Flask to corresponding small-scale shake flask controls as well as products from other suppliers. The data indicate that with minor modifications to the ExpiCHO Expression System protocol, control levels of cell growth and protein expression are obtained with the Nalgene 5 L angled-bottom flask. The gentle mixing imparted by the angled-bottom shake flasks is an exceptional choice for ExpiCHO-S cells and may be useful for the scale-up of other sensitive cell types as well. The results also show that the innovative and proprietary design of the Nalgene 5 L Angled-Bottom Shake Flask enables scale-up for ExpiCHO-S cells where products from other suppliers do not.

Materials

Materials used for protein expression with Nalgene 5 L Angled-Bottom Shake Flasks and the ExpiCHO Expression System are shown in Table 1. For more details, please see the **ExpiCHO Expression System user guide (Pub. No. MAN0014337)**.

Subculture of ExpiCHO-S cells

ExpiCHO-S cells are capable of achieving high cell densities; therefore, we recommend that the cells attain a minimum density of 4–6 x 10^6 viable cells/mL at the time of subculturing.

Using the viable cell density, the volume of cell suspension required to seed a new shake flask was calculated according to the recommended seeding densities in Table 2 and the recommended culture volumes in Table 3.

Table 1. Materials for protein expression.

Component	Storage	Cat. No.
Nalgene 5 Liter Shake Flask, Angled Bottom	Room temperature	4115-5000
ExpiCHO-S Cells	Liquid nitrogen	A29127
ExpiCHO Expression Medium	2–8°C, protect from light	A2910002
ExpiFectamine CHO Transfection Kit (1 L), contains: ExpiFectamine CHO Reagent ExpiCHO Feed ExpiFectamine CHO Enhancer	2–8°C, protect from light	A29129
OptiPRO SFM	2–8°C, protect from light	12309019

Table 2. Recommended seeding densities for routine cell culture maintenance and transfection in Nalgene 5 L Angled-Bottom Shake Flasks.

Subculture timing	Recommended seeding density
To obtain cells ready at 3 days post-subculture	0.2–0.3 x 10^6 viable cells/mL
To obtain cells ready at 4 days post-subculture	0.15–0.2 x 10^6 viable cells/mL

Table 3. Recommended conditions for routine cell culture maintenance and transfection in Nalgene 5 L Angled-Bottom Shake Flasks.

Parameter	Condition
Culture volume for cell growth	2,000–3,000 mL
Culture volume to be transfected	1,500 mL
Target shake speed*	115 rpm (19 mm shaking diameter) 110 rpm (25 mm shaking diameter) 110 rpm (50 mm shaking diameter)
Flask type	Angled bottom, vented

* Due to slight differences in shakers, optimal speeds may differ slightly from the target shake speeds shown. Optimize shake speeds to attain maximal protein expression comparable to small-scale shake flask controls.

Transfection of ExpiCHO-S cells

Preparation of cells for transfection in Nalgene 5 L Angled-Bottom Shake Flasks

1. ExpiCHO cells were cultured in Nalgene shake flasks as directed in the ExpiCHO Expression System user guide (Pub. No. MAN0014337) and Tables 2 and 3.
2. Six days prior to transfection (day –6), cells were seeded into 220 mL of culture medium in a Nalgene 1 L shake flask, to a density of 0.3 x 10^6 viable cells/mL, and grown until the cells reached a density of 4–6 x 10^6 viable cells/mL (Figure 1).
3. Three days prior to transfection (day –3), cells were seeded into 1 L of culture medium in a Nalgene 2.8 L shake flask, to a density of 1 x 10^6 viable cells/mL, and grown until the cells reached a density of 4–6 x 10^6 viable cells/mL (Figure 1).
4. One day prior to transfection (day –1), cells were seeded into 2 L of culture volume in a Nalgene 5 L Angled-Bottom Shake Flask to a density of 3.5–4 x 10^6 viable cells/mL (Figure 1).
5. On the day of transfection (day 0), cells should have reached a density of approximately 8–10 x 10^6 viable cells/mL, with viability of 95–99%, appropriate for proceeding with transfection. In a new 5 L angled-bottom flask, cells were diluted with fresh ExpiCHO Expression Medium (pre-warmed to 37ºC) to a final density of 6 x 10^6 viable cells/mL in 1.5 L total volume, and swirled gently to mix (Figure 1). The cells were then ready for transfection.

