USER GUIDE

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by life technologies™

FreeStyle™ MAX 293 Expression System

For large-scale transfection of suspension 293 cells in a defined, serum-free medium

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life technologies™

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

Contents

Kit Contents and Storage

Shipping/Storage

The components of the FreeStyle™ MAX 293 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™ 293-F Cells	Dry ice	Liquid nitrogen
FreeStyle™ MAX Reagent	Blue ice	+4°C
FreeStyle™ 293 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

FreeStyle™ 293-F Cells

Storage conditions: Liquid nitrogen

Amount supplied: One vial containing 1×10^7 cells

Composition: 1 mL of cells in 90% FreeStyle™ 293 Expression Medium and 10% DMSO.

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.

FreeStyle™ 293 Expression Medium

Storage conditions: +4°C, in the dark

Amount supplied: 1 liter

Composition: Proprietary, defined, serum-free medium formulated with GlutaMAX™-I supplement

OptiPRO™ SFM

Storage conditions: +4°C, in the dark

Amount supplied: 100 mL

Composition: Proprietary, defined, serum-free medium

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

Product Use

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

Accessory Products

Introduction

The products listed in this section may be used with the FreeStyle™ MAX 293 Expression System. For more information, refer to our website (**www.lifetechnologies.com**) or call Technical Support (see page 16).

Accessory Products

The following reagents supplied in the FreeStyle™ MAX 293 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™ 293-F Cells	1 vial (1×10^7 cells)	R790-07
FreeStyle™ 293 Expression Medium	1 L	12338-018
	6 × 1 L	12338-026
FreeStyle™ MAX Reagent	1 mL	16447-100
OptiPRO™ SFM	100 mL	12309-050
	1000 mL	12309-019
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® *lacZ*/Galactosidase Quantitation Kit	1000 assays	F-2905
Antibiotic-Antimycotic (100x)	100 mL	15240-062
Pluronic® F-68, 10% (100X)	100 mL	24040-032
pCEP4	20 µg	V044-50
pcDNA™3.2/V5-DEST	6 µg	12489-019
pcDNA™4/HisMax A, B, & C	20 µg each	V864-20

FreeStyle™ MAX CHO 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 CHO Expression System.

Item	Amount	Catalog No.
FreeStyle™ MAX CHO Expression System	25 reactions (30 mL cultures)	K9000-20
FreeStyle™ CHO-S® Cells	1 vial (1×10^7 cells)	R800-07
FreeStyle™ CHO Expression Medium	1 L	12651-014
	6 × 1 L	12651-022

Introduction

Overview

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

This manual supports the FreeStyle™ MAX 293 Expression System.

Note: For information on the FreeStyle™ MAX CHO Expression System, see the FreeStyle™ MAX CHO Expression System manual available from our website (**www.lifetechnologies.com**) or Technical Support (see page 16).

FreeStyle™ MAX 293 Expression System

The FreeStyle™ MAX 293 Expression System includes FreeStyle™ 293-F cells that have been adapted to serum-free, suspension culture in FreeStyle™ 293 Expression Medium. Transfection and expression experiments may be performed directly in FreeStyle™ 293 Expression Medium without the need to change media. The complete FreeStyle™ MAX 293 Expression Kit 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.

Components of the FreeStyle™ MAX 293 Expression System

The FreeStyle™ MAX 293 Expression System includes the following major components:

- **FreeStyle™ MAX Reagent:** This transfection reagent provides high transfection efficiency in suspension FreeStyle™ 293-F cells and FreeStyle™ CHO-S® cells (see below for more information).
- **FreeStyle™ 293-F cells:** This cell line is adapted to high density, serum-free suspension culture in FreeStyle™ 293 Expression Medium and is capable of producing high levels of recombinant protein (see page 3 for more information).
- **FreeStyle™ 293 Expression Medium:** This is a defined, serum-free medium formulated specifically to allow growth **and** large-scale transfection of suspension FreeStyle™ 293-F 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).

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™ 293-F Cells and FreeStyle™ CHO-S® Cells.

Continued on next page

Overview, continued

OptiPRO™ SFM

OptiPRO™ Serum Free Medium is included with the FreeStyle™ MAX 293 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**) or call Technical Support (see page 16).

