invitrogen

FreeStyle™ 293-F cells

USER GUIDE

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B.0	31 January 2020	Converted to CCMS. Rebranded. Updated contents to state liquid nitrogen vapor phase. Added the Important guidelines for thawing and storing cells topic.
A.0	21 April 2015	Remove RUO statement, add LULL 561, update trademark language and branding

Contents

FreeStyle™ 293-F cells

Contents and storage

Shipping and storage

This manual is shipped with FreeStyle™ 293-F Cells. FreeStyle™ 293-F Cells are shipped on dry ice. Upon receipt, store in **liquid nitrogen vapor-phase**.

Contents

Storage conditions: Liquid nitrogen vapor-phase

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

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

CAUTION! Handle as potentially biohazardous material under at least Biosafety Level 2 containment.

WARNING! GENERAL CHEMICAL HANDLING. For every chemical, read the Safety Data Sheets (SDSs) and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves. Safety Data Sheets (SDSs) are available from **www.lifetechnologies.com/support**.

This product contains Dimethyl Sulfoxide (DMSO); components of the product may be absorbed into the body through the skin.

Important guidelines for thawing and storing cells

- Upon receipt, immediately thaw cells or place into vapor-phase liquid nitrogen storage until ready to use. **Do not store the cells at –80°C**.
- Avoid short-term extreme temperature changes. When storing cells in liquid nitrogen after shipping on dry ice, allow the cells to remain in liquid nitrogen for 3-4 days before thawing.
- Prior to starting experiments, ensure you have established cells and have frozen stocks on hand. Upon receipt, grow and freeze multiple vials of cells to ensure that you have an adequate supply of early-passage cells.

Introduction

Overview

Introduction

The FreeStyle™ 293-F cell line is derived from the 293 cell line (see **"Parental cell line" on page 6**) and is intended for use with the FreeStyle™ MAX 293 Expression System (Cat. no. K9000-10) or the FreeStyle™ 293 Expression System (Cat. no. K9000-01). 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 **"Thaw and establish cells" on page 9**).

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 expressed in these cells participates in transactivation of some viral promoters, allowing these cells to produce very high levels of protein.

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

FreeStyle™ 293-F cells

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.

- 293-F Master Cell Bank cultures have been tested by an independent service and found to be negative for HBV, HCV, HTLV-I & -II, and HIV-1 & -2
- Adapted to high density, serum-free, suspension growth and can be 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 large volumes.

FreeStyle™ 293 expression medium

We recommend using FreeStyle™ 293 Expression Medium to grow, maintain, and transfect FreeStyle™ 293-F cells. FreeStyle™ 293 Expression Medium available from Thermo Fisher Scientific (see "Accessory products" on page 21 for ordering information) is a defined, serum-free formulation specifically developed for the high density, suspension culture and transfection of 293 cells. The medium contains no human or animal origin components and is formulated with GlutaMAX™-I to increase stability and maximize shelf life. For more information, see our website (**www.lifetechnologies.com**) or call Technical Support (see).

Growth 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 22–28 hours (doubling time can exceed 28 hours during the first few passages after the cells have been thawed. However, cells may perform poorly if the doubling time is consistently greater than 30 hours.)
- 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 cell cultures to approach the plateau of culture density before transfection, as this will result in a decrease of transfection efficiency and protein expression.

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

FreeStyle™ MAX reagent

FreeStyle™ MAX Reagent is a proprietary, animal origin-free formulation for the highly efficient transfection of plasmid DNA into eukaryotic cells. The main benefits of FreeStyle™ MAX Reagent are:

- Specifically formulated to achieve the **highest expression levels** and **lowest cytotoxicity** in suspension FreeStyle™ 293-F Cells and FreeStyle™ CHO-S™ Cells
- No media change required
- Complex formation can be performed in animal-origin free OptiPRO™ SFM™

For more information, visit **www.lifetechnologies.com** or call Technical Support (see).

FreeStyle™ MAX 293 expression system

The FreeStyle™ 293-F Cells are part 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.
- Enables transient expression of protein for up to 9 days post-transfection. Peak values vary depending on protein expressed.

For more information, visit **www.lifetechnologies.com** or call Technical Support (see).

Methods

Important guidelines

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 (<30 passages). Upon receipt of the cells from Thermo Fisher Scientific, 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 1×10^6–3×10^6 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 **"Determine cell density and viability" on page 9**). 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.

CAUTION! 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.

