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Growth and maintenance of the Flp-In™ T-REx™ cell line

USER GUIDE

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Growth and maintenance of the Flp-In™ T-REx™ cell line

Important information

Shipping/Storage

The Flp-In™ T-REx™-293 cell line is shipped on dry ice. Store in liquid nitrogen vapor-phase 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.

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 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" on page 6 and "Generation of the Flp-In™ T-REx™-293 cell line" on page 7 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). 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

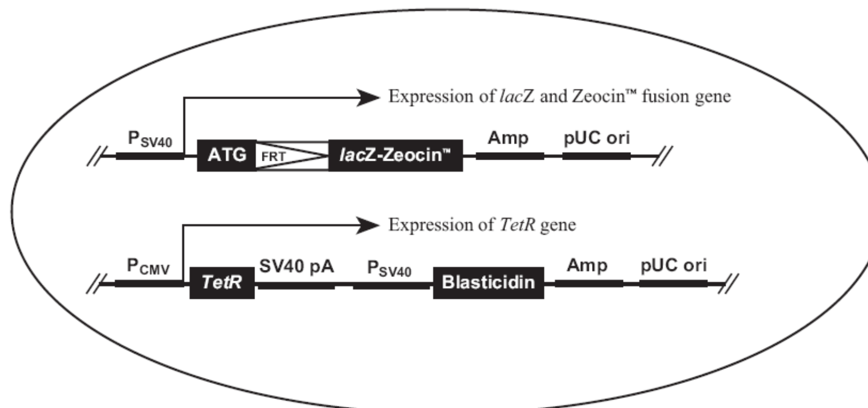
The Flp-In™ T-REx™-293 cell line is derived from 293 human embryonic kidney cells (Graham et al., 1977). The 293 parental cell line was obtained from the American Type Culture Collection (ATCC®). For more information about the 293 parental cell line, see the ATCC® website (**www.atcc.org**) and refer 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 "Generation of the Flp-In™ T-REx™-293 cell line" on page 7).
- 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" on page 15)
- 0.4% Trypan blue in PBS and hemacytometer (for counting cells)
- Reagents to prepare complete medium
- Freezing Medium (see "Media for cell line" on page 7 and "Freezing cells" on page 11)
- 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 in “Media for cell line” on page 7).
7. Incubate the cells and check them daily until the cells are 80–90% confluent (2–7 days).
8. Proceed to “Passaging the cells” on page 10.

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 on page 10 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 on page 10–8 on page 11 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 × *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℃ per minute.

9. Transfer™ vials to liquid nitrogen vapor-phase for long-term storage.

   Note: You may check the viability and recovery of frozen cells 24 hours after storing cryovials in liquid nitrogen vapor-phase by following the procedure outlined in "Thawing cells" on page 10.

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 "Selection and induction agents" on page 16 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 37℃ 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℃ 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). 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

Product	Amount	Catalog No.
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

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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

Documentation and support

Customer and technical support

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- Product documentation
  - User guides, manuals, and protocols
  - Certificates of Analysis
  - Safety Data Sheets (SDSs; also known as MSDSs)

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

Limited product warranty

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