Advantages of the FreeStyle™ MAX 293 Expression System

Using the FreeStyle™ MAX 293 Expression System for protein production in mammalian cells provides the following advantages:

- Uses 293 human embryonal kidney cells, for very high expression levels of recombinant protein in human cells
- 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

Suitable Expression Vectors

Suitable expression vectors for the FreeStyle™ MAX 293 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 polyhistidine tag

FreeStyle™ 293-F Cells

Introduction

The FreeStyle™ 293-F cell line is supplied with the FreeStyle™ MAX 293 Expression System and is derived from the 293 cell line (see below). FreeStyle™ 293-F cells are adapted to suspension culture in FreeStyle™ 293 Expression Medium. Frozen cells are supplied in and may be thawed directly into FreeStyle™ 293 Expression Medium (see **Thawing and Establishing Cells**, page 6).

Parental Cell Line

The 293 cell line is a permanent line established from primary embryonal human kidney transformed with sheared human adenovirus type 5 DNA (Graham *et al.*, 1977; Harrison *et al.*, 1977). The E1A adenovirus gene is expressed in these cells and participates in transactivation of some viral promoters, allowing these cells to produce very high levels of protein.

The FreeStyle™ 293-F cell line supplied with the FreeStyle™ MAX 293 Expression System is a variant of the 293 cell line that has been adapted to suspension growth in FreeStyle™ 293 Expression Medium. The FreeStyle™293-F cell line was obtained from Robert Horlick at Pharmacopeia.

Characteristics of FreeStyle™ 293-F Cells

The FreeStyle™ 293-F cell line exhibits the following characteristics:

- Prepared from low passage Master Cell Bank cultures derived from parental 293-F cells that were re-cloned by limiting dilution. The 293 clonal derived cultures are maintained in serum-free conditions for only 30–35 total passages.
- Adapted to high density, serum-free, suspension growth and maintained in FreeStyle™ 293 Expression Medium.
- Demonstrates high transfection efficiencies with FreeStyle™ MAX Reagent.
- Suspension cultures may be transfected in FreeStyle™ 293 Expression Medium without the need to change media.
- Permits transfection of cells at small and large volumes using scalable conditions.

Note

Other 293 cell lines may be used with the FreeStyle™ MAX 293 Expression System. Before these cell lines may be used for transfection studies, however, they must be adapted to serum-free, suspension culture in FreeStyle™ 293 Expression Medium and evaluated for transfection and expression.

FreeStyle™ 293 Expression Medium

Introduction

FreeStyle™ 293 Expression Medium is a defined, serum-free medium specifically developed for the high-density, suspension culture and transfection of 293 cells. The medium contains **NO** human or animal origin components.

Features of the Medium

FreeStyle™ 293 Expression Medium exhibits the following features:

- An optimized, serum-free and protein-free formulation designed to support the high-density culture and transfection of 293 cells (e.g., FreeStyle™ 293-F cells) in suspension. The medium is **not** recommended for **adherent** 293 cell culture.
- Prepared ready-to-use, with no supplementation required.
- Contains no human or animal-origin products.
- Formulated with GlutaMAX™-I supplement (see below) to increase stability and maximize shelf life.
- Supports small-scale growth of FreeStyle™ 293-F cells in shaker flasks, and large-scale growth of FreeStyle™ 293-F cells in bioreactors.

GlutaMAX™-I Supplement

GlutaMAX™-I media contain the dipeptide, L-alanyl-L-glutamine, a stabilized form of L-glutamine. GlutaMAX™-I media have the following characteristics:

- L-glutamine does not degrade in storage or during incubation
- Ammonia build-up is minimized
- Glutamine delivery is controlled

L-glutamine does not need to be added at the time of use.

Note: GlutaMAX™-I supplement is only removed from the medium by cell metabolism. There is no accumulation of toxic metabolites due to spontaneous breakdown.

