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Expi293™ Expression System: Structural Biology and Inducible Expression Modules

USER GUIDE

For scalable transfection of Expi293F™ GnTI- and Expi293F™ Inducible cell lines in a chemically defined, serum-free medium, using the ExpiFectamine™ 293 Transfection Kit.

For scalable metabolic protein labeling using Expi293F™ cell lines in a chemically defined, serum-free, methionine-free medium using a methionine-deficient ExpiFectamine™ 293 Transfection Kit.

Catalog Numbers A39250, A39250CN, A39251, A14527CN, A39252, A41276CN, A41249, A14524, A14525, A14526, A39249

Publication Number MAN0007814

Revision C.0

ThermoFisher SCIENTIFIC

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Revision history: Pub. No. MAN0007814

Revision	Date	Description
C.0	10 February 2023	Update thaw and cryopreservation protocols.
B.0	29 May 2020	Update to reflect inducible expression and structural biology kit configurations.
A.0	14 June 2019	Update of user guide to bring it up to current standards and styles.Addition of GNTI cells, Inducible cells, and Inducible GNTI cells.Updating of protocols to incorporate the new cell lines added..

The information in this guide is subject to change without notice.

Contents

1 Product information

IMPORTANT! Before using this product, read and understand the information in the "Safety" appendix in this document.

Product description

The Gibco™ Expi293™ Expression System is a high-yield transient expression system based on suspension-adapted Human Embryonic Kidney (HEK) cells. As part of the structural biology and inducible expression modules, we offer 3 System Starter Kits for structural biology or inducible expression and additional reagents to allow metabolic labelling of proteins. Each Expi293™ Expression System Starter Kit provides cells, culture medium, and reagents to express a total of 1 liter production volume.

The structural biology and inducible expression modules of the Expi293™ Expression System includes four Expi293F™ cell lines that can be used to express your protein of interest.

- Use Expi293F™ cells for metabolic protein labeling.
- Use Expi293F™ GnTI- Cells for expression of proteins with a uniform glycosylation pattern.
- Use Expi293F™ Inducible Cells for regulated expression of proteins through tetracycline mediated induction.
- Use Expi293F™ Inducible GnTI- Cells for regulated expression of proteins with a uniform glycosylation pattern.

Each of these cell lines display the same growth kinetics, follow the same transfection protocols, and can be used in metabolic protein labeling studies.

For metabolic (methionine) protein labeling, Expi293™ Met (-) Expression Medium and the ExpiFectamine™ 293 Met (-) Transfection Kit should be used to metabolically label proteins of interest with L-Methionine (Methyl-^{13}C) or L-Selenomethionine.

Expi293™ Met (-) Expression Medium and the ExpiFectamine™ 293 Met (-) Transfection Kit can be ordered separately or together as part of the Expi293™ Met (-) Protein Labeling Kit. L-Methionine (Methyl-^{13}C) and L-Selenomethionine can be ordered separately.

For further instruction on metabolically labeling proteins using the Expi293™ Expression System, see "Transfect Expi293F™ cell lines using ExpiFectamine™ 293 Met (-) Transfection Kit for metabolic protein labeling" on page 23.

Contents and storage

Table 1 Expi293™ GnTI- Expression System Kit (Cat. No. A39250 or A39250CN)

Contents	Amount	Cat. No.	Storage
Expi293F™ GnTI- Cells[1] (1 × 10^7 cells/mL)	1 mL	A39240	Liquid nitrogen[2]
Expi293™ Expression Medium	1 L	A1435101	2°C to 8°C Protect from light
ExpiFectamine™ 293 Transfection Kit: • ExpiFectamine™ 293 Reagent • ExpiFectamine™ 293 Transfection Enhancer 1 • ExpiFectamine™ 293 Transfection Enhancer 2	1 kit for 1 L of culture	A14524	
Opti-Plex™ Complexation Buffer	100 mL	A4096801	
pRABBIT IgG IRES-EmGFP Positive Control Vector (at 1 mg/mL in TE[3] Buffer, pH 8.0)	150 µg	A39243	–20°C
PNGase F Glycan Cleavage Kit	1 Kit	A39245	–20°C

[1] Cells are cryopreserved in 90% Expi293™ Expression Medium and 10% DMSO.
[2] Store the frozen cells in liquid nitrogen immediately upon receipt and until ready to use. Do not store the cells at –80°C.
[3] TE buffer, pH 8.0: 10 mM Tris-HCl, 1 mM EDTA, pH 8.0.