Media preparation

For suspension growth and transfection applications, use:

- FreeStyle™ 293 Expression Medium as is. Supplementation is not required.
- Antibiotics are **not** recommended; however, 5 mL/L of Antibiotic-Antimycotic containing penicillin, streptomycin, and amphotericin B may be used if required (see "Accessory products" on page 21 for ordering information).

Important

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

Determine 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 "Accessory products" on page 21 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.

Thaw and establish 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 (see "Accessory products" on page 21 for ordering information).

Materials needed

1. FreeStyle™ 293-F cells (supplied with the kit; store frozen cells in liquid nitrogen until ready to use)
2. 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.
3. 125-mL polycarbonate, disposable, sterile Erlenmeyer flask with vented cap (available from VWR™, Radnor PA, Cat. no. 30180-036)
4. Orbital shaker in 37°C incubator with a humidified atmosphere of 8% CO_2
5. Reagents to determine viable and total cell counts (see **"Determine cell density and viability" on page 9**)

Thaw 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 in "Determine cell density and viability" on page 9). 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 0.3×10^6 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! Subculture cells a minimum of 5 passages before use in transfection experiments to allow opportunity for recovery from thawing. To subculture cells, see "Passage cells" on page 11.

Subculture cells

Passage 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 in "Determine cell density and viability" on page 9).
2. Using the cell density determined in Step 1 on page 11, calculate the split ratio needed to seed the new shaker flask at 0.1 × 10^6–0.2 × 10^6 viable cells/mL.
3. Dilute the cells in fresh, pre-warmed FreeStyle™ 293 Expression Medium to give a final cell density of 0.1 × 10^6–0.2 × 10^6 viable cells/mL in the desired final volume.
4. Incubate flasks in a 37℃ incubator containing a humidified atmosphere of 8% CO_2 in air on an orbital shaker platform rotating at 135 rpm.
5. Repeat Steps 1 on page 11–4 on page 11 as necessary to maintain or expand cells.

Note: 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.

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). For culture volumes **above 400 mL**, lower the speed of the orbital shaker if foam is generated. In 1 L cultures, we recommend 90 rpm.

Flask Volume	Culture Volume	Manufacturer	Catalog No.
125-mL	25–40 mL	VWR™, Radnor, PA	30180-036
250-mL	50–80 mL	VWR™, Radnor, PA	30180-044
500-mL	100–200 mL	VWR™, Radnor, PA	30180-052
1-L	200–400 mL	VWR™, Radnor, PA	82013-164
3-L	600–1000 mL	Corning™, Acton, MA	431252

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.

Other cell culture systems

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 Thermo Fisher Scientific, the optimum spinner speed was 100–130 rpm and 70–100 rpm impeller speed in Celligen™ stirred tank bioreactors. We recommend seeding cells at 0.3×10^6 –0.5×10^6 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.

Note: 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, Cat. no. 24040) to avoid sheer stress in the culture.

Cryopreservation

Introduction

You may freeze FreeStyle™ 293-F cells directly in FreeStyle™ 293 Expression Medium (50:50 ratio of fresh to conditioned 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 45% fresh FreeStyle™ 293 Expression Medium, 45% conditioned FreeStyle™ 293 Expression Medium and 10% DMSO.

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

Prepare 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

Fresh	0.45 mL
Conditioned	0.45 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.

Freeze 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 0.5×10^6 –1×10^6 viable cells/mL. Transfer™ cells to a sterile, conical centrifuge tube.
2. Determine the viable and total cell counts (see protocol in "Determine cell density and viability" on page 9) 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 × *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 **"Thaw and establish cells" on page 9**.

Transfect cells

Introduction

To transfect suspension FreeStyle™ 293-F cells, you will use the cationic lipid-based transfection reagent, FreeStyle™ MAX Reagent, complex in animal-origin free OptiPRO™ SFM™, while cells stay in FreeStyle™ 293 Expression Medium, which does not inhibit cationic lipid-mediated transfection. FreeStyle™ 293 Expression Medium is formulated to allow high transfection efficiency of suspension FreeStyle™ 293-F cells **without** the need to change media. Transient transfection experiments may be performed in a large volume, allowing large-scale protein production.

FreeStyle™ 293 Expression Medium, FreeStyle™ MAX Reagent and OptiPRO™ SFM™ are available separately from Thermo Fisher Scientific (see "Accessory products" on page 21 for ordering information). For more information, see "FreeStyle™ MAX 293 expression system" on page 7, available at **www.lifetechnologies.com** or call Technical Support (see).