Growth Characteristics of FreeStyle™ 293-F Cells in the Medium

Typically, FreeStyle™ 293-F cells cultured in FreeStyle™ 293 Expression Medium demonstrate the following:

- Doubling time in the range of 20–25 hours (doubling time can exceed 25 hours during the first few passages after the cells have been thawed.)
- Cell densities of up to 3×10^6 cells/mL in shaker or spinner culture
- Cell densities of up to 4×10^6 cells/mL in bioreactor culture
- Do not allow FreeStyle™ 293-F cells to reach a cell density above 3×10^6 cells/mL before transfection, as this will result in a decrease of transfection efficiency.

Note: Individual culturing and passaging techniques coupled with cellular heterogeneity inherent within the FreeStyle™ 293-F cell population may result in experimental variability.

Methods

General Information

General Cell Handling

Follow the general guidelines below to grow and maintain FreeStyle™ 293-F 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 (below 30 passages). Upon receipt of the cells from Life Technologies, grow and freeze multiple vials of the FreeStyle™ 293-F Cells to ensure that you have an adequate supply of early-passage cells.
- For general maintenance of cells, pass FreeStyle™ 293-F cells when they reach a density in between 1–3 × 10^6 viable cells/mL (generally every 48–72 hours). Do not dilute below 0.1 × 10^6 viable cells/mL.
- Use trypan blue exclusion to determine cell viability (see below). Log phase cultures should be >90% viable.
- When thawing or subculturing cells, transfer cells into pre-warmed medium.

Important

It is very important to have healthy, well-growing FreeStyle™ 293-F cells to get high yields of protein expression. Strictly follow the guidelines for culturing FreeStyle™ 293-F cells in this manual for the best results.

As with other human cell lines, when working with FreeStyle™ 293-F cells, handle as potentially biohazardous material under at least Biosafety Level 2 containment.

Preparing Media

For suspension growth and transfection applications, use:

- FreeStyle™ 293 Expression Medium as is. No supplementation is required.
- Antibiotics are **not** recommended; however, 5 mL/L of Antibiotic-Antimycotic containing penicillin, streptomycin, and amphotericin B may be used when required (see page vi for ordering information).

Note: FreeStyle™ 293 Expression Medium is extremely sensitive to light. For optimal results, use and store media protected from light.

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. Vigorously vortex for 10–30 seconds to break up cell clumps.
4. Determine cell density electronically using a Coulter Counter or manually using a hemacytometer.

Thawing and Establishing Cells

Introduction

Follow the protocol below to thaw FreeStyle™ 293-F cells to initiate cell culture. The FreeStyle™ 293-F cell line is supplied in a vial containing 1 mL of cells at 1×10^7 viable cells/mL in 90% FreeStyle™ 293 Expression Medium and 10% DMSO. Thaw FreeStyle™ 293-F cells directly into the FreeStyle™ 293 Expression Medium supplied with the kit.

Materials Needed

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

- FreeStyle™ 293-F cells (supplied with the kit; store frozen cells in liquid nitrogen until ready to use)
- FreeStyle™ 293 Expression Medium (supplied with the kit; pre-warm to 37°C before use)

  Note: We do not recommend adding antibiotics to media as this may negatively impact cell growth.
- 125-mL polycarbonate, disposable, sterile Erlenmeyer flask with vented cap
- Orbital shaker in 37°C incubator with a humidified atmosphere of 8% CO_2
- Reagents to determine viable and total cell counts (see **Determining Cell Density and Viability**, page 5)

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™ 293 Expression Medium.
3. Incubate cells in a 37°C incubator containing a humidified atmosphere of 8% CO_2 in air on an orbital shaker platform rotating at 135 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™ 293-F cells 24–48 hours after thawing by seeding shaker flasks at 3×10^5 viable cells/mL in pre-warmed FreeStyle™ 293 Expression Medium. 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 of 5 passages before use in transfection experiments to allow opportunity for recovery from thawing. To subculture cells, see the procedure on the next page.

Subculturing Cells

Passaging Cells

Subculture cells when the density is in between 1–3 × 10^6 viable cells/mL, typically every 48–72 hours. When maintaining FreeStyle™ 293-F 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 1–2 × 10^5 viable cells/mL.
3. Dilute the cells in fresh, pre-warmed FreeStyle™ 293 Expression Medium to give a final cell density of 1–2 × 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_2 in air on an orbital shaker platform rotating at 135 rpm.
5. Repeat Steps 1–5 as necessary to maintain or expand cells. Monitor the degree of cell clumping (see below).