Table 2 Expi293™ Inducible Expression System Kit (Cat. No. A39251 or A14527CN)

Contents	Amount	Cat. No.	Storage
Expi293F™ Inducible Cells[1] (1 × 10^7 cells/mL)	1 mL	A39241	Liquid nitrogen[2]
Expi293™ Expression Medium	1 L	A1435101	2°C to 8°C Protect from light
ExpiFectamine™ 293 Transfection Kit: • ExpiFectamine™ 293 Reagent • ExpiFectamine™ 293 Transfection Enhancer 1 • ExpiFectamine™ 293 Transfection Enhancer 2	1 kit for 1 L of culture	A14524	
Opti-Plex™ Complexation Buffer	100 mL	A4096801	
pRABBIT IgG IRES-EmGFP Positive Control Vector (at 1 mg/mL in TE[3] Buffer, pH 8.0)	150 µg	A39243	–20°C
pcDNA™5/TO Mammalian Expression Vector	20 µg	V103320	
Tetracycline Hydrochloride[4]	500 mg	A39246	Protect from light and moisture Room temperature

[1] Cells are cryopreserved in 90% Expi293™ Expression Medium and 10% DMSO.
[2] Store the frozen cells in liquid nitrogen immediately upon receipt and until ready to use. Do not store the cells at –80°C.
[3] TE buffer, pH 8.0: 10 mM Tris-HCl, 1 mM EDTA, pH 8.0.
[4] Not included in Cat. No. A41275.

Table 3 Expi293™ Inducible GnTI- Expression System Kit (Cat. No. A39252 or A41276CN)

Contents	Amount	Cat. No.	Storage
Expi293F™ Inducible GnTI- Cells[1] (1 × 10^7 cells/mL)	1 mL	A39242	Liquid nitrogen[2]
Expi293™ Expression Medium	1 L	A1435101	2°C to 8°C Protect from light
ExpiFectamine™ 293 Transfection Kit: • ExpiFectamine™ 293 Reagent • ExpiFectamine™ 293 Transfection Enhancer 1 • ExpiFectamine™ 293 Transfection Enhancer 2	1 kit for 1 L of culture	A14524	
Opti-Plex™ Complexation Buffer	100 mL	A4096801	
pRABBIT IgG IRES-EmGFP Positive Control Vector (at 1 mg/mL in TE[3] Buffer, pH 8.0)	150 µg	A39243	–20°C
pcDNA™5/TO Mammalian Expression Vector	20 µg	V103320	
Tetracycline Hydrochloride[4]	500 mg	A39246	Protect from light and moisture Room temperature
PNGase F Glycan Cleavage Kit	1 kit	A39245	–20°C

[1] Cells are cryopreserved in 90% Expi293™ Expression Medium and 10% DMSO.
[2] Store the frozen cells in liquid nitrogen immediately upon receipt and until ready to use. Do not store the cells at –80°C.
[3] TE buffer, pH 8.0: 10 mM Tris-HCl, 1 mM EDTA, pH 8.0.
[4] Not included in Cat. No. A41276.

Table 4 Expi293™ Met (-) Protein Labeling Kit (Cat. No. A41249)

Contents	Amount	Cat. No.	Storage
ExpiFectamine™ 293 Met (-) Transfection Kit • ExpiFectamine™ 293 Reagent • ExpiFectamine™ 293 Transfection Enhancer 1 • ExpiFectamine™ 293 Met (-) Transfection Enhancer 2 • Opti-Plex™ Complexation Buffer	1 kit for 1 L culture	A39249	2°C to 8°C Protect from light.
Expi293™ Met (-) Expression Medium	1 L	A4096701	

Table 5 ExpiFectamine™ 293 Transfection Kit (Cat. No. A14524, A14525