USER GUIDE

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Growth and Maintenance of the Flp-In™ T-REx™ Cell Line

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Contents

Important Information

Shipping/Storage	The Flp-In™ T-REx™-293 cell line is shipped on dry ice. Store in liquid nitrogen upon receipt.
Contents	The Flp-In™ T-REx™-293 cell line is supplied as one vial containing 1×10^7 frozen cells in 1 mL of Freezing Medium.

Introduction

Overview

Introduction

The Flp-In™ T-REx™-293 cell line stably expresses the *lacZ*-Zeocin™ fusion gene and the Tet repressor, and is designed for use with the Flp-In™ T-REx™ System (Cat. no. K6500-01) available from Life Technologies. The cell line expresses the Tet repressor from the pcDNA™6/TR regulatory plasmid and contains a single integrated Flp Recombination Target (FRT) site from pFRT/*lac*Zeo as confirmed by Southern blot analysis. See **Parental Cell Line** and page 6 for information about the generation of the Flp-In™ T-REx™-293 cell line. For more information about the Flp-In™ T-REx™ System and its components, refer to the Flp-In™T-REx™ Core Kit manual, visit www.lifetechnologies.com, or call Technical Support (see page 13). The Flp-In™ T-REx™ Core Kit manual is also available for downloading at www.lifetechnologies.com.

You may use the Flp-In™ T-REx™-293 cell line as a host to generate a tetracycline-inducible Flp-In™ T-REx™ expression cell line by cotransfecting the pcDNA™5/FRT/TO expression vector containing your gene of interest and the Flp recombinase expression plasmid, pOG44 (O'Gorman et al., 1991). Flp recombinase mediates insertion of your pcDNA™5/FRT/TO expression construct into the genome at the integrated FRT site through site-specific DNA recombination (O'Gorman et al., 1991; Sauer, 1994). Once a stable cell line has been generated, expression of your gene of interest can be induced with tetracycline. For more information about FRT sites, Flp recombinase-mediated DNA recombination, and the mechanism of tetracycline regulation in the Flp-In™ T-REx™ System refer to the Flp-In™ T-REx™ Core Kit manual.

Parental Cell Line

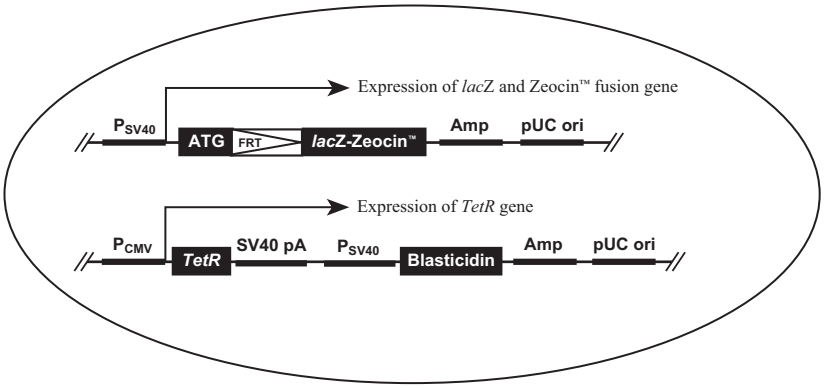
The Flp-In™ T-REx™-293 cell line is derived from 293 human embryonic kidney cells (Graham et al., 1977). The 293 parental cell line was obtained from the American Type Culture Collection (ATCC). For more information about the 293 parental cell line, see the ATCC website (www.atcc.org) and refer to ATCC number CRL-1573.

Generation of the Flp-In™ T-REx™-293 Cell Line

The Flp-In™ T-REx™-293 cell line contains two stably, independently integrated plasmids which exhibit the following features:

- The pFRT/*lac*Zeo plasmid introduces a single FRT site into the genome and stably expresses the *lacZ*-Zeocin™ fusion gene under the control of the SV40 early promoter (see the following diagram). The location of the FRT site in the Flp-In™ T-REx™-293 cell line has not been mapped, but is presumed to have integrated into a transcriptionally active genomic locus as determined by generation of a Flp-In™ T-REx™-293 expression cell line containing the pcDNA™5/FRT/TO/CAT control plasmid.
- The pcDNA™6/TR plasmid stably expresses the Tet repressor gene under the control of the constitutive human cytomegalovirus (CMV) immediate-early enhancer/ promoter (Andersson et al., 1989; Boshart et al., 1985; Nelson et al., 1987).