Day 0: transfection

6. Complexes of Gibco™ ExpiFectamine™ CHO Reagent with plasmid DNA were prepared using cold (4°C) reagents, as described in steps 6a–6d. It is not necessary to keep reagents on ice during complexation; the reagents can be removed from refrigeration for DNA complexation at room temperature.

   Note: The volumes given in steps 6a–6c will generate a complexation reaction sufficient to transfect 1.5 L of culture in a single Nalgene 5 L Angled-Bottom Shake Flask. Addition of all reagents to this starting volume will result in a final volume of approximately 2 L.

   a. In a 250 mL shake flask, plasmid DNA was diluted by adding 1.2 mL of DNA (assuming a 1 mg/mL plasmid stock) to 60 mL of cold Gibco™ OptiPRO™ SFM, and mixed by swirling.

   b. The ExpiFectamine CHO Reagent bottle was inverted gently 4–5 times to mix the contents.

   c. In a separate 250 mL shake flask, 4.8 mL of ExpiFectamine CHO Reagent was added to 55.5 mL of cold OptiPRO SFM, and mixed by swirling.

   d. The diluted ExpiFectamine CHO Reagent was transferred to the diluted plasmid DNA and mixed by gently swirling.




	Day –6	Day –3	Day –1	Day 0
Flask type	1 L	2.8 L	5 L angled-bottom	5 L angled-bottom
Culture volume	220 mL	1 L	2 L	1.5 L
Seeding density	0.3 x 10^6 cells/mL	1 x 10^6 cells/mL	3.5 x 10^6 cells/mL	6 x 10^6 cells/mL

Figure 1. Strategy for scaling up cell cultures prior to transfection in Nalgene 5 L Angled-Bottom Shake Flasks. For these experiments, 9 x 10^9 viable cells are needed per 5 L shake flask to be transfected at a 1.5 L transfection volume.

7. The ExpiFectamine CHO Reagent/plasmid DNA complex from step 6d was allowed to incubate at room temperature for 1–5 minutes, followed by transfer of the complexation mix to the shake flask containing cultured cells from step 5, swirling the flask gently during addition.

8. Transfected cultures were incubated in a 37°C incubator with a humidified atmosphere of 8% CO_2 in air on an orbital shaker (refer to Table 4 for recommended shake speeds) until the addition of feed and enhancer on day 1 post-transfection.

Day 1: enhancer and feed addition

Note: To attain maximum performance for protein expression using the ExpiCHO system at the 5 L flask scale, it is optimal to divide the feeds into multiple, smaller additions. For example, instead of a single feed addition of 24%, the 5 L Standard and High Titer protocols in the user guide recommend using two feed additions of 12% each on days 1 and 4 post-transfection. Similarly, instead of adding two feeds of 16% each, the 5 L Max Titer protocol recommends three feeds at 10% volume on days 1, 4, and 8 post-transfection. While the one- and two-feed protocols given in the ExpiCHO system user guide are still possible for the relevant protocols, the addition of smaller amounts of feed across multiple additions helps achieve optimal results.

Note: For the day 1 addition, ExpiFectamine CHO Enhancer and ExpiCHO Feed may be mixed together immediately prior to adding to the flasks.

9. On day 1 (18–22 hours post-transfection), 9 mL of ExpiCHO Enhancer was added to the 5 L flask. At the same time, the first feed was added to the flask. Refer to Table 5 for ExpiCHO Feed addition volumes for the 5 L flask scale corresponding to the expression protocol chosen (i.e., Standard Titer, High Titer, or Max Titer).

