USER GUIDE

invitrogen™
by life technologies™

Growth and Maintenance of Flp-In™ Cell Lines

Catalog Numbers R750-07, R752-07, R758-07, R760-07, R761-07, R762-07

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Contents

Product information

Contents and storage

Shipping and storage

All cell lines are shipped on dry ice. Store in liquid nitrogen upon receipt.

Contents

This manual is supplied with the following cell lines:

Cell Line	Catalog no.
Flp-In™-293	R750-07
Flp-In™-CV-1	R752-07
Flp-In™-CHO	R758-07
Flp-In™-BHK	R760-07
Flp-In™-3T3	R761-07
Flp-In™-Jurkat	R762-07

All cell lines are supplied as one vial containing 1×10^7 frozen cells in 1 mL of Freezing Medium.

Description of the system

Introduction

The Flp-In™ cell lines stably express the *lacZ*-Zeocin™ fusion gene and are designed for use with the Flp-In™ System (Cat. nos. K6010-01 and K6010-02). Each cell line contains a single integrated Flp Recombination Target (FRT) site from pFRT/*lac*Zeo or pFRT/*lac*Zeo2 as confirmed by Southern blot analysis. See **Parental cell lines** and page 6 for information about the generation of the Flp-In™ cell lines. For more information about the Flp-In™ System and its components, refer to the Flp-In™ System manual, visit **www.lifetechnologies.com**, or contact Technical Support (see page 17). The Flp-In™ System manual is also available from our website.

Generation of Flp-In™ expression cell lines requires cotransfection of the Flp-In™ cell line with a Flp-In™ 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 Flp-In™ expression construct into the genome at the integrated FRT site through site-specific DNA recombination (O'Gorman et al., 1991; Sauer, 1994). Stable cell lines expressing your gene of interest from the Flp-In™ expression vector can be generated by selection using hygromycin B. For more information about FRT sites and Flp recombinase-mediated DNA recombination, refer to the Flp-In™ System manual.

Parental cell lines

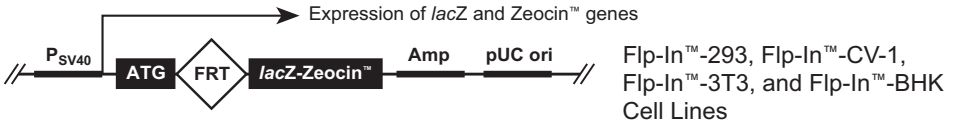
The table below provides a brief description of the source of the parental cell line used to generate each Flp-In™ cell line. The parental cell lines were obtained from the American Type Culture Collection (ATCC). The ATCC number for each cell line is included. For further information about the parental cell lines, refer to the ATCC website (**www.atcc.org**).

Cell Line	Characteristic	Source	ATCC Number
293	Adherent	Human embryonic kidney (Graham et al., 1977)	CRL-1573
CV-1	Adherent	African Green Monkey kidney (Kit et al., 1965)	CCL-70
CHO-K1	Adherent	Chinese Hamster ovary (Kao and Puck, 1968)	CCL-61
BHK	Adherent	Baby hamster kidney (Talavera and Basilico, 1977)	CCL-10
NIH/3T3	Adherent	Mouse (NIH Swiss) embryonic fibroblast (Jainchill et al., 1969)	CRL-1658
Jurkat	Suspension	Human T-cell leukemia (Weiss et al., 1984)	TIB-152

Description of Flp-In™ cell lines

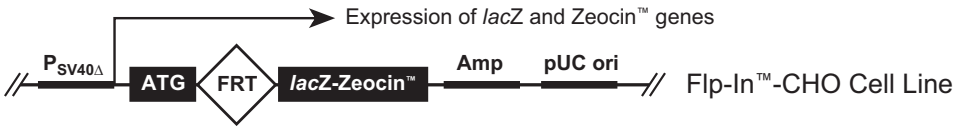
All of the Flp-In™ cell lines (except Flp-In™-CHO; see the following section) contain a single integrated FRT site and stably express the *lacZ*-Zeocin™ fusion gene from the pFRT/*lac*Zeo plasmid under the control of the SV40 early promoter (see the following diagram). The location of the FRT site in each Flp-In™ 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™ expression cell line containing the pcDNA™5/FRT/CAT or pEF5/FRT/GW-CAT control plasmid. The Flp-In™ cell lines should be maintained in medium containing Zeocin™ Selection Antibiotic (see **Media for cell lines,** page 7).

