

USER GUIDE

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by *life* technologies™

# FreeStyle™ MAX CHO Expression System

For large-scale transfection of suspension CHO cells  
in a defined, serum-free medium

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MAN0000566

**For Research Use Only. Not intended for any animal or human  
therapeutic or diagnostic use.**

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## Kit Contents and Storage

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### Shipping/Storage

The components of the FreeStyle™ MAX CHO Expression System are shipped and should be stored as listed in the table below. For more information about the amount supplied and composition of each reagent, see below.

Contents	Shipping	Storage
FreeStyle™ CHO-S® Cells	Dry ice	Liquid nitrogen
FreeStyle™ MAX Reagent	Blue ice	+4°C
FreeStyle™ CHO Expression Medium	Room Temperature	+4°C, in the dark
OptiPRO™ SFM	Room Temperature	+4°C, in the dark
pCMV SPORT-βgal	Blue ice	-20°C

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### FreeStyle™ CHO-S® Cells

**Storage conditions: Liquid nitrogen**

Amount supplied: One vial containing  $1 \times 10^7$  cells

Composition: 1 mL of cells in 90% FreeStyle™ CHO Expression Medium and 10% DMSO.

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### FreeStyle™ MAX Reagent

**Storage conditions: +4°C. Do not freeze.**

Amount supplied: 1 mL (sufficient for 25 transfections and one control in a volume of 30 mL using 37.5 μL of FreeStyle™ MAX Reagent per transfection)

Composition: Proprietary.

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### FreeStyle™ CHO Expression Medium

**Storage conditions: +4°C, in the dark**

Amount supplied: 1 liter

Composition: Proprietary, defined, serum-free medium.

**Note:** Add 8 mM L-glutamine before use

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### OptiPRO™ SFM

**Storage conditions: +4°C, in the dark**

Amount supplied: 100 mL

Composition: Proprietary, defined, serum-free medium

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### pCMV SPORT-βgal

**Storage conditions: -20°C**

Amount supplied: 25 μg

Composition: 0.5 μg/μL in 10 mM Tris-HCl, pH 7.4, 5 mM NaCl, 0.1 mM EDTA

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### Product Use

**For Research Use Only.** Not intended for any human or animal diagnostic or therapeutic uses.

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## Accessory Products

### Introduction

The products listed in this section may be used with the FreeStyle™ MAX CHO Expression System. For more information, refer to our website ([www.lifetechnologies.com](http://www.lifetechnologies.com)) or call Technical Support (see page 17).

### Accessory Products

The following reagents supplied in the FreeStyle™ MAX CHO Expression System and other reagents suitable for use with the kit are available separately from Life Technologies. Ordering information is provided below.

Item	Amount	Catalog No.
FreeStyle™ CHO-S® Cells	1 vial (1 × 10 <sup>7</sup> cells)	R800-07
FreeStyle™ CHO Expression Medium	1 L	12651-014
	6 × 1 L	12651-022
FreeStyle™ MAX Reagent	1 mL	16447-100
OptiPRO™ SFM	100 mL	12309-050
	1000 mL	12309-019
L-glutamine-200 mM, liquid	100 mL	25030-081
PureLink™ HiPure Plasmid Midiprep Kit	25 preps	K2100-04
PureLink™ HiPure Plasmid Maxiprep Kit	10 preps	K2100-06
PureLink™ HiPure Plasmid Filter Maxiprep Kit	10 preps	K2100-16
PureLink™ HiPure Plasmid Megaprep Kit	4 preps	K2100-08
Trypan Blue Stain	100 mL	15250-061
FluoReporter® <i>lacZ</i> /Galactosidase Quantitation Kit	1000 assays	F-2905
Penicillin-Streptomycin, liquid	100 mL	15140-122
pCEP4	20 µg	V044-50
pcDNA™3.2/V5-DEST	6 µg	12489-019
pcDNA™4/HisMax A, B, & C	20 µg e.a.	V864-20

### FreeStyle™ MAX 293 Expression System

Protein production using the FreeStyle™ MAX Expression Systems can be performed in CHO cells or 293 cells. Below the ordering information is provided for the reagents specific for the FreeStyle™ MAX 293 Expression System.

Item	Amount	Catalog no.
FreeStyle™ MAX 293 Expression System	25 reactions (30 mL cultures)	K9000-10
FreeStyle™ 293-F Cells	1 vial (1 × 10 <sup>7</sup> cells)	R790-07
FreeStyle™ 293 Expression Medium	1 L	12338-018
	6 × 1 L	12338-026

# Introduction

## Overview

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### Introduction

The FreeStyle™ MAX Expression Systems are designed to allow large-scale transient transfection and protein expression in defined, serum-free medium. The reagents provided are completely animal origin free. The systems are optimized to drive protein expression employing the following cell lines:

- For expression in suspension Chinese Hamster Ovary (CHO) cells, use the FreeStyle™ MAX CHO Expression System
- For expression in suspension human embryonic kidney 293 cells, use the FreeStyle™ MAX 293 Expression System

This manual supports the FreeStyle™ MAX CHO Expression System.

**Note:** For information on the FreeStyle™ MAX 293 Expression System, see the FreeStyle™ MAX 293 Expression System manual available from our website ([www.lifetechnologies.com](http://www.lifetechnologies.com)) or Technical Support (see page 17).

