



## FreeStyle™ 293 Expression Medium

*A serum and protein-free medium specifically developed for the ability to support the growth and transfection of FreeStyle 293-F cells under suspension type culture conditions. FreeStyle 293 SFM is a complete medium, ready to use and is totally free of animal proteins or animal-origin components.*

**Cat. No. 12338-018 FreeStyle 293 Expression Medium 1L**

**Cat. No. 12338-026 FreeStyle 293 Expression Medium 6x1L**

**Storage Conditions: 2-8°C, in the dark**

### Advantages of FreeStyle 293 Expression Medium

- FreeStyle 293 Expression Medium has been specifically developed as a multi-functional medium to support both the suspension growth and the transfection of FreeStyle 293-F cells. FreeStyle 293-F cells are a high-producing clonal variant of 293 cells and are available separately as a catalog item from Invitrogen (see Related Products).
- FreeStyle 293 Expression Medium is ready-to-use and is able to support both suspension cell growth and transfection, requiring significantly less media changes and culture manipulations.
- The unique proprietary formulation of FreeStyle 293 Expression Medium eliminates concerns surrounding the presence of animal proteins or animal-origin components. There are **NO** animal proteins or animal-origin constituents in FreeStyle 293 Expression Medium.
- FreeStyle 293 Expression Medium is prepared ready-to-use, with no supplementation required. An L-Glutamine substitute, Glutamax-1 is present in the medium and is suitable for growth and transfection of all 293 clonal variants thusfar examined.
- FreeStyle 293 Expression Medium is able to save significant time and costs associated with adaptation of cell cultures to serum-free conditions. FreeStyle 293-F cells (available separately, see Related Products) have been pre-adapted to growth under serum-free conditions.

**Advantages to FreeStyle 293-F cells (FreeStyle 293-F cells are available as part of the FreeStyle 293 Expression System Kit or separately as a 2mL cryogenic vial containing 7.5-15x10<sup>6</sup> total cells in 1.5mL of serum-free medium. See Related Products, Cat. No. R790-07)**

- FreeStyle 293-F cells are derived from parental 293-F which were re-cloned by limiting dilution in serum-free medium. The FreeStyle 293-F cells you receive are 30 to 35 total passages post-cloning.
- FreeStyle 293-F cells have been adapted to serum-free growth under suspension culture conditions and may be thawed directly into FreeStyle 293 Expression Medium.
- FreeStyle 293-F cells exhibit superior transfection efficiencies with 293fectin Transfection Reagent available separately (See Related Products).
- FreeStyle 293-F cells are performance tested for viability and cell growth post-recovery from cryopreservation.

**Note:** All components, FreeStyle 293 Expression Medium, FreeStyle 293-F cells, 293fectin Transfection Reagent, a reporter plasmid (pCMV SPORT-βgal) and OptiMEM Complexing Medium are available individually or in kit form. (See FreeStyle 293 Expression System in Related Products). The FreeStyle 293 Expression Protocol Manual is available electronically from the Invitrogen website or through Invitrogen Technical Services.

### INTRODUCTION

The HEK-293 cell line is a permanent cell line originally established from human primary embryonic kidney tissue which was transformed with sheared human adenovirus type 5 DNA.<sup>1,2</sup> 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.

Invitrogen obtained the 293-F cell line from Robert Horlick at Pharmacopoeia. The 293-F parental cell line was adapted to serum-free culture conditions and re-cloned by limiting dilution. Resulting clones were screened and selected for their superior serum-free cell growth and transfection efficiencies.<sup>3</sup> An expansion of the best available clone selected has resulted in a population of cells bearing the FreeStyle 293-F cells designation.

### CELL CULTURE PROCEDURES

#### General Information

Parental 293 cells have historically been grown as monolayers in a serum-supplemented version of a complex medium. The ability to grow these cells under serum-free conditions substantially reduces many downstream purification issues. Serum-free growth also reduces the risks associated with possible contamination by adventitious agents and lot-to-lot inconsistencies due to inherent serum differences. The FreeStyle 293-F clonal variant of 293 cells has been successfully adapted to high-density growth<sup>4</sup> in optimized serum-free medium. Under our experimental conditions FreeStyle 293-F cells normally exhibit doubling times ranging between 20 and 40 hours. FreeStyle 293-F cells demonstrate cell density maxima in excess of 2.0x10<sup>5</sup> viable cells/mL in shaker flask cultures. The prophylactic administration of antibiotics is not recommended and may negatively impact cell growth.

#### Recovery of Cryopreserved FreeStyle 293-F Cells

Cryopreserved FreeStyle 293-F cells are shipped on dry ice and for continued storage should be kept in liquid nitrogen until use.