For more information about pFRT/*lac*Zeo, refer to the Flp-In™ System manual. For information about pcDNA™5/FRT/CAT or pEF5/FRT/GW-CAT, refer to the pcDNA™5/FRT or pEF5/FRT/V5-DEST™ manuals, respectively.



Flp-In™-CHO cell line

The Flp-In™-CHO cell line contains a single integrated FRT site and stably expresses the *lacZ*-Zeocin™ fusion gene from the pFRT/*lac*Zeo2 plasmid. Note that pFRT/*lac*Zeo2 contains a mutated SV40 early promoter ($P_{SV40\Delta}$) which is severely abrogated in its activity. The SV40Δ early promoter in pFRT/*lac*Zeo2 exhibits approximately 60-fold less activity than the wild-type SV40 early promoter in pFRT/*lac*Zeo. Because of the minimal activity of the SV40Δ promoter, we expect that stable transfectants expressing the *lacZ*-Zeocin™ gene from pFRT/*lac*Zeo2 should contain FRT sites which have integrated into the most transcriptionally active genomic loci. The location of the FRT site in the Flp-In™-CHO cell line has not been mapped, but has been demonstrated to have integrated into a highly transcriptionally active genomic locus as determined by generation of a Flp-In™ expression cell line containing the pcDNA™5/FRT/luc (luciferase-expressing) control plasmid. The Flp-In™-CHO cell line should be maintained in medium containing Zeocin™ Selection Antibiotic (see **Media for cell lines,** page 7). For more information about pFRT/*lac*Zeo2 and the SV40Δ early promoter, refer to the pFRT/*lac*Zeo2 manual.



Media for cell lines

The following table lists the recommended complete medium, freezing medium, and antibiotic concentration required to maintain and culture each Flp-In™ cell line.

Cell Line	Complete Medium	[Antibiotic]	Freezing Medium
Flp-In™-293	D-MEM (high glucose) 10% FBS* 2 mM L-glutamine 1% Pen-Strep (optional)	100 µg/mL Zeocin™ Selection Antibiotic	90% complete medium 10% DMSO
Flp-In™-CV-1	D-MEM (high glucose) 10% FBS* 2 mM L-glutamine 1% Pen-Strep (optional)	100 µg/mL Zeocin™ Selection Antibiotic	90% complete medium 10% DMSO
Flp-In™-CHO	Ham's F12 10% FBS* 2 mM L-glutamine 1% Pen-Strep (optional)	100 µg/mL Zeocin™ Selection Antibiotic	90% complete medium 10% DMSO
Flp-In™-BHK	D-MEM (high glucose) 10% FBS* 2 mM L-glutamine 1% Pen-Strep (optional)	100 µg/mL Zeocin™ Selection Antibiotic	90% complete medium 10% DMSO
Flp-In™-3T3	D-MEM (high glucose) 10% donor calf serum 2 mM L-glutamine 1% Pen-Strep (optional)	100 µg/mL Zeocin™ Selection Antibiotic	90% complete medium 10% DMSO
Flp-In™-Jurkat	RPMI 1640 10% FBS* 2 mM L-glutamine 1% Pen-Strep (optional)	100 µg/mL Zeocin™ Selection Antibiotic	90% complete medium 10% DMSO