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### FreeStyle™ MAX CHO Expression System

The FreeStyle™ MAX CHO Expression System includes FreeStyle™ CHO-S® cells that have been adapted to serum-free, suspension culture in FreeStyle™ CHO Expression Medium. Transfection and expression experiments may be performed directly in FreeStyle™ CHO Expression Medium without the need to change media. The complete FreeStyle™ MAX CHO Expression System provides enough reagents to perform 25 transfections and one control transfection in a 30 mL volume, but larger volume transfections may be performed using simple scale-up of reagents.

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### Components of the FreeStyle™ MAX CHO Expression System

The FreeStyle™ MAX CHO Expression System includes the following major components:

- **FreeStyle™ MAX Reagent:** This transfection reagent provides high transfection efficiency in suspension FreeStyle™ CHO-S® cells (see below for more information).
  - **FreeStyle™ CHO-S® cells:** This cell line is adapted to high density, serum-free suspension culture in FreeStyle™ CHO Expression Medium and is capable of producing high levels of recombinant protein (see page 3 for more information).
  - **FreeStyle™ CHO Expression Medium:** This is a defined, serum-free medium formulated specifically to allow growth **and** large-scale transfection of suspension FreeStyle™ CHO-S® cells (see page 4 for more information).
  - **OptiPRO™ Serum Free Medium** to facilitate optimal formation of DNA-lipid complexes (see next page for more information).
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### FreeStyle™ MAX Reagent

FreeStyle™ MAX Reagent is a proprietary, animal origin-free formulation for the highly efficient transfection of plasmid DNA into eukaryotic cells. FreeStyle™ MAX Reagent is specifically formulated to achieve the **highest expression levels** and **lowest cytotoxicity** in suspension FreeStyle™ CHO-S® Cells and FreeStyle™ 293-F Cells.

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## Overview, continued

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### OptiPRO™ SFM

OptiPRO™ Serum Free Medium is included with the FreeStyle™ MAX CHO Expression System to facilitate optimal formation of DNA-lipid complexes. OptiPRO™ SFM is a serum free medium which is devoid of any components of animal or human origin. OptiPRO™ SFM has an ultra-low protein concentration of 7.5 µg/mL. OptiPRO™ SFM is available separately from Life Technologies (see page vi for ordering information). For more information, see our website ([www.lifetechnologies.com](http://www.lifetechnologies.com)) or call Technical Support (see page 17).

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### Advantages of the FreeStyle™ MAX CHO Expression System

Using the FreeStyle™ MAX CHO Expression System for protein production in mammalian cells provides the following advantages:

- Uses Chinese Hamster Ovary cells, the most widely used cell line for expression of recombinant protein in mammalian cells. This may streamline the process for clinical applications.
  - The FreeStyle™ MAX Reagent offers high recombinant protein yield with low cytotoxicity
  - Provides a rapid transient transfection protocol for expression of your target protein.
  - Uses suspension culture to easily scale up to large amounts of culture.
  - All reagents are completely animal-origin free, including the defined, serum-free medium, which may be imperative for regulatory requirements
  - CHO cells are known to provide stable and accurate glycosylation (Sheeley *et al.*, 1997; Werner *et al.*, 1998), and are more likely to yield accurate glycoproteins.
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### Suitable Expression Vectors

Suitable expression vectors for the FreeStyle™ MAX CHO Expression System generally express the recombinant protein under control of a CMV promoter. Other strong promoters may also be used. Below are suitable CMV-promoter driven expression vectors available from Life Technologies (see page vi for ordering information):

- pCEP4, which will express the recombinant protein under control of a CMV promoter without tag
  - pcDNA™3.2/V5-DEST, which will express the recombinant protein under control of a CMV promoter with a C-terminal V5-tag
  - pcDNA™4/HisMax A, B, & C, which will express the recombinant protein under control of a CMV promoter with an N-terminal HIS-tag
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# FreeStyle™ CHO-S® Cells

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## Introduction

The FreeStyle™ CHO-S® cell line is supplied with the FreeStyle™ MAX CHO Expression System and is derived from the CHO cell line (see below). FreeStyle™ CHO-S® cells are adapted to suspension culture in FreeStyle™ CHO Expression Medium. Frozen cells are supplied in and may be thawed directly into FreeStyle™ CHO Expression Medium (see **Thawing and Establishing Cells**, page 6).

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## Parental Cell Line

Chinese Hamster Ovary (CHO) cells are among the most commonly used cell lines for transfection, expression and large-scale production of recombinant proteins. The CHO-S® cell line is a stable aneuploid cell line established from the ovary of an adult Chinese hamster (Puck, 1958). The cell line has been distinguished as a separate sub-clone from the common CHO K1 cell line, and its history and stability have been extensively described (D'Anna, 1996; D'Anna *et al.*, 1997; Deaven & Petersen, 1973).