FreeStyle™ 293-F 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.

Scaling Up Cell Culture

It is possible to scale up the FreeStyle™ 293-F cultures in spinner flasks or bioreactors. The appropriate spinner or impeller speed and seeding density should be determined and optimized for each system. At Life Technologies, the optimum spinner speed was 100–130 rpm and 70–100 rpm impeller speed in Celligen™ stirred tank bioreactors. We recommend seeding cells at 3–5 × 10^5 viable cells/mL.

Note: If the split ratio of cells to fresh media is less than 1:2, you may want to spin down the cell suspension and resuspend the cell pellet in fresh, pre-warmed FreeStyle™ 293 Expression Medium prior to inoculating the spinner or bioreactor culture. Monitor cell viability and the degree of cell clumping. Note that extensive cell clumping may reduce transfection efficiency.

At high stirring speeds (i.e., greater than 130 rpm) and/or depending on the impeller design, you may want to supplement the FreeStyle™ 293 Expression Medium with additional Pluronic® F-68 (2.5–5 mL/L of 10% Pluronic® F-68, Catalog No. 24040) to avoid sheer stress in the culture.

Freezing Cells

Introduction

You may freeze FreeStyle™ 293-F cells directly in FreeStyle™ 293 Expression Medium with 10% DMSO. When freezing the FreeStyle™ 293-F cell line, we recommend the following:

Freeze cells at a density of 1×10^7 viable cells/mL.

Use a freezing medium composed of 90% fresh FreeStyle™ 293 Expression Medium and 10% DMSO.

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

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™ 293 Expression Medium	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™ 293-F cells in shaker flasks, harvesting when the cell density reaches $0.5–1 \times 10^6$ 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 1×10^7 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°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.

Transfecting Cells

Introduction

To transfect suspension FreeStyle™ 293-F 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™ 293 Expression Medium does not inhibit cationic lipid-mediated transfection. FreeStyle™ 293 Expression Medium is specifically formulated to allow high transfection efficiency of suspension FreeStyle™ 293-F cells **without** the need to change or add media. Transient transfection experiments may be performed in a large volume, allowing large-scale protein production.

FreeStyle™ MAX Reagent

FreeStyle™ MAX Reagent is a proprietary formulation suitable for transfection of DNA into eukaryotic cells. In the FreeStyle™ MAX 293 Expression System, use of FreeStyle™ MAX Reagent to transfect FreeStyle™ 293-F cells provides the following advantages:

- FreeStyle™ MAX Reagent demonstrates high transfection efficiency and protein yield in suspension FreeStyle™ 293-F cells (cultured in FreeStyle™ 293 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**) or call Technical Support (see page 16).

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 293 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.

Positive Control

pCMV SPORT-βgal is provided as a positive control vector for transfection and expression in FreeStyle™ 293-F cells. The gene encoding β-galactosidase is expressed in FreeStyle™ 293-F cells under the control of the human cytomegalo-virus (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 15.

Continued on next page

Assay for β-galactosidase Activity

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

Materials to Have on Hand

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

- Suspension FreeStyle™ 293-F cells cultured in FreeStyle™ 293 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 90% viable before proceeding to transfection.
- Purified plasmid DNA of interest (1 mg/mL)
- FreeStyle™ MAX Reagent (supplied with the kit; store at +4°C until use)
- OptiPRO™ SFM (supplied with the kit; warm to room temperature)
- FreeStyle™ 293 Expression Medium (supplied with the kit; pre-warmed to 37°C)

  Note: Do not add antibiotics to media during transfection as this may decrease transfection activity.
- 125-mL polycarbonate, disposable, sterile Erlenmeyer flasks with vented cap.
- Orbital shaker in 37°C incubator with a humidified atmosphere of 8% CO_2
- Reagents to determine viable and total cell counts
- Sterile, disposable, polycarbonate snap-cap tubes

Optimal Conditions for 30 mL Transfection

To transfect suspension FreeStyle™ 293-F cells in a 30 mL volume, we recommend using the following optimized conditions:

- **Final transfection volume:** 30 mL
- **Number of cells to transfect:** 3×10^7 cells (cell density at time of transfection of 1×10^6 cells/mL)
- **Amount of plasmid DNA:** 37.5 µg (starting point; can vary from 24–42 µg)
- **FreeStyle™ MAX Reagent:** 37.5 µL (starting point; can vary from 24–42 µL)

Note

If you are using other 293 cells, you may want to test varying amounts of FreeStyle™ MAX Reagent and plasmid DNA (e.g., 24, 30, 35, 40, 45, 50 µL lipid with 24, 30, 35, 40, 45, 50 µg DNA) to determine the optimal conditions for transfection.