*FBS = fetal bovine serum

Important guidelines

- FBS does not need to be heat inactivated for use with these cell lines.
- Cell lines should be maintained in medium containing Zeocin™ Selection Antibiotic at the concentrations listed in the previous section.
- If adherent cells (e.g., Flp-In™-293, Flp-In™-CV-1, Flp-In™-CHO, Flp-In™-3T3, Flp-In™-BHK) are split at a 1:5 to 1:10 dilution, they will generally reach 80–90% confluence in 3–4 days.
- Suspension Flp-In™-Jurkat cells will demonstrate optimal growth characteristics if maintained at a cell density between 1×10^5 cells/mL and 1×10^6 cells/mL.
- When maintaining Flp-In™-Jurkat cells in suspension culture, do not allow the medium to turn yellow; this indicates that cells have reached too high a density or that the medium is depleted of nutrients. If this occurs, either add fresh complete media to the cells or passage them.

Methods

Culturing Flp-In™ cell lines

General cell handling

Follow the guidelines below 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 particular 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 12).
- For general maintenance of cells, pass all cell lines when they are 80–90% confluent (for adherent cells) or when they reach a density of $2–4 \times 10^6$ cells/mL (for suspension cells).
- 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 (see page 15 for ordering information)
- 0.4% Trypan blue in PBS and hemacytometer (for counting cells)
- Reagents to prepare the appropriate complete medium (see page 7)
- Freezing Medium (see pages 7 and 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 adherent cells

The following protocol is designed to help you thaw adherent cells to initiate cell culture. All cell lines are supplied in vials containing 1×10^7 cells in 1 mL of Freezing Medium.

1. Remove the vial 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, and transfer the cells to a T-75 flask containing 12 mL of complete medium without Zeocin™ Selection Antibiotic.
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™ Selection Antibiotic.
5. Incubate cells overnight at 37°C.
6. The next day, aspirate off the medium and replace with fresh, complete medium containing Zeocin™ Selection Antibiotic (at the recommended concentration listed 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 adherent cells**, page 10.

Thawing suspension cells

The following protocol is designed to help you thaw suspension cells to initiate cell culture. All cell lines are supplied in vials containing 1×10^7 cells in 1 mL of Freezing Medium.

1. Remove the vial 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, and transfer the cells to a sterile, conical tube containing 5 mL of complete medium without Zeocin™ Selection Antibiotic.
3. Centrifuge for 3 minutes at 750 × *g* at room temperature.
4. Aspirate off the medium and resuspend the cells in 12 mL of fresh, complete medium without Zeocin™ Selection Antibiotic.
5. Transfer the cells to a T-75 flask and incubate cells overnight at 37°C.
6. The next day, add Zeocin™ Selection Antibiotic to the cells (at the recommended concentration listed on page 7).
7. Incubate the cells and count them daily until the cells reach a density ranging from $2–4 \times 10^6$ cells/mL (2–7 days).

   Note: You may add fresh, complete medium containing Zeocin™ Selection Antibiotic to the cells every few days.
8. Proceed to **Passaging suspension cells**, page 10.

Passaging adherent 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. Add 5 mL of complete medium to stop trypsinization.
5. Briefly pipet the solution up and down to break up clumps of cells.
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 containing Zeocin™ Selection Antibiotic. If you want the cells to reach confluency sooner, split the cells at a lower dilution (e.g., 1:4).

   Note: To expand cells into 175-cm^2 flasks, add 28 mL of fresh, complete medium containing Zeocin™ Selection Antibiotic 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.
7. Incubate flasks in a humidified, 37°C, 5% CO_2 incubator.
8. Repeat Steps 1–7 as necessary to maintain or expand cells.

Passaging suspension cells

1. Passage suspension cells when they reach a density of 2–4 × 10^6 cells/mL.
2. To maintain cells in 75-cm^2 flasks, transfer 1–1.5 mL of cell suspension from Step 1 to a new 75-cm^2 flask containing 13–14 mL of fresh, complete medium with Zeocin™ Selection Antibiotic.

   Note: You may split the cells at a lower dilution (e.g., 1:4), if desired.
3. To expand cells into 175-cm^2 flasks, add 28 mL of fresh, complete medium containing Zeocin™ Selection Antibiotic 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.