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## Characteristics of FreeStyle™ CHO-S® Cells

The FreeStyle™ CHO-S® cell line exhibits the following characteristics:

- Prepared from low passage Master Cell Bank cultures derived from parental CHO-S® cells that were re-cloned by limiting dilution, and selected for their superior serum-free cell growth and transfection efficiencies. The clonally derived cultures are maintained in serum-free conditions for only 20–25 total passages.
  - Adapted to serum-free suspension growth in FreeStyle™ CHO Expression Medium, a serum-free, protein-free, and chemically defined medium, formulated with no components of animal or human origin (Gorfien, 1998). **Note:** Cells also grow well in traditional media supplemented with serum.
  - Demonstrates high transfection efficiencies with FreeStyle™ MAX Reagent.
  - Suspension cultures may be transfected in FreeStyle™ CHO Expression Medium without the need to change media.
  - Permits transfection of cells at large volumes, from shake flasks to bioreactors.
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# FreeStyle™ CHO Expression Medium

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## Introduction

FreeStyle™ CHO Expression Medium is a defined, serum-free medium specifically developed for the high-density, suspension culture and transfection of CHO cells. The medium contains **NO** human or animal origin components.

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## Features of the Medium

FreeStyle™ CHO Expression Medium exhibits the following features:

- Chemically defined, containing no proteins or peptide components of animal, plant or synthetic origin. There are also no undefined hydrolysates or lysates in the formulation.
  - Superior growth of wild-type and recombinant CHO cells in suspension culture when compared to serum-free, undefined formulations. The medium is **not** recommended for **adherent** CHO cell culture.
  - Supports the small scale growth of FreeStyle™ CHO-S® cells in shaker flasks, and the large-scale, high-density growth in bioreactors.
  - Allows transfection of suspension FreeStyle™ CHO-S® cells without medium change.
  - Formulated without L-glutamine to avoid problems associated with L-glutamine degradation, including ammonia accumulation. The FreeStyle™ CHO Expression Medium should be supplemented with L-glutamine to a final concentration of 8 mM before use.
  - Formulated without Phenol red to minimize potential for estrogen-like effects of phenol red.
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## Growth Characteristics of FreeStyle™ CHO-S® Cells in the Medium

Typically, FreeStyle™ CHO-S® cells cultured in FreeStyle™ CHO Expression Medium demonstrate the following:

- Doubling time in the range of 16–22 hours (doubling time can exceed 22 hours during the first few passages after the cells have been thawed.)
- Cell densities of up to  $7 \times 10^6$  cells/mL in shaker or spinner culture
- Cell densities of up to  $4 \times 10^6$  cells/mL in bioreactor culture

For general cell culture, keep FreeStyle™ CHO-S® cells in between  $5 \times 10^4$  and  $1.5 \times 10^6$  cells/mL before transfection (or in between  $5 \times 10^5$  and  $2.0 \times 10^6$  cells/mL if passaging daily). A cell density that is too high will result in a decrease of transfection efficiency.

If large numbers of cells are needed, you can seed cultures at  $0.5 \times 10^6$  cells/mL and use cells **as soon as** they reach a density of  $5 \times 10^6$  cells/mL (3–4 days). Do not let cultures that have reached a cell density of  $5 \times 10^6$  cells/mL grow any further, as this will result in a decrease of transfection efficiency.

**Note:** Individual culturing and passaging techniques coupled with cellular heterogeneity inherent within the FreeStyle™ CHO-S® cell population may result in experimental variability.

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# Methods

## General Information

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### General Cell Handling

Follow the guidelines below to grow and maintain FreeStyle™ CHO-S® cells.

- **All solutions and equipment that come in contact with the cells must be sterile.** Always use proper sterile technique and work in a laminar flow hood.
  - Before starting experiments, be sure to have cells established (at least 5 passages) and also have some frozen stocks on hand. We recommend using early-passage cells for your experiments. Upon receipt of the cells from Life Technologies, grow and freeze multiple vials of the FreeStyle™ CHO-S® cell line to ensure that you have an adequate supply of early-passage cells.
  - For general maintenance of cells, pass FreeStyle™ CHO-S® cells when they reach a density of approximately  $1\text{--}1.5 \times 10^6$  viable cells/mL (generally every 48–72 hours).
  - Alternatively, cells can be passed every day. If passing cells every day, pass FreeStyle™ CHO-S® cells when they reach a density of  $2 \times 10^6$  viable cells/mL.
  - If large numbers of cells are needed, you can seed cultures at  $0.5 \times 10^6$  cells/mL and use cells as soon as they reach a density of  $5 \times 10^6$  cells/mL (3–4 days). Do not let cultures that have reached a cell density of  $5 \times 10^6$  cells/mL grow any further, as this will result in a decrease of transfection efficiency.
  - Use trypan blue exclusion to determine cell viability (see below). Log phase cultures should be >95% viable.
  - When thawing or subculturing cells, transfer cells into pre-warmed medium.
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As with other human cell lines, when working with FreeStyle™ CHO-S® cells, handle as potentially biohazardous material under at least Biosafety Level 2 containment.

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### Preparing Media

For suspension growth and transfection applications, use:

- FreeStyle™ CHO Expression Medium. This medium should be supplemented with L-glutamine to a final concentration of 8 mM before use (e.g. 40 mL of 200 mM stock to one liter of medium).
  - 5 mL/L of Penicillin/Streptomycin (0.5X Pen-Strep) may be used when required (see page vi for ordering information).
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### Important

FreeStyle™ CHO Expression Medium is extremely sensitive to light. For optimal results, use and store media protected from light.