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Transfection Procedure

Follow the procedure below to transfect suspension FreeStyle™ 293-F cells in a 30 mL volume. Remember that you may keep the cells in FreeStyle™ 293 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™ 293-F cells at 6–7 × 10^5 cells/mL. Place the flask(s) on an orbital shaker platform rotating at 135 rpm at 37°C, 8% CO_2.
2. On the day of transfection, the cell density should be about 1.2–1.5 × 10^6/mL. Dilute the cells to 1 × 10^6 /mL. To ensure high transfection results, viability of cells must be over 90%. 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 Reagent in OptiPRO™ SFM to a total volume of 0.6 mL and mix gently by inverting the tube (do not vortex). Immediately add diluted FreeStyle™ MAX Reagent to diluted DNA solution to obtain a total volume of 1.2 mL and mix gently.
5. Incubate the DNA-lipid mixture 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-lipid mixture into the 125-mL flask containing cells while slowly swirling the flask.
7. Incubate transfected cell cultures at 37°C, 8% CO_2 on an orbital shaker platform rotating at 135 rpm. There is no need to change or supplement the culture medium during the first 6–7 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, depending on the protein expressed.

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Optimizing Protein Expression

- When expressing a protein for the first time, perform a time course experiment between days 1 and 9 post-transfection to identify the peak of protein production, and to monitor cell viability.
- Test varying amounts of plasmid DNA and FreeStyle™ MAX Reagent. For 30 mL cultures, try between 24–42 µg DNA and 24–42 µL FreeStyle™ MAX Reagent.
- For secreted IgG protein production, we have observed peak yields at 5–7 days post-transfection.
- 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).

Scaling Up Transfections

If you wish to transfect suspension FreeStyle™ 293-F 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™ 293-F 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 in between these values to optimize transfection.

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	3×10^7	37.5 µg	24–42 µg	to 0.6 mL	37.5 µL	24–42 µL	to 0.6 mL
250 mL	1 liter	2.5×10^8	312.5 µg	200–350 µg	to 5 mL	312.5 µL	200–350 µL	to 5 mL
1 liter	3 liter	1×10^9	1.25 mg	0.8–1.4 mg	to 20 mL	1.25 mL	0.8–1.4 mL	to 20 mL

*Cell density of 1×10^6 cells/mL on day of transfection

Adjustments for 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 90 rpm.

Note

The transfection efficiency may decrease as the volume increases. Optimizing transfection conditions for large volume is recommended.

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 1×10^7 viable cells/mL.
		Use a freezing medium composed of 90% fresh FreeStyle™ 293 Expression Medium and 10% DMSO.
		Use low-passage cells to make your own stocks.
		Follow procedures in **Freezing Cells** (page 8) exactly.
		Obtain new FreeStyle™ 293-F Cells.
	Thawing medium not correct	Use FreeStyle™ 293 Expression Medium (pre-warm before use).
		Do not add antibiotics to media as this may negatively impact cell growth.
	Shaker not set up correctly	Shake on an orbital shaker at 135 rpm in 37°C incubator with a humidified atmosphere of 8% CO_2.
	Cells to diluted	Spin down culture and grow cells in a smaller culture volume.
Cells grow slowly	Growth medium not correct	Use FreeStyle™ 293 Expression Medium (pre-warm before use).
		Do not add antibiotics to media as this may negatively impact cell growth.
	Shaker not set up correctly	Shake on an orbital shaker at 135 rpm in 37°C incubator with a humidified atmosphere of 8% CO_2.
	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™ 293-F cells under passage 30; do not overgrow.
	Cell culture clumpy	Prevent this by sufficient agitation of the culture, a regular and frequent cell passage schedule, and maintenance cells at recommended densities.