For more information about pFRT/*lac*Zeo, pcDNA™6/TR, and pcDNA™5/FRT/TO/CAT plasmids, refer to the Flp-In™ T-REx™ Core Kit manual.



Flp-In™ T-REx™-293 Cell Line

Media for Cell Line

The following table provides the recommended complete medium, freezing medium, and antibiotic concentration required to maintain and culture the Flp-In™ T-REx™-293 cell line.

Complete Medium	[Antibiotic]	Freezing Medium
D-MEM (high glucose) 10% FBS 2 mM L-glutamine 1% Pen-Strep (optional)	100 µg/mL Zeocin™ 15 µg/mL blasticidin	90% complete medium 10% DMSO

Important Guidelines

Consider the following when working with Flp-In™ T-REx™-293 cells:

- FBS does not need to be heat inactivated for use with this cell line.
- The cell line should be maintained in medium containing Zeocin™ selective reagent and blasticidin at the concentrations listed.
- If cells are split at a 1:5 to 1:10 dilution, they will generally reach 80–90% confluence in 3–4 days.

Methods

Culturing the Flp-In™ T-REx™-293 Cell Line

General Cell Handling

Follow the guidelines provided to successfully grow and maintain your 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 and also have some frozen stocks on hand. We recommend that you always use early-passage cells for your experiments. Upon receipt of the cells from Life Technologies, grow and freeze multiple vials of the cell line to ensure that you have any adequate supply of early-passage cells.
- Cells should be at the appropriate confluence (approximately 60%) and >90% viability prior to transfection (see page 6).
- For general maintenance of the cell line, pass the cells when they are 80–90% confluent (3–4 days if split at a 1:5 to 1:10 dilution).
- Use trypan blue exclusion to determine cell viability. Log phase cultures should be >90% viable.

Before Starting

Be sure to have the following solutions and supplies available:

- 15-mL sterile, conical tubes
- 5-, 10-, and 25-mL sterile pipettes
- Cryovials
- Phosphate-Buffered Saline (PBS) (see **Recipes**, page 12)
- 0.4% Trypan blue in PBS and hemacytometer (for counting cells)
- Reagents to prepare complete medium
- Freezing Medium (see pages 6 and 9)
- Table-top centrifuge
- 75-cm^2 flasks, 175-cm^2 flasks and other appropriately-sized tissue culture flasks or plates
- Trypsin/versene (EDTA) solution or other trypsin solution

Thawing Cells

The following protocol is designed to help you thaw cells to initiate cell culture. The Flp-In™ T-REx™-293 cell line is supplied in a vial containing 3×10^7 cells in 1 mL of Freezing Medium.

1. Remove the vial of cells from the liquid nitrogen and thaw quickly at 37°C.
2. Just before the cells are completely thawed, decontaminate the outside of the vial with 70% ethanol, and transfer the cells to a T-75 flask containing 12 mL of complete medium without Zeocin™ selective reagent and blasticidin.
3. Incubate the flask at 37°C for 2–4 hours to allow the cells to attach to the bottom of the flask.
4. Aspirate off the medium and replace with 12 mL of fresh, complete medium without Zeocin™ selective reagent and blasticidin.
5. Incubate cells overnight at 37°C.
6. The next day, aspirate off the medium and replace with fresh, complete medium containing Zeocin™ selective reagent and blasticidin (at the recommended concentrations listed on page 6).
7. Incubate the cells and check them daily until the cells are 80–90% confluent (2–7 days).
8. Proceed to **Passaging the Cells**.