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### Determining Cell Density and Viability

Follow the procedure below to determine viable and total cell counts.

1. Transfer a small aliquot of the cell suspension to a microcentrifuge tube.
  2. Determine viability and the amount of cell clumping using the trypan blue dye exclusion method (see page vi for ordering information).
  3. If cells are clumping, vortex vigorously for 10–30 seconds.
  4. Determine cell density electronically using a Coulter Counter or manually using a hemacytometer.
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# Thawing and Establishing Cells

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## Introduction

Follow the protocol below to thaw FreeStyle™ CHO-S® cells to initiate cell culture. The FreeStyle™ CHO-S® cell line is supplied in a vial containing 1 mL of cells at  $1 \times 10^7$  viable cells/mL in 90% FreeStyle™ CHO Expression Medium and 10% DMSO. Thaw FreeStyle™ CHO-S® cells directly into the FreeStyle™ CHO Expression Medium supplied with the kit.

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## Materials Needed

You will need to have the following reagents on hand before beginning:

- FreeStyle™ CHO-S® cells (supplied with the kit; store frozen cells in liquid nitrogen until ready to use)
- FreeStyle™ CHO Expression Medium (supplied with the kit; pre-warm at 37°C before use). Make sure that 8 mM L-glutamine has been added.

**Note:** We do not recommend adding antibiotics to media at this point as this may negatively impact cell growth.

- 125-mL polycarbonate, disposable, sterile Erlenmeyer flask with vented cap (available from VWR, West Chester PA, cat. no. 30180-036)
  - Orbital shaker in 37°C incubator with a humidified atmosphere of 8% CO<sub>2</sub>
  - Reagents to determine viable and total cell counts (see **Determining Cell Density and Viability**, page 5)
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## Thawing Procedure

Store frozen cells in liquid nitrogen until ready to use. To thaw and establish cells:

1. Remove the cryovial of cells from the liquid nitrogen and thaw quickly in a 37°C water bath.
2. Just before the cells are completely thawed, decontaminate the outside of the vial with 70% ethanol. Gently break up clumps and cell pellet if present and transfer the entire contents of the cryovial into a 125-mL polycarbonate, disposable, sterile Erlenmeyer shaker flask containing 30 mL of pre-warmed FreeStyle™ CHO Expression Medium supplemented with 8 mM L-glutamine.
3. Incubate cells in a 37°C incubator containing a humidified atmosphere of 8% CO<sub>2</sub> in air on an orbital shaker platform rotating at 125 rpm.
4. Next day, determine viable and total cell counts (see protocol on page 5). Generally, viability is >70%; a bit lower is no reason for concern, but if viability is less than 60% thaw a new batch of cells.
5. Subculture the FreeStyle™ CHO-S® cells 24–48 hours after thawing by seeding shaker flasks at  $0.3 \times 10^6$  viable cells/mL in pre-warmed FreeStyle™ CHO Expression Medium supplemented with 8 mM L-glutamine. We generally use 125- or 250-mL polycarbonate, disposable, sterile, Erlenmeyer flasks containing 40 or 80 mL total working volume of cell suspension, respectively.

**Important Note:** Subculture cells a minimum 5 passages before use in transfection experiments to allow opportunity for recovery from thawing. To subculture cells, see the procedure on the next page.

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# Subculturing Cells

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## Passaging Cells Every 48–72 hours

Subculture cells when the density is approximately  $1\text{--}1.5 \times 10^6$  viable cells/mL, typically every 48–72 hours. When maintaining FreeStyle™ CHO-S® cells, we generally use a 125- or 250-mL polycarbonate, disposable, sterile Erlenmeyer flask with vented cap containing 25–40 mL or 50–80 mL total working volume of cell suspension, respectively.

**Note:** Glass flasks without baffles may be used, but thorough cleaning after each use is essential to avoid potential toxicity which is more problematic in serum-free cultures.

1. Determine viable and total cell counts (see protocol on page 5).
2. Using the cell density determined in Step 1, calculate the split ratio needed to seed the new shaker flask at  $0.5\text{--}2 \times 10^5$  viable cells/mL.
3. Dilute the cells in fresh, pre-warmed FreeStyle™ CHO Expression Medium supplemented with 8 mM L-glutamine to give a final cell density of  $0.5\text{--}2 \times 10^5$  viable cells/mL in the desired final volume.
4. Incubate flasks in a 37°C incubator containing a humidified atmosphere of 8% CO<sub>2</sub> in air on an orbital shaker platform rotating at 120–135 rpm.
5. Repeat Steps 1–5 as necessary to maintain or expand cells.

**Note:** If you are passaging cells every 2–3 days, do not let cells grow above a density of  $1.5 \times 10^6$  viable cells/mL, or you may experience reduced transfection efficiency.

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## Alternative: Passaging Cells Every 24 hours

Alternatively, cells may be passaged every 24 hours; split cells to approximately  $0.5 \times 10^6$  viable cells/mL as described above. The next day, cell density should be  $1.3\text{--}1.4 \times 10^6$  viable cells/mL. Split cells every 24 hours.