10. Immediately return the flask to either a 37°C incubator with a humidified atmosphere of 8% CO_2 if following the Standard Titer protocol or a 32°C incubator with a humidified atmosphere of 5% CO_2 if following either the High Titer or Max Titer protocols.

11. The 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 Titer protocol:** 8–10 days post-transfection
    - **High Titer protocol:** 10–12 days post-transfection
    - **Max Titer protocol:** 12–14 days post-transfection

Table 4. Summary of recommended conditions for transfection in Nalgene 5 L Angled-Bottom Shake Flasks for the ExpiCHO Expression System.

Parameter	Condition
Flask size/type	5 L Nalgene Angled-Bottom Shake Flask
Number of cells required	9.0×10^9
Culture volume to transfect	1.5 L
Shake speed	115 rpm (19 mm shaking diameter) 110 rpm (25 mm shaking diameter) 110 rpm (50 mm shaking diameter)
Volume of plasmid DNA*	1.2 mL
OptiPRO SFM**	60 mL
ExpiFectamine CHO Reagent	4.8 mL
OptiPRO SFM†	55.5 mL
ExpiCHO Enhancer	9 mL

* Assuming a plasmid DNA stock concentration of 1 mg/mL and a final concentration of 0.8 µg plasmid DNA per mL of culture volume to be transfected.

** Volume of OptiPRO SFM used to dilute plasmid DNA.

† Volume of OptiPRO SFM used to dilute ExpiFectamine CHO Reagent.

Table 5. Recommended volumes of ExpiCHO Feed for Standard, High, and Max Titer protocols.

Protocol	Day 1 feed addition	Day 4 feed addition*	Day 8 feed addition
Standard Titer (2 feeds)	180 mL (12%)	180 mL (12%)	NA
High Titer (2 feeds)	180 mL (12%)	180 mL (12%)	NA
Max Titer (3 feeds)	150 mL (10%)	150 mL (10%)	150 mL (10%)

* For the Standard Titer protocol, it is important to add the second feed no later than day 4. For the High Titer protocol, the second feed may be added on day 4 or 5.

Results

Growth of ExpiCHO-S cells in Nalgene 5 L Angled-Bottom Shake Flasks

To assess ExpiCHO-S cell growth dynamics in Nalgene 5 L Angled-Bottom Shake Flasks, ExpiCHO-S cells were seeded to a density of 0.3 x 10^6 viable cells/mL and shaken at 110 rpm on a 25 mm orbital shaker followed by assessment of viable cell density and percent viability over 6 days. Compared to control cultures grown in Nalgene 125 mL flasks, ExpiCHO-S cells cultured at either 2 L or 3 L culture volumes in Nalgene 5 L Angled-Bottom Shake Flasks showed comparable growth dynamics and percent viability, with the maximal cell density trending slightly higher for the 5 L flasks under either culture volume condition tested (Figure 2).