FreeStyle™ MAX reagent

FreeStyle™ MAX Reagent is a proprietary formulation suitable for transfection of DNA into eukaryotic cells grown in suspension and provides the following advantages:

- FreeStyle™ MAX Reagent demonstrates high transfection efficiency with minimal cytotoxicity 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

Plasmid preparation

Plasmid DNA for transfection into eukaryotic cells must be clean, sterile and free from contamination with phenol and NaCl. Contaminants may kill the cells, and salt will interfere with complexing, decreasing transfection efficiency. We recommend isolating DNA using the PureLink™ HiPure Plasmid Kits, which are validated for use with the FreeStyle™ MAX 293 Expression System (see "Accessory products" on page 21).

Note: Make sure your DNA preparation is sterile. We recommend performing filtration before use through a 0.22-µm filter.

Materials needed

- 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 95% viable before proceeding to transfection.
- Purified plasmid DNA of interest (1 mg/mL).
- FreeStyle™ MAX Reagent (store at 4°C until use).
- OptiPRO™ SFM™ to dilute DNA and transfection reagent (pre-warmed to room temperature). See "Accessory products" on page 21 for ordering information.
- FreeStyle™ 293 Expression Medium (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.
- Orbital shaker in 37°C incubator with a humidified atmosphere of 8% CO_2.
- Reagents to determine viable and total cell counts.

FreeStyle™ 293 Expression Medium, OptiPRO™ SFM™ and FreeStyle™ MAX Reagent are available separately from Thermo Fisher Scientific (see "Accessory products" on page 21 for ordering information).

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 (final cell density 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)

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 0.6×10^6–0.7×10^6 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 1.2×10^6–1.5×10^6/mL. Dilute the cells to 1×10^6 cells/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 1–7 days post-transfection, depending on the protein expressed.

Optimize 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 **"Scale up transfections" on page 16** in the following section.

Note: Cells that are transfected with high efficiency grow slower than untransfected cells. This is a good thing.

Scale up transfections

If you transfect suspension FreeStyle™ 293-F cells in a larger volume, consider the following points:

- Scale up the volume of each reagent in proportion to the culture volume.
- For culture volumes **above 40 mL**, lower the speed of the orbital shaker if foam is generated. In 1 L cultures, we recommend 90 rpm.
- 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.
- 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.

Troubleshooting

Culture cells

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

Problem	Cause	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 45% fresh FreeStyle™ 293 Expression Medium, 45% conditioned FreeStyle™ 293 Expression Medium and 10% DMSO.
		Use low-passage cells to make your own stocks.
		Follow procedures in **"Cryopreservation" on page 12**.
		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 properly	See "Subculture cells" on page 11 for proper settings for orbital shaker.
	Cells too diluted	Spin down culture and grow cells in a smaller culture volume.

Problem	Cause	Solution
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 properly	See "Subculture cells" on page 11 for proper settings for orbital shaker.
	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 of cells at recommended densities.
	Cell density too low when passing cells	Spin down cells at 100 × *g* for 5 minutes at room temperature, aspirate media and dilute cells at higher density.
	Cells transfected	Cells transfected at high efficiency grow slower than untransfected cells. This is a desired outcome.

Transfection and protein production

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

Problem	Cause	Solution
Low Transfection Efficiency and/or Low Protein Yield	Cells cultured for too many passages (≥30)	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 **"Subculture cells" on page 11** section.
	Cells transfected in media containing antibiotics	Do not add antibiotics during transfection.
	FreeStyle™ Max™ Reagent handled incorrectly	Store at 4℃. **Do not freeze.**
		Mix gently by inversion. **Do not vortex.**
	Used poor quality expression construct plasmid DNA	**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 SPORTβ- gal to assess your transfection conditions.
		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 "Optimize protein expression" on page 15)
	DNA not sterile	Sterilize DNA (see "Plasmid preparation" on page 14).

Problem	Cause	Solution
Low Transfection Efficiency and/or Low Protein Yield	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 7 post-transfection to identify the peak of protein production, and to monitor cell viability.

Appendix

Accessory products

Additional products

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

Item	Quantity	Catalog no.
FreeStyle™ MAX 293 Expression System	1 kit	K9000-10
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

FreeStyle™ MAX CHO™ expression system

Protein production using the FreeStyle™ MAX Expression Systems can be performed in CHO™ cells or 293 cells. The following table lists ordering information for the reagents specific to the FreeStyle™ MAX CHO™ Expression System. Note that the FreeStyle™ MAX Reagent is used with both the FreeStyle™ MAX 293 and CHO™ Systems.