If expansion is needed, cells can be split at  $0.8\text{--}1.0 \times 10^6$  viable cells/mL. Next day, cell density should be  $2.0 \times 10^6$  viable cells/mL. Split cells every 24 hours.

**Note:** If you are passaging cells every day, do not let cells grow above a density of  $2.0 \times 10^6$  viable cells/mL, or you may experience reduced transfection efficiency.

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## Passaging Large Numbers of Cells

If large numbers of cells are needed, you can seed cultures at  $0.5 \times 10^6$  cells/mL and use cells as soon as they reach a density of  $5 \times 10^6$  cells/mL (3–4 days). Do not let cultures that have reached a cell density of  $5 \times 10^6$  cells/mL grow any further, as this will result in a decrease of transfection efficiency.

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Keep monitoring the degree of cell clumping. Occasionally FreeStyle™ CHO-S® suspension cultures may grow as 2–10 cell clusters. Vigorous vortexing for 10–30 seconds may be required at each subculture for a number of passages until the cultures grow predominantly as single cells.

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## Subculturing Cells, continued

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### Shake Flasks

The cells can be grown in many different culture volumes. We generally use the following polycarbonate, disposable, sterile Erlenmeyer flask with vented cap (other flasks with the same characteristics may be used):

Flask Volume	Culture Volume	Manufacturer	Catalog No.
125 mL	25–40 mL	VWR, West Chester, PA	30180-036
250 mL	50–80 mL	VWR, West Chester, PA	30180-044
500 mL	100–150 mL	VWR, West Chester, PA	30180-052
1 liter	200–300 mL	VWR, West Chester, PA	82013-164
3 liter	600–1000 mL	Corning, Acton, MA	431252

### Other Cell Culture Systems

It is possible to scale up the FreeStyle™ CHO-S® cultures in spinner flasks or bioreactors. The appropriate spinner or impeller speed and seeding density should be determined and optimized for each system. We recommend seeding cells at  $0.2 \times 10^6$  viable cells/mL.

**Note:** Monitor cell viability and the degree of cell clumping. Note that extensive cell clumping may reduce transfection efficiency.

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# Freezing Cells

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## Introduction

You may freeze FreeStyle™ CHO-S® cells directly in FreeStyle™ CHO Expression Medium with 10% DMSO. When freezing the FreeStyle™ CHO-S® cell line, we recommend the following:

- Freeze cells at a density of  $\geq 1 \times 10^7$  viable cells/mL.
- Use a freezing medium composed of 90% fresh growth medium and 10% DMSO.

Guidelines to prepare freezing medium and to freeze cells are provided in this section.

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## Preparing Freezing Medium

Prepare freezing medium immediately before use.

1. In a sterile, conical centrifuge tube, mix together the following reagents for every 1 mL of freezing medium needed:

FreeStyle™ CHO Expression Medium (supplemented with 8 mM L-glutamine)	0.9 mL
DMSO	0.1 mL
  2. Filter-sterilize the freezing medium and place the tube on ice until use. Discard any remaining freezing medium after use.
- 

## Freezing Cells

Before starting, label cryovials and prepare freezing medium. Keep the freezing medium on ice.

1. Grow the desired quantity of FreeStyle™ CHO-S® cells in shaker flasks, harvesting when the cell density reaches  $1 \times 10^6$  viable cells/mL. Transfer cells to a sterile, conical centrifuge tube.
2. Determine the viable and total cell counts (see protocol on page 5) and calculate the volume of freezing medium required to yield a final cell density of  $1 \times 10^7$  viable cells/mL.
3. Centrifuge cells at  $100 \times g$  for 5 minutes at room temperature and carefully aspirate the medium.
4. Resuspend the cells in the pre-determined volume of chilled freezing medium.
5. Place cryovials in a microcentrifuge rack and aliquot 1 mL of the cell suspension into each cryovial.
6. Freeze cells in an automated or manual, controlled-rate freezing apparatus following standard procedures. For ideal cryopreservation, the freezing rate should be a decrease of  $1^\circ\text{C}$  per minute.
7. Transfer frozen vials to liquid nitrogen for long-term storage.

**Note:** You may check the viability and recovery of frozen cells 24 hours after storing cryovials in liquid nitrogen by following the procedure outlined in **Thawing and Establishing Cells**, page 6.

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# Transfecting Cells

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## Introduction

To transfect suspension FreeStyle™ CHO-S® cells, you will use the cationic lipid-based transfection reagent, FreeStyle™ MAX Reagent, included with the kit. Unlike some other serum-free media formulations, FreeStyle™ CHO Expression Medium does not inhibit cationic lipid-mediated transfection. FreeStyle™ CHO Expression Medium is specifically formulated to allow high transfection efficiency of suspension FreeStyle™ CHO-S® cells **without** the need to change or add media. Transient transfection experiments may be performed in a large volume, allowing large-scale protein production.