Passaging the Cells

1. When cells are ~80–90% confluent, remove all medium from the flask.
2. Wash cells once with 10 mL PBS to remove excess medium and serum. Serum contains inhibitors of trypsin.
3. Add 5 mL of trypsin/versene (EDTA) solution to the monolayer and incubate 1–5 minutes at room temperature until cells detach. Check the cells under a microscope and confirm that most of the cells have detached. If cells are still attached, incubate a little longer until most of the cells have detached.
4. Once the cells have detached, briefly pipet the solution up and down to break up clumps of cells.
5. Add 5 mL of complete medium to stop trypsinization.
6. To maintain cells in 75-cm^2 flasks, transfer 1 mL of the 10 mL cell suspension from Step 5 to a new 75-cm^2 flask and add 15 mL fresh, complete medium containing Zeocin™ selective reagent and blasticidin.

   Note: If you want the cells to reach confluency sooner, split the cells at a lower dilution (i.e., 1:4).
7. To expand cells, add 28 mL of fresh, complete medium containing Zeocin™ selective reagent and blasticidin to each of three 175-cm^2 flasks, then transfer 2 mL of the cell suspension to each flask to obtain a total volume of 30 mL.
8. Incubate flasks in a humidified, 37°C, 5% CO_2 incubator.
9. Repeat Steps 1–8 as necessary to maintain or expand cells.

Freezing Cells

Introduction

When freezing the Flp-In™ T-REx™-293 cell line, we recommend the following:

- Freeze cells at a density of **at least** 3×10^6 cells/mL.
- Use a freezing medium composed of 90% complete medium and 10% DMSO. Complete medium is medium containing serum.

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

Preparing Freezing Medium

Freezing medium should be prepared fresh immediately before use.

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

Fresh complete medium	0.9 mL
DMSO	0.1 mL

2. Place the tube on ice. Discard any remaining freezing medium after use.

Freezing the Cells

Cells should be approximately 80% confluent at the time of freezing. Before starting, label cryovials and prepare freezing medium (see above). Keep the freezing medium on ice.

1. Remove the medium and wash the cells once with PBS (10 mL for a 175 cm^2 flask).
2. Add trypsin/versene (EDTA) solution (5 mL for a 175 cm^2 flask) and incubate for 1–5 minutes until cells detach.
3. Once cells have detached, briefly pipet solution up and down to break up clumps of cells.
4. Add 5 mL of complete medium to stop trypsinization. Count the cells.
5. Pellet cells at $250 \times g$ for 5 minutes in a table top centrifuge at room temperature and carefully aspirate off the medium.
6. Resuspend the cells at a density of **at least** 3×10^6 cells/mL in chilled freezing medium.
7. Place vials in a microcentrifuge rack and aliquot 1 mL of the cell suspension into each cryovial.
8. 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.
9. Transfer 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 Cells**, page 8.

Transfection

Transfection Methods

Flp-In™ T-REx™-293 cells are generally amenable to transfection using standard methods including calcium phosphate precipitation (Chen and Okayama, 1987; Wigler et al., 1977), lipid-mediated transfection (Felgner et al., 1989; Felgner and Ringold, 1989), and electroporation (Chu et al., 1987; Shigekawa and Dower, 1988). We typically use Lipofectamine® 2000 Reagent to transfect the Flp-In™ T-REx™-293 cells. Other transfection reagents may be suitable.

Note: Lipofectamine® 2000 Reagent (Cat. no. 11668-027) is available from Life Technologies.

Generation of Stable Expression Cell Lines

Stable Flp-In™ T-REx™-293 expression cell lines can be generated by cotransfection of your pcDNA™5/FRT/TO expression construct and the pOG44 plasmid. Stable transfectants are selected using hygromycin B. Before transfection, you may want to test the sensitivity of the Flp-In™ T-REx™-293 cell line to hygromycin B to more accurately determine the hygromycin B concentration to use for selection. We generally use 100–200 µg/mL hygromycin B to select for the pcDNA™5/FRT/TO expression vector. For more information, refer to the Flp-In™ T-REx™ Core Kit manual. Hygromycin B may be obtained from Life Technologies (see page 13 for ordering information).