#### Procedure

- Rapidly thaw frozen vial in a 37°C water bath. Triturate and transfer the entire contents of the cryovial into a T-75cm<sup>2</sup> tissue culture flask containing 17mL of pre-warmed FreeStyle 293 Expression Medium. Incubate flask(s) in a 37°C incubator containing a humidified atmosphere of 8% CO<sub>2</sub> in air. Loosen caps a quarter turn from snug to allow oxygenation.
- Once culture has reached >5x10<sup>5</sup> viable cells/mL (normally, 3 to 5 days) displace the cells from the flask's surface by rapping the flask sharply against your hand. Transfer the cell suspension aseptically into a centrifuge tube and vortex for 10 seconds.
- Determine viable and total cell counts. Note: determine viability (i.e. by trypan blue dye exclusion method) and determine amount of clumping. Vortex for 10 to 30 seconds depending on amount of clumping observed and then determine cell density, either electronically by using a Coulter Counter or by manual means such as with a hemacytometer chamber.
- Expand 293 cultures by seeding additional shaker flasks at 3x10<sup>5</sup> viable cells/mL in pre-warmed FreeStyle 293 Expression Medium.

#### Maintenance of FreeStyle 293 cells in Shake Flasks:

Maintain 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.

The standard flask recommended is the 125 or 250mL polycarbonate disposable sterile Erlenmeyer flask containing a total working volume of 20mL in a 125mL flask or 40mL of cell suspension in a 250mL flask.

#### Subculture of FreeStyle 293 cells in Shake Flasks:

- Determine viable and total counts. (See Procedure step #3 above).
- Seed shaker flasks at 3x10<sup>5</sup> viable cells/mL, diluting cells in pre-warmed FreeStyle 293 medium. Position flasks on the orbital shaker in the incubator with caps loosened a quarter turn from snug to allow for oxygenation.
- Sub-culture every 3 to 4 days.

**Note:** FreeStyle 293 suspension cultures may grow as 2 to 10 cell clusters, therefore vigorous vortexing for 10 to 30 seconds may be required at each subculture for a number of passages until the cultures grow in a more single-cell suspension fashion.

#### Scale-up of FreeStyle 293-F Cells:

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. In our laboratory, the optimum spinner speed was 100 to 130 rpm and 70-100 rpm impeller speed in Celligen™ stirred tank bioreactors. Note: at higher stirring speeds and/or depending on the impeller design, it may be necessary to supplement FreeStyle 293 Expression Medium with additional PLURONIC® F-68 (2.5 to 5mL/L of 10% PLURONIC® F-68, Cat. No. 24040) to avoid shear stress in the culture. Optimal seeding densities are 3 to 5x10<sup>5</sup> viable cells/mL. Note: if the split ratio of cells to fresh media is <1:2, it may be necessary to spin down the cell suspension and resuspend

the cell pellet in fresh FreeStyle 293 Expression Medium prior to inoculation of the spinner or bioreactor culture.

### Cryopreservation of Serum-free Cultures:

#### Procedure:

1. Grow desired quantity of 293 cells in shaker, spinner or bioreactor vessels. Harvest when the cell density is in the range of 0.5 to  $1.0 \times 10^6$  viable cells/mL. Determine viable and total cell counts and calculate the required volume of cryopreservation medium required to yield a final cell density of 5 to  $10 \times 10^6$  viable cells/mL.
2. Prepare the required volume of cryopreservation medium consisting of 90% fresh FreeStyle 293 Expression Medium and DMSO to a final concentration of 10%. Sterile filter and chill the cryopreservation medium at 4°C until use.
3. Centrifuge cells from cell suspension obtained in step 1 at 100xg for 5 to 10 minutes. Aseptically decant supernatant and resuspend cell pellet in the pre-determined volume of chilled cryopreservation medium.
4. Dispense aliquots of this suspension (mixing frequently to maintain a homogeneous cell suspension) into cryovials according to manufacturer's specifications (i.e. 1.5mL in a 2mL cryovial).
5. Achieve cryopreservation in an automated or manually controlled rate freezing apparatus following standard procedures. Quality results are obtained when freezing rates decrease at a rate of 1°C per minute.
6. Following completion of cryopreservation, immediately transfer frozen ampules to liquid nitrogen storage (-125 to -200°C).
7. Viability and recovery of cryopreserved cells should be checked 24 hours after storage of vials in liquid nitrogen and at intervals determined by the user to insure efficacy.

### TRANSFECTION PROCEDURE

The components in the FreeStyle System allow the transient transfection of 293 suspension cultures for expression of recombinant proteins of interest. Follow the procedure below to transfect a suspension of FreeStyle 293-F cells in a 30mL volume. You may keep the cells in Freestyle™ 293 Expression Medium during transfection. We recommend including a positive and negative control in your experiment to help you evaluate your results.