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## FreeStyle™ MAX Reagent

FreeStyle™ MAX Reagent is a proprietary formulation suitable for transfection of DNA into eukaryotic cells. In the FreeStyle™ MAX CHO Expression System, use of FreeStyle™ MAX Reagent to transfect FreeStyle™ CHO-S® cells provides the following advantages:

- FreeStyle™ MAX Reagent demonstrates high transfection efficiency in suspension FreeStyle™ CHO-S® cells (cultured in FreeStyle™ CHO Expression Medium)
- DNA- FreeStyle™ MAX Reagent complexes can be added directly to cells in culture medium
- It is not necessary to remove complexes or change or add medium following transfection

FreeStyle™ MAX Reagent is available separately from Life Technologies (see page vi for ordering information). For more information, see our website ([www.lifetechnologies.com](http://www.lifetechnologies.com)) or call Technical Support (see page 17).

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## Plasmid Preparation

Plasmid DNA for transfection into eukaryotic cells must be clean, sterile and free from phenol and sodium chloride. Contaminants may kill the cells, and salt will interfere with complexing, decreasing transfection efficiency. We recommend isolating plasmid DNA using one of the Purelink™ HiPure Plasmid Kits, which have been validated for use with the FreeStyle™ MAX CHO Expression System (see page vi for ordering information).

**Note:** Make sure your DNA preparation is sterile, for instance by performing filtration through a 0.22 µm filter before use.

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## Positive Control

pCMV SPORT-βgal is provided as a positive control vector for transfection and expression in FreeStyle™ CHO-S® cells. The gene encoding β-galactosidase is expressed in FreeStyle™ CHO-S® cells under the control of the human cytomegalovirus (CMV) promoter. Successful transfection will result in β-galactosidase expression that is easily assayed (see the next page). For a map of pCMV SPORT-βgal, see page 16.

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## Transfecting Cells, continued

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### Assay for $\beta$ -galactosidase Activity

You may evaluate  $\beta$ -galactosidase expression by activity assay using cell-free lysates (Miller, 1972). Life Technologies offers the FluoReporter<sup>®</sup> *lacZ*/Galactosidase Quantitation Kit (Cat. no. F-2905) for fast and easy detection of  $\beta$ -galactosidase expression.

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### Materials to Have on Hand

You will need to have the following reagents on hand before beginning:

- Suspension FreeStyle<sup>™</sup> CHO-S<sup>®</sup> cells cultured in FreeStyle<sup>™</sup> CHO Expression Medium  
**Recommendation:** Calculate the number of cells that you will need for your transfection experiment and expand cells accordingly. Make sure that the cells are healthy and greater than 95% viable before proceeding to transfection.
  - Purified plasmid DNA of interest (1 mg/mL)
  - FreeStyle<sup>™</sup> MAX Reagent (supplied with the kit; store at +4°C until use)
  - OptiPRO<sup>™</sup> SFM (supplied with the kit; pre-warmed to room temperature)
  - FreeStyle<sup>™</sup> CHO Expression Medium, supplemented with L-glutamine to a final concentration of 8 mM (supplied with the kit; pre-warmed to 37°C)  
**Note:** Do not add more than 5 mL/L of Penicillin/ Streptomycin (0.5X Pen-Strep) to media during transfection as this may decrease transfection activity.
  - 125-mL polycarbonate, disposable, sterile Erlenmeyer flasks
  - Orbital shaker in 37°C incubator with a humidified atmosphere of 8% CO<sub>2</sub>
  - Reagents to determine viable and total cell counts
  - Vortex mixer
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### Optimal Conditions for 30 mL Transfection

To transfect suspension FreeStyle<sup>™</sup> CHO-S<sup>®</sup> cells in a 30 mL volume, we recommend using the following optimized conditions:

- **Final transfection volume:** 30 mL
  - **Number of cells to transfect:**  $3 \times 10^7$  cells (cell density at time of transfection  $1 \times 10^6$  cells/mL)
  - **Amount of plasmid DNA:** 37.5  $\mu$ g (starting point; can vary from 30–45  $\mu$ g)
  - **FreeStyle<sup>™</sup> MAX Reagent:** 37.5  $\mu$ L (starting point; can vary from 30–45  $\mu$ L)
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### Note

If you are using other CHO cells, you may want to test varying amounts of FreeStyle<sup>™</sup> MAX Reagent and plasmid DNA (e.g., 30, 35, 40, 45, 50  $\mu$ L with 30, 35, 40, 45, 50  $\mu$ g) to determine the optimal conditions for transfection.

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## Transfecting Cells, continued

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### Transfection Procedure

Follow the procedure below to transfect suspension FreeStyle™ CHO-S® cells in a **30 mL volume**. Remember that you may keep the cells in FreeStyle™ CHO Expression Medium during transfection. We recommend including a positive control (pCMV SPORT-βgal) and a negative control (no DNA, no FreeStyle™ MAX Reagent) in your experiment to help you evaluate your results.