   Note: You may also expand cells into a spinner flask, if desired.
4. Incubate flasks in a humidified, 37°C, CO_2 incubator.
5. Repeat Steps 1–4 as necessary to maintain or expand cells.

Freezing cells

Introduction

When freezing the Flp-In™ cell lines, 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

Before starting, label cryovials and prepare freezing medium (see **Preparing freezing medium**). Keep the freezing medium on ice.

1. To collect cells, perform the following:
   - For adherent cells, follow Steps 1–5 of **Passaging adherent cells**, page 10.
   - For suspension cells, transfer cells to a sterile, conical centrifuge tube.
2. Count the cells.
3. Pellet cells at $250 \times g$ for 5 minutes in a table top centrifuge at room temperature and carefully aspirate off the medium.
4. Resuspend the cells at a density of **at least** 3×10^6 cells/mL in chilled freezing medium.
5. Place vials 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 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 adherent cells** or **Thawing suspension cells**, page 9, as appropriate.

Transfecting cells

Introduction

To generate stable Flp-In™ expression cell lines, you will cotransfect your Flp-In™ expression construct and the pOG44 plasmid into the Flp-In™ cell line and select for stable transfectants using hygromycin B. General guidelines and recommendations for transfection are provided in this section. We recommend that you read through this section before beginning.

Transfection methods

The Flp-In™ cell lines 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). Although other transfection reagents may be suitable, we recommended using Lipofectamine® 2000 Reagent (see page 15 for ordering information) to introduce Flp-In™ expression constructs into the following Flp-In™ cell lines:

- Flp-In™-293
- Flp-In™-CV-1
- Flp-In™-CHO
- Flp-In™-Jurkat
- Flp-In™-3T3
- Flp-In™-BHK

Important

We have observed down-regulation of the viral CMV promoter and subsequent loss of gene expression when pcDNA™5/FRT-based expression constructs are introduced into Flp-In™-3T3 or Flp-In™-BHK cells. This behavior is not observed with pEF5/FRT-based expression constructs. If you are generating Flp-In™ expression cell lines using the Flp-In™-3T3 or Flp-In™-BHK cell line, we recommend that you express your gene of interest from a pEF5/FRT-based plasmid (e.g., pEF5/FRT/V5-DEST™).

Generating stable expression cell lines

To generate Flp-In™ expression cell lines, cotransfect your Flp-In™ expression construct and the pOG44 plasmid into the Flp-In™ cell line of choice, and select for stable transfectants using hygromycin B. Before transfection, you may want to test the sensitivity of the Flp-In™ cell line to hygromycin B to more accurately determine the hygromycin B concentration to use for selection. A suggested range of hygromycin B concentrations to use for selection of your Flp-In™ expression vector is listed below. For more information, refer to the Flp-In™ System manual. Hygromycin B may be obtained from Life Technologies (see page 15 for ordering information).

Important: Following cotransfection, your Flp-In™ expression clones should become sensitive to Zeocin™ Selection Antibiotic; therefore, your selection medium should NOT contain Zeocin™ Selection Antibiotic.

Cell Line (After Transfection with Flp-In™ Expression Vector)	Estimated Hygromycin B Concentration (µg/mL)
Flp-In™-293	100–200
Flp-In™-CV-1	100–200
Flp-In™-CHO	500–600
Flp-In™-BHK	100–200
Flp-In™-3T3	100–200
Flp-In™-Jurkat	200–400

Note

When transfecting Flp-In™-CHO cells, we recommend following these guidelines:

- 48 hours after transfection, split the cells directly into medium containing the appropriate concentration of hygromycin B.
- Split the cells such that they are no more than 25% confluent. If the cells are too dense, the antibiotic will not kill the cells. Antibiotics work best on actively dividing cells.