Item	Quantity	Catalog no.
FreeStyle™ MAX CHO™ Expression System	1 kit	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

References

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

Harrison, T., Graham, F., and Williams, J. (1977) Host-range Mutants of Adenovirus Type 5 Defective for Growth in HeLa Cells. Virology *77*, 319-329

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Safety

WARNING! GENERAL SAFETY. Using this product in a manner not specified in the user documentation may result in personal injury or damage to the instrument or device. Ensure that anyone using this product has received instructions in general safety practices for laboratories and the safety information provided in this document.

- Before using an instrument or device, read and understand the safety information provided in the user documentation provided by the manufacturer of the instrument or device.
- Before handling chemicals, read and understand all applicable Safety Data Sheets (SDSs) and use appropriate personal protective equipment (gloves, gowns, eye protection, and so on). To obtain SDSs, see the "Documentation and Support" section in this document.

Chemical safety

WARNING! GENERAL CHEMICAL HANDLING. To minimize hazards, ensure laboratory personnel read and practice the general safety guidelines for chemical usage, storage, and waste provided below. Consult the relevant SDS for specific precautions and instructions:

- Read and understand the Safety Data Sheets (SDSs) provided by the chemical manufacturer before you store, handle, or work with any chemicals or hazardous materials. To obtain SDSs, see the "Documentation and Support" section in this document.
- Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (for example, safety glasses, gloves, or protective clothing).
- Minimize the inhalation of chemicals. Do not leave chemical containers open. Use only with sufficient ventilation (for example, fume hood).
- Check regularly for chemical leaks or spills. If a leak or spill occurs, follow the manufacturer cleanup procedures as recommended in the SDS.
- Handle chemical wastes in a fume hood.
- Ensure use of primary and secondary waste containers. (A primary waste container holds the immediate waste. A secondary container contains spills or leaks from the primary container. Both containers must be compatible with the waste material and meet federal, state, and local requirements for container storage.)
- After emptying a waste container, seal it with the cap provided.
- Characterize (by analysis if needed) the waste generated by the particular applications, reagents, and substrates used in your laboratory.
- Ensure that the waste is stored, transferred, transported, and disposed of according to all local, state/provincial, and/or national regulations.
- **IMPORTANT!** Radioactive or biohazardous materials may require special handling, and disposal limitations may apply.

WARNING! HAZARDOUS WASTE (from instruments). Waste produced by the instrument is potentially hazardous. Follow the guidelines noted in the preceding General Chemical Handling warning.

WARNING! 4L Reagent and Waste Bottle Safety. Four-liter reagent and waste bottles can crack and leak. Each 4-liter bottle should be secured in a low-density polyethylene safety container with the cover fastened and the handles locked in the upright position.

Biological hazard safety

WARNING! Potential Biohazard. Depending on the samples used on this instrument, the surface may be considered a biohazard. Use appropriate decontamination methods when working with biohazards.

WARNING! BIOHAZARD. Biological samples such as tissues, body fluids, infectious agents, and blood of humans and other animals have the potential to transmit infectious diseases. Conduct all work in properly equipped facilities with the appropriate safety equipment (for example, physical containment devices). Safety equipment can also include items for personal protection, such as gloves, coats, gowns, shoe covers, boots, respirators, face shields, safety glasses, or goggles. Individuals should be trained according to applicable regulatory and company/ institution requirements before working with potentially biohazardous materials. Follow all applicable local, state/provincial, and/or national regulations. The following references provide general guidelines when handling biological samples in laboratory environment.

- U.S. Department of Health and Human Services, *Biosafety in Microbiological and Biomedical Laboratories (BMBL)*, 5th Edition, HHS Publication No. (CDC) 21-1112, Revised December 2009; found at:
  https://www.cdc.gov/labs/pdf/CDC-BiosafetymicrobiologicalBiomedicalLaboratories-2009-P.pdf
- World Health Organization, *Laboratory Biosafety Manual*, 3rd Edition, WHO/CDS/CSR/LYO/2004.11; found at:
  www.who.int/csr/resources/publications/biosafety/Biosafety7.pdf

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    Note: For SDSs for reagents and chemicals from other manufacturers, contact the manufacturer.

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