1. Approximately 24 hrs before transfection, pass FreeStyle™ CHO-S® cells at  $5\text{--}6 \times 10^5$  cells/mL. Place the flask(s) on an orbital shaker platform rotating at 120–135 rpm at 37°C, in a humidified atmosphere of 8% CO<sub>2</sub> in air.
2. On the day of transfection, the cell density should be about  $1.2\text{--}1.5 \times 10^6$ /mL. Dilute the cells to  $1 \times 10^6$  /mL. To ensure high transfection results, viability of cells must be over 95%. Add 30 mL of cells into each 125 mL shake flask.
3. Gently invert the tube of FreeStyle™ MAX Transfection Reagent several times to mix. Do not vortex.
4. Dilute 37.5 μg of plasmid DNA into OptiPRO™ SFM to a total volume of 0.6 mL and mix. In a separate tube, dilute 37.5 μL of FreeStyle™ MAX Transfection Reagent in Opti-Pro™ SFM to a total volume of 0.6 mL and mix gently by inverting the tube (do not vortex). Immediately add diluted FreeStyle™ MAX Transfection Reagent to diluted DNA solution to obtain a total volume of 1.2 mL and mix gently.
5. Incubate the DNA-FreeStyle™ MAX mix for 10 minutes at room temperature to allow complexes to form. Do not incubate for longer than 20 minutes.
6. Slowly add 1.2 mL of DNA-FreeStyle™ MAX Reagent complex into the 125-mL flask containing cells while slowly swirling the flask.
7. Incubate transfected cell cultures at 37°C, in a humidified atmosphere of 8% CO<sub>2</sub> in air on an orbital shaker platform rotating at 135 rpm. There is no need to change or supplement the culture medium during the first 6 to 7 days.
8. Protein expression may be detectable within 4–8 hours of transfection, with maximal protein yield usually between 1 and 7 days post-transfection.

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## Transfecting Cells, continued

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### Optimizing Protein Expression

- When expressing a protein for the first time, perform a time course experiment between days 1 and 7 post-transfection to identify the peak of protein production, and to monitor cell viability.
- We have observed peak yields for IgG protein production at 5–7 days post-transfection.
- Test varying amounts of plasmid DNA and FreeStyle™ MAX Reagent. For 30-mL cultures, try between 30–45 µg DNA and 30–45 µL FreeStyle™ MAX Reagent.
- To assess transfection efficiency via expression of a GFP-type fluorescent protein, we recommend monitoring the cultures starting at 24 hours post-transfection.
- In some cases, transfection efficiency may go down during the course of the experiment, although protein production is still going up. Never evaluate results by transfection efficiency only; always measure the protein production.

For optimizing protein expression while scaling up culture volumes, see **Scaling up Transfections** (below).

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### Scaling Up Transfections

If you wish to transfect suspension FreeStyle™ CHO-S® cells in a larger volume, scale up the volume of each reagent in proportion to the culture volume. The table below lists suggested conditions to use when transfecting FreeStyle™ CHO-S® cells in a 30 mL, 250 mL or 1 liter volume. The transfection conditions may vary depending on the type of culture vessel used and the growth conditions of your cells; therefore, you may want to perform pilot studies to optimize your transfection conditions. A suitable range for optimization is given; vary the amounts of FreeStyle™ MAX Reagent and plasmid DNA.

**Note:** Often, the conditions for transfection of large culture volumes need to be adjusted to the higher side of the range, while those for small culture volumes may tend towards the lower side of the range.

Cell Culture			DNA			FreeStyle™ MAX Reagent		
Culture Volume	Culture Flask	Total Number Cells*	Starting Point	Range for Optimizing	Dilution Volume	Starting Point	Range for Optimizing	Dilution Volume
30 mL	125 mL	$3 \times 10^7$	37.5 µg	30–45 µg	to 0.6 mL	37.5 µL	30–45 µL	to 0.6 mL
250 mL	1 liter	$2.5 \times 10^8$	312.5 µg	250–375 µg	to 5 mL	312.5 µL	250–375 µL	to 5 mL
1 liter	3 liter	$1 \times 10^9$	1.25 mg	1.0–1.5 mg	to 20 mL	1.25 mL	1.0–1.5 mL	to 20 mL

\*Cell density of  $1 \times 10^6$  cells/mL on day of transfection

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### Adjustments Large Culture Volumes

For culture volumes **above 30 mL**, lower the speed of the orbital shaker if foam is generated. In 1 L cultures, we recommend 70–80 rpm.

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# Troubleshooting

## Culturing Cells

The table below lists some potential problems and possible solutions that may help you troubleshoot your cell culture problems.

Problem	Reason	Solution
No viable cells after thawing stock	Stock not stored correctly	Order new stock and store in liquid nitrogen. Keep in liquid nitrogen until thawing.
	Home-made stock not viable	Freeze cells at a density of $1 \times 10^7$ viable cells/mL.
		Use a freezing medium composed of 90% fresh FreeStyle™ CHO Expression Medium and 10% DMSO.
		Use low-passage cells to make your own stocks
		Follow procedures in <b>Freezing Cells</b> (page 9) exactly.
	Thawing medium not correct	Use FreeStyle™ CHO Expression Medium supplemented with 8 mM L-glutamine (pre-warm before use).
		Do not add antibiotics to media as this may negatively impact cell growth.
Shaker not set up properly	Shake on an orbital shaker at 125 rpm in 37°C incubator with a humidified atmosphere of 8% CO <sub>2</sub> in air.	
Cells too diluted	Spin down culture and grow cells in a smaller culture volume.	
Cells grow slowly	Growth medium not correct	Use FreeStyle™ CHO Expression Medium supplemented with 8 mM L-glutamine (pre-warm before use).
		Do not add more than 5 mL/L of Penicillin/Streptomycin (0.5X Pen-Strep) to media as this may impact cell growth.
	Shaker not set up properly	Shake on an orbital shaker at 125 rpm in 37°C incubator with a humidified atmosphere of 8% CO <sub>2</sub> in air.
	Medium foamy	Lower the shaker speed slightly till no foam forms.
	Flasks too small	Use flasks that are at least 2.5 times bigger than the culture volume.
	Cells too old	Use healthy FreeStyle™ MAX CHO-S® cells under passage 25; do not overgrow.
	Cell culture clumpy	Prevent this by sufficient agitation of the culture, a regular and frequent cell passage schedule, and maintenance of cells at recommended densities.