**Note:** For larger- or smaller-scale transfection experiments, scale the volumes of reagents up, or down, accordingly.

1. The day before transfection, determine the number of cells that you will need for your experiment, and expand the cells accordingly. For a 30mL transfection, you will need  $3 \times 10^7$  cells at a final cell density of  $1 \times 10^6$  cells/mL.
2. On the day of transfection, transfer the suspension culture from the culture vessel(s) into as many sterile, conical centrifuge tubes with screw caps as necessary. Centrifuge at 1000 rpm for 5 minutes at room temperature.
3. Resuspend the cell pellet in fresh FreeStyle 293 Expression Medium and vortex the cells vigorously for 10 to 30 seconds to break up cell clumps. **Note:** To achieve a more optimal transfection result, it is better to have a single cell suspension.
4. Count the cells to determine viability and total cell counts.
5. Calculate the volume of cell suspension containing the number of cells needed for transfection. Add the appropriate volume of cell suspension to a sterile 125mL shaker flask and bring the total volume to 30mL with pre-warmed Freestyle 293 Expression Medium.
6. Place the shaker flask in a 37°C incubator with a humidified atmosphere of 8% CO<sub>2</sub> in air on an orbital shaker rotating at 125 rpm.
7. For each transfection sample, prepare the 293fectin-DNA complexes in the following manner:
  - Dilute 30µg of plasmid DNA in OPTI-MEM® I (see Related Products) to a total volume of 1mL. Mix gently.

**Note:** The formation of the DNA-293fectin complex can be inhibited by the ingredients of some media. Opti-MEM I is an excellent support medium for the complexing of DNA and 293fectin and is recommended for use in this regard.

  - Dilute 30µL of 293fectin (see Related Products) in Opti-MEM® I to a total volume of 1mL. Mix gently.
  - Incubate for 5 minutes at room temperature.

**Note:** Once the 293fectin is diluted, combine it with the diluted DNA within 30 minutes. Longer incubation times may result in decreased activity.

- Combine the diluted DNA with the diluted 293fectin to obtain a total volume of 2mL. Mix gently.
  - Incubate for 20-30 minutes at room temperature to allow the DNA-293fectin complexes to form.
8. Add the DNA-293fectin complexes (2mL) to the shaker flask and mix gently.
  9. Incubate the cells in a 37°C incubator with a humidified atmosphere of 8% CO<sub>2</sub> in air on an orbital shaker rotating at 125 rpm.
  10. Remove aliquots at 24, 48, 72, and 96 hours post-transfection to assay for recombinant transgene or protein expression. It is not necessary to remove the complexes or change the medium.

**Note:** Under the conditions described here for 30mL transfections, maximal protein outputs post-transfection were determined to be in the 24 to 48 hour time range. Since the individual parameters and gene products will differ, it is recommended that a time course be conducted to determine production optima. Transfections taking place at volumes other than 30mL should be scaled accordingly.

### References:

1. Graham, F.L., Smiley, J., Russell, W.C. and Nairn, R. (1977) *J. Gen. Virol.* 36,59.
2. Harrison, T., Graham, F., and Williams, J. (1977) *Virology* 77,319
3. Ciccarone, V., Chu, Y., Schifferli, K., Pichet, J.P., Hawley-Nelson, P., Evans, K., Roy, L., and Bennett, S. (1999) *Focus* 21,54
4. Epstein, D.A., Godwin, G.P., Gruber, D.F. and Grefrath, P.I. (1999) *Focus*, 21,22.

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For further information on this or other GIBCO™ products, contact Technical Services at the following:

United States TECH-LINE<sup>SM</sup>: 1 800 955 6288  
Canada TECH-LINE: 1 800 757 8257

Outside the U.S. and Canada, refer to the GIBCO product catalogue for the TECH-LINE in your region.

You may also contact your Invitrogen Sales Representative or our World Wide Web site at [www.invitrogen.com](http://www.invitrogen.com).

**For research use only.  
Not intended for human or animal  
diagnostic or therapeutic uses.**

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### Related Products:

FreeStyle 293 Expression System	K9000-01
• FreeStyle 293 Expression Medium	12338-018
• FreeStyle 293-F Cells	R790-07
• 293fectin Transfection Reagent	12347-019
• OptiMEM Complexing Medium	31985-062
• Reporter gene (pCMV SPORT-βgal)	10586-014
• FreeStyle 293 Expression System Manual	25-0439
GLUTAMAX™-1	35050-061
PLURONIC® F-68 10% Solution	24040-032
GENETICIN®	10131-035