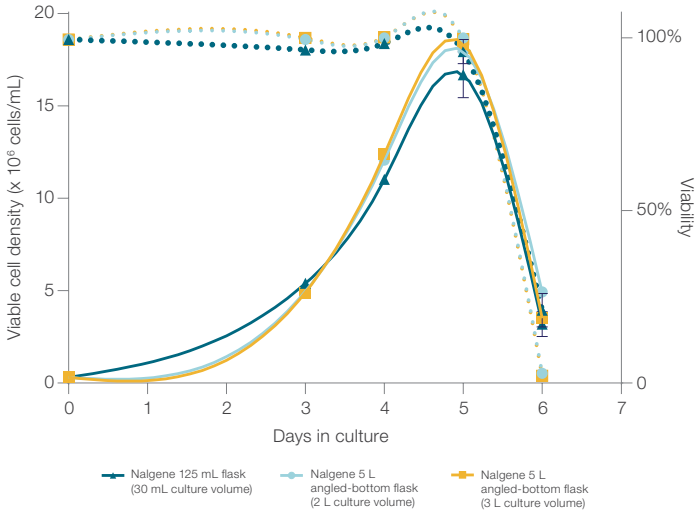

Figure 2. Growth kinetics of ExpiCHO cells. Viable cell density (solid lines) and viability (dotted lines) for ExpiCHO-S cells cultured at 2 L or 3 L volumes in Nalgene 5 L Angled-Bottom Shake Flasks were compared to controls cultured in Nalgene 125 mL shake flasks.

Protein expression of ExpiCHO-S cells in Nalgene 5 L Angled-Bottom Shake Flasks

To assess protein expression in Nalgene 5 L Angled-Bottom Shake Flasks, ExpiCHO cells were transfected according to the method above (steps 6–8) at 6 x 10^6 viable cells/mL in a volume of 1.5 L in Nalgene 5 L angled-bottom flasks. Transfected cultures were then incubated for 10–14 days per the relevant protocols.

Standard Titer (37°C) protocol: Compared to control cultures transfected in Nalgene 125 mL flasks, ExpiCHO cells transfected in Nalgene 5 L Angled-Bottom Shake Flasks by the Standard Titer protocol expressed comparable levels of human IgG1 monoclonal antibody at harvest on day 10 (Figure 3). Here, the benefits of a two-feed strategy are evident: with a single addition of feed at 24% of the volume of cells transfected, titers are reduced by roughly half compared to the two-feed approach in which two feeds of 12% each are added on days 1 and 4 post-transfection.

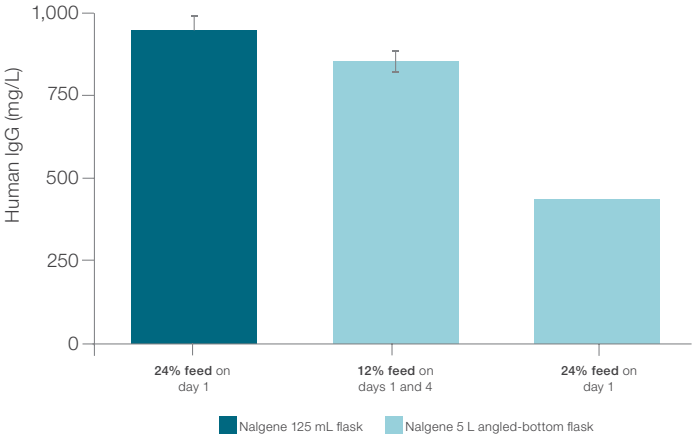

Figure 3. Protein expression using the Standard Titer protocol. Protein titers obtained from ExpiCHO cells transfected in a 1.5 L starting volume using the Standard Titer (37°C) protocol in Nalgene 5 L Angled-Bottom Shake Flasks with either a one-feed or two-feed strategy were compared to Nalgene 125 mL shake flask controls. Cultures were harvested at day 10 post-transfection to determine protein titers.

Max Titer (32°C) protocol: Compared to control cultures transfected in Nalgene 125 mL flasks, ExpiCHO cells transfected in Nalgene 5 L Angled-Bottom Shake Flasks by the modified Max Titer protocol expressed comparable levels of human IgG1 monoclonal antibody at harvest on day 14 (Figure 4). Here, the benefits of a three-feed strategy are also evident: using a two-feed strategy with addition of two feeds at 16% of the volume of cells transfected on days 1 and 4 post-transfection, titers are reduced by roughly 30% compared to the three-feed approach in which three feeds of 10% each are added on days 1, 4, and 8 post-transfection.