*Continued on next page*

## Troubleshooting, continued

### Transfection and Protein Production

The table below lists some potential problems and possible solutions that may help you troubleshoot your transfection and protein production experiments.

Problem	Reason	Solution	
Low Transfection Efficiency and/or Low Protein Yield	Cells cultured for too many passages (over 25 passages)	Thaw a new batch of early-passage cells	
	Cells not passed 24 hours before transfection	Approximately 24 hours before transfection, pass cells at $5\text{--}6 \times 10^5$ cells/mL.	
	Improperly cultured FreeStyle™ CHO-S® cells	Exactly follow procedures as outlined in <b>Subculturing Cells</b> section (page 7).	
	Cells transfected in media containing too much antibiotics	Do not add more than 5 mL/L of Penicillin/Streptomycin (0.5X Pen-Strep) to media.	
	FreeStyle™ Max Reagent handled incorrectly		Store at +4°C. <b>Do not freeze.</b>
			Mix gently by inversion. <b>Do not vortex.</b>
	Used poor quality expression construct plasmid DNA ( <i>i.e.</i> plasmid DNA from a mini-prep)	<b>Do not</b> use mini-prep plasmid DNA for transfection. Use a PureLink™ HiPure Plasmid Kit to prepare plasmid DNA with low endotoxin contamination.	
	Suboptimal transfection conditions		Perform transfections with positive control plasmid pCMV SPORT-βgal to assess your transfection conditions (see page 10)
			Assess transfection efficiency via expression of a GFP-type fluorescent protein (we recommend monitoring the cultures starting at 24 hours post-transfection.)
			Vary the amounts of DNA and FreeStyle™ MAX Reagent used (see page 13).
	DNA not sterile		Sterilize DNA (see page 10)
	Gene of interest is toxic to cells		Do not generate constructs containing activated oncogenes or harmful genes.
			Try FreeStyle™ MAX 293 Expression System
Protein harvested too early or too late		When expressing a protein for the first time, perform a time course experiment between days 1 and 7 post-transfection to identify the peak of protein production, and to monitor cell viability.	

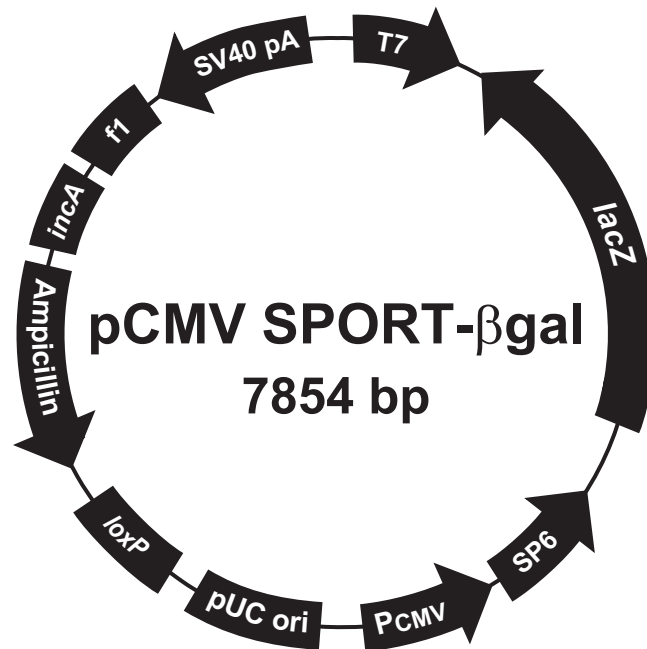
## Appendix

### pCMV SPORT-βgal

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#### Description

pCMV SPORT-βgal is included in the FreeStyle™ MAX CHO Expression System for use as a transfection and expression control, and contains the *lacZ* gene cloned into pCMV SPORT1. The plasmid uses the human cytomegalovirus (CMV) promoter to control expression of β-galactosidase. **The complete sequence of pCMV SPORT-βgal is available for downloading from our website ([www.lifetechnologies.com](http://www.lifetechnologies.com)) or by calling Technical Support (see page 17).**



#### Comments for pCMV SPORT-βgal: 7854 nucleotides

SV40 small T intron and polyA signal: bases 193-555 (complementary strand)

T7 promoter: bases 645-664

*lacZ* ORF: bases 1009-4149 (complementary strand)

SP6 promoter: bases 4259-4278 (complementary strand)

CMV promoter: bases 4308-4901 (complementary strand)

pUC origin: bases 5390-6063 (complementary strand)

*loxP*: bases 6115-6148

Ampicillin (*bla*) resistance gene: bases 6250-7110 (complementary strand)

*incA*: bases 7134-7306

f1 intergenic region: bases 7579-7854 (complementary strand)

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