Continued on next page

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.

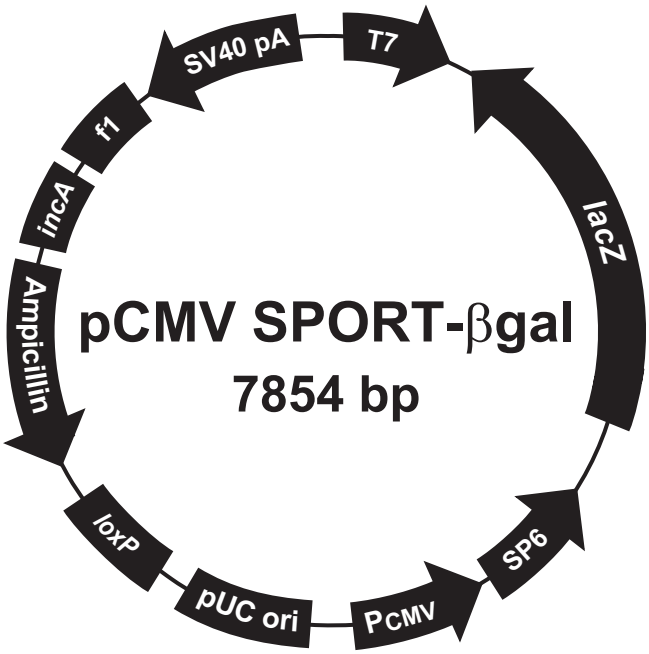
Problem	Reason	Solution
Low Transfection Efficiency and/or Low Protein Production	Cells cultured for too many passages (over 30 passages)	Thaw a new batch of early-passage cells.
	Cells not passed 24 hours before transfection	Approximately 24 hours before transfection, pass cells at 6–7 × 10^5 cells/mL.
	Improperly cultured FreeStyle™ 293-F cells	Exactly follow procedures as outlined in **Subculturing Cells** section (page 7).
	Cells transfected in media containing antibiotics	Do not add antibiotics during transfection.
	FreeStyle ™ Max Reagent handled incorrectly	Store at +4°C. **Do not freeze.**
		Mix gently by inversion. **Do not vortex.**
	Used poor quality expression construct plasmid DNA (i.e., plasmid DNA from a mini-prep)	**Do not** 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 9).
		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 12).
	DNA not sterile	Sterilize DNA (see page 9).
	Gene of interest is toxic to cells	Do not generate constructs containing activated oncogenes or harmful genes.
		Try FreeStyle™ MAX CHO 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 9 post-transfection to identify the peak of protein production, and to monitor cell viability.

Appendix

pCMV SPORT-βgal

Description

pCMV SPORT-βgal is included in the FreeStyle™ MAX 293 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**) or by calling Technical Support (see page 16).**



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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For the latest services and support information for all locations, go to **www.lifetechnologies.com**.

At the website, you can:

- Access worldwide telephone and fax numbers to contact Technical Support and Sales facilities
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- Submit a question directly to Technical Support (**techsupport@lifetech.com)**
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- Download software updates and patches

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Purchaser Notification

Introduction

Use of the FreeStyle™ MAX 293 Expression System is covered under a number of different licenses including those detailed below.

Information for European Customers

The FreeStyle™ 293-F Cell Line is genetically modified and carries human adenovirus type 5 DNA. As a condition of sale, this product must be in accordance with all applicable local legislation and guidelines including EC Directive 90/219/EEC on the contained use of genetically modified organisms.

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Graham, F. L., Smiley, J., Russell, W. C., and Nairn, R. (1977) Characteristics of a Human Cell Line Transformed by DNA from Human Adenovirus Type 5. J. Gen. Virol. *36*, 59-74

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Miller, J. H. (1972) *Experiments in Molecular Genetics*, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York

Notes

Headquarters
5791 Van Allen Way | Carlsbad, CA 92008 USA
Phone +1 760 603 7200 | Toll Free in USA 800 955 6288

For support visit
www.invitrogen.com/support or email techsupport@invitrogen.com

www.lifetechnologies.com

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