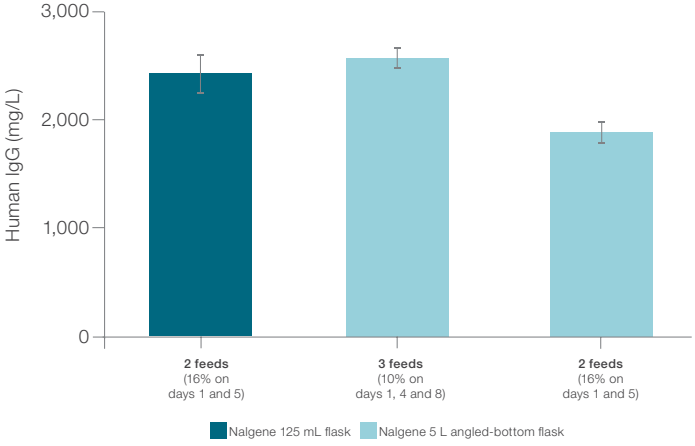

Figure 4. Protein expression attained using the Max Titer protocol. Protein titers from ExpiCHO cells transfected at a 1.5 L starting volume using the Max Titer (32°C) protocol in Nalgene 5 L Angled-Bottom Shake Flasks, with either a two-feed or three-feed strategy, were compared to those of Nalgene 125 mL shake flask controls. Cultures were harvested on day 14 post-transfection to determine protein titers.

Comparison of 5 L shake flasks: The Nalgene 5 L Angled-Bottom Shake Flask was tested against 5 L shake flasks from three other suppliers using the Max Titer protocol to show its ability to support scale-up for ExpiCHO-S cells (Figure 5).

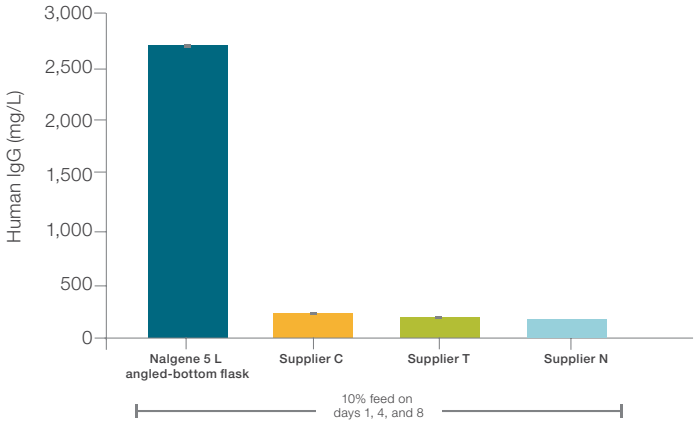

Figure 5. Protein expression levels in 5 L shake flasks from different suppliers. Protein titers were determined for ExpiCHO cells transfected in a 1.5 L starting volume using the Max Titer (32°C) protocol with a three-feed strategy in 5 L shake flasks (N = 2).

Conclusions

Nalgene 5 L Angled-Bottom Shake Flasks allow for the gentle culture of ExpiCHO-S cells to generate maximum protein titers upon transfection at a final culture volume of approximately 2 L. Minor optimizations of the ExpiCHO Expression System protocol were introduced (i.e., splitting the feeds into smaller additions) to attain protein titers equivalent to those of small-scale controls. The gentle culture conditions enabled by the Nalgene 5 L angled-bottom flasks also make them an excellent choice for protein expression using other non-CHO cell types (e.g., insect, HEK293) as well as for cultivation of other sensitive cell lines. The Nalgene 5 L Angled-Bottom Shake Flask was the only flask tested that was found capable of generating these larger protein titers.

Ordering information

Product	Quantity	Cat. No.
Nalgene 5 Liter Shake Flask, Angled Bottom	4/case	4115-5000
ExpiCHO-S Cells	1 mL	A29127
ExpiCHO Expression Medium	6 x 1 L	A2910002
ExpiFectamine CHO Transfection Kit	For 1 L of culture	A29129
OptiPRO SFM	1,000 mL	12309019
CO_2 Resistant Shaker	1	88881101

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