Important: Following cotransfection, your Flp-In™ T-REx™-293 expression clones should become sensitive to Zeocin™; therefore, your selection medium should not contain Zeocin™. Your selection medium should still contain 15 µg/mL blasticidin to select for the pcDNA™6/TR plasmid.

Polyclonal Selection of Isogenic Cell Lines

Because the Flp-In™ T-REx™-293 cells contain a single integrated FRT site, all of the hygromycin-resistant foci that you obtain after cotransfection with the pcDNA™5/FRT/TO expression vector and pOG44 should be isogenic (i.e., the pcDNA™5/FRT/TO expression vector should integrate into the same genomic locus in every clone; therefore, all clones should be identical). To obtain stable expression cell lines, you may perform "polyclonal" selection and screening of your hygromycin-resistant cells. After hygromycin selection, simply pool the hygromycin-resistant foci and screen the entire population of cells for the following phenotypes:

- Zeocin™ antibiotic sensitivity
- Lack of β-galactosidase activity
- Blastidicin resistance
- Tetracycline-regulated gene expression

Selection of Individual Cell Lines

If desired, single hygromycin-resistant, blasticidin-resistant foci can be isolated and expanded to generate individual clonal cell lines. To isolate individual clones, simply pick 5–20 hygromycin-resistant, blasticidin-resistant foci and expand the cells. You may verify that your pcDNA™5/FRT/TO expression construct has integrated into the FRT site by testing each clone for Zeocin™ selective reagent sensitivity and lack of β-galactosidase activity. The proper clones should exhibit the following phenotypes:

- Hygromycin resistance
- Zeocin™ antibiotic sensitivity
- Lack of β-galactosidase activity
- Blastidicin resistance
- Tetracycline-regulated gene expression

Note

In rare instances, it is possible to generate a Flp-In™ T-REx™-293 expression cell line in which the pcDNA™5/FRT/TO plasmid has undergone both Flp recombinase-mediated integration into the FRT site and random integration into a second genomic site. In this case, clones will still exhibit hygromycin resistance. To test for these "second site integrants", transfect the cells with the pOG44 plasmid and select for Zeocin™ selective reagent resistance. The Flp recombinase should mediate excision of the Flp-In™ expression plasmid at the FRT site and restore the *lacZ*-Zeocin™ fusion gene. The resulting cells should exhibit:

- β-galactosidase activity
- Zeocin™ antibiotic resistance
- Blasticidin resistance

Alternatively, you may perform Southern blot analysis to identify second site integrants if suitable restriction enzymes are selected.

Tetracycline Induction

Once you have generated your Flp-In™ T-REx™-293 expression cell line, you will induce expression of the gene of interest with tetracycline. We generally add tetracycline to a final concentration of 1 µg/mL and incubate the cells for 24 hours at 37ºC before harvesting. Since expression conditions may vary depending upon the nature of your protein, we recommend that you perform a time course of tetracycline induction to optimize expression of your protein.

For more detailed protocols and guidelines to prepare tetracycline and induce expression of your protein of interest, refer to the Flp-In™ T-REx™ Core Kit manual.

Appendix

Recipes

Phosphate-Buffered Saline (PBS)

For washing cells only.

137 mM NaCl
2.7 mM KCl
10 mM Na_2HPO_4
1.8 mM KH_2PO_4

1. Dissolve the following in 800 mL deionized water:

   8 g NaCl
   0.2 g KCl
   1.44 g Na_2HPO_4
   0.24 g KH_2PO_4

2. Adjust pH to 7.4 with concentrated HCl.
3. Bring the volume to 1 liter and autoclave for 20 minutes on liquid cycle.
4. Store at 4°C or room temperature.

Accessory Products

Flp-In™ T-REx™ Products

The plasmids required to generate Flp-In™ T-REx™ host cell lines and expression cell lines are available separately from Life Technologies. The pcDNA™5/FRT/TO vector is also available adapted with topoisomerase I (pcDNA™5/FRT/TO-TOPO® TA Expression Kit) to facilitate rapid cloning of *Taq*-amplified PCR products. For more information about the features of each vector, visit www.lifetechnologies.com or call Technical Support (see page 13). Ordering information is provided in the following table.

Product	Amount	Catalog No.
pFRT/*lac*Zeo	20 µg	V6015-20
pFRT/*lac*Zeo2	20 µg	V6022-20
pcDNA™6/TR	20 µg	V1025-20
pOG44	20 µg	V6005-20
pcDNA™5/FRT/TO Vector Kit	20 µg	V6520-20
pcDNA™5/FRT/TO TOPO® TA Expression Kit	20 reactions	K6510-20

Selection and Induction Agents

The selection agents needed for maintenance and growth of the Flp-In™ T-REx™-293 cell line are available separately from Life Technologies (see the following table). Hygromycin B is also available from Life Technologies for selection of your Flp-In™ T-REx™ expression construct after cotransfection with the pOG44 plasmid into Flp-In™ T-REx™ host cell lines. Tetracycline is available to induce expression of your gene of interest after generation of your Flp-In™ T-REx™ expression cell line. For additional information about these selection and induction agents, see the Flp-In™ T-REx™ Core Kit manual or visit www.lifetechnologies.com.

Antibiotic	Amount	Catalog No.
Zeocin™ Selection Reagent	1 g 5 g	R250-01 R250-05
Blasticidin S HCl	50 mg	R210-01
Hygromycin B	1 g	R220-05
Tetracycline	5 g	Q100-19

Cell Culture Reagents

Gibco™ cell culture products are available from Life Technologies to facilitate growth and maintenance of the Flp-In™ T-REx™-293 cell line. Ordering information is provided in the following table.

Product	Amount	Catalog No.
Dulbecco's Modified Eagle Medium (D-MEM)	500 mL	11965-092
Fetal Bovine Serum	500 mL	16000-044
200 mM L-Glutamine	100 mL	25030-081
Penicillin-Streptomycin	100 mL	15070-063

Technical Support

Obtaining Support

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
- Search through frequently asked questions (FAQs)
- Submit a question directly to Technical Support (**techsupport@lifetech.com**)
- Search for user documents, SDSs, vector maps and sequences, application notes, formulations, handbooks, certificates of analysis, citations, and other product support documents
- Obtain information about customer training
- Download software updates and patches

Safety Data Sheets (SDS)

Safety Data Sheets (SDSs) are available at **www.lifetechnologies.com/support**.

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Introduction

Use of the Flp-In™ T-REx™ System and its components ("System") is covered under a number of different licenses including those detailed in the following sections.

Information for European Customers

The Flp-In™ T-REx™-293 cell line is genetically modified and carries the pUC-derived plasmids, pFRT/*lac*Zeo and pcDNA™6/TR. 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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Purchaser Notification, Continued

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- any use of the System or Expression Products in the manufacture of a Commercial Product;
- any sale of the System or Expression Products;
- any use of the System or Expression Products to facilitate or advance research or development of a Commercial Product; and
- any use of the System or Expression Products to facilitate or advance any research or development program the results of which will be applied to the development of a Commercial Product.

"Expression Products" means products expressed with the System, or with the use of any vectors or host strains in the System. "Commercial Product" means any product intended for sale or commercial use.

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Access to the System must be limited solely to those officers, employees and students of your entity who need access to perform the aforementioned research. Each such officer, employee and student must be informed of these terms and conditions and agree, in writing, to be bound by same. You may not distribute the System or the vectors or host strains contained in it to others. You may not transfer modified, altered, or original material from the System to a third party without written notification to, and written approval from Life Technologies. You may not assign, sub-license, rent, lease or otherwise transfer any of the rights or obligations set forth herein, except as expressly permitted by Life Technologies.

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Zeocin™

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Life Technologies Corporation
5791 Van Allen Way
Carlsbad, CA 92008
Phone: 760-603-7200
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