Polyclonal selection of isogenic cell lines

Because every Flp-In™ cell line contains a single integrated FRT site, all of the hygromycin-resistant foci that you obtain after cotransfection with the Flp-In™ expression vector and pOG44 should be isogenic (i.e., the Flp-In™ 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 B selection, simply pool the hygromycin-resistant foci and screen the entire population of cells for the following phenotypes:

- Zeocin™ Selection Antibiotic sensitivity
- Lack of β-galactosidase activity
- Expression of the gene of interest

Select individual cell lines

If desired, single hygromycin-resistant foci can be isolated and expanded to generate individual clonal cell lines. To isolate individual clones, simply pick 5–20 hygromycin-resistant foci and expand the cells. You may verify that your Flp-In™ expression construct has integrated into the FRT site by testing each clone for Zeocin™ Selection Antibiotic sensitivity and lack of β-galactosidase activity. Select those clones that are hygromycin-resistant, Zeocin™ Selection Antibiotic-sensitive, and lack β-galactosidase activity, and assay for expression of your gene of interest.

Note

Note that in rare instances, it is possible to generate a Flp-In™ expression cell line in which the Flp-In™ expression 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 B resistance. To test for these "second site integrants", transfect the cells with the pOG44 plasmid and select for Zeocin™ Selection Antibiotic 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™ Selection Antibiotic resistance, and continued expression of the gene of interest. Alternatively, you may perform Southern blot analysis to identify second site integrants if suitable restriction enzymes are selected.

Appendix A: Ordering information

Accessory products

Introduction

The products listed in this section are intended for use with the Flp-In™ Cell Lines and the Flp-In™ System. For more information, refer to **www.lifetechnologies.com** or contact Technical Support (see page 17).

Cell culture reagents

A large variety of Gibco™ cell culture products are available from Life Technologies to facilitate growth and maintenance of the Flp-In™ cell lines. For more information about the products listed below, refer to **www.lifetechnologies.com** or contact Technical Support (see page 17).
Note: Reagents are available in other sizes.

Item	Amount	Catalog no.
Dulbecco's Modified Eagle Medium (D-MEM)	500 mL	11960-044
Ham's F-12	500 mL	11765-054
RPMI Medium 1640	500 mL	11875-093
Fetal Bovine Serum	500 mL	16000-044
Donor Calf Serum	500 mL	16030-074
L-Glutamine, 200 mM (100X)	100 mL	25030-081
Penicillin-Streptomycin	100 mL	15070-063
0.05% Trypsin-EDTA (1X), Phenol Red	100 mL	25300-054

Additional reagents

The products listed below may be used with the Flp-In™ Cell Lines. Zeocin™ Selection Antibiotic is available for maintenance and growth of the Flp-In™ cell lines. For more information, refer to **www.lifetechnologies.com** or contact Technical Support (see page 17).

Item	Amount	Catalog no.
Zeocin™ Selection Antibiotic	8 × 1.25 mL	R250-01
	50 mL	R250-05
Hygromycin B, 50 mg/mL	20 mL	10687-010
Lipofectamine® 2000 Reagent	0.75 mL	11668-027
	1.5 mL	11668-019
Lipofectamine® Reagent	1 mL	18324-012
Plus™ Reagent	0.85 mL	11514-015
Phosphate-Buffered Saline (PBS), pH 7.4	500 mL	10010-023

Flp-In™ products

The plasmids required to generate Flp-In™ host cell lines and expression cell lines are available separately from Life Technologies. For more information about the features of each vector, refer to **www.lifetechnologies.com** or contact Technical Support (see page 17). Ordering information is provided below.

Product	Amount	Catalog no.
pFRT/*lac*Zeo	20 µg	V6015-20
pFRT/*lac*Zeo2	20 µg	V6022-20
pOG44	20 µg	V6005-20
pcDNA™5/FRT	20 µg	V6010-20
pcDNA™5/FRT/V5-His TOPO® TA Expression Kit	20 reactions	K6020-01
pSecTag/FRT/V5-His TOPO® TA Expression Kit	20 reactions	K6025-01
pEF5/FRT/V5-DEST™ Gateway® Vector Pack	6 µg	V6020-20

Documentation and support

Obtaining support

Technical support

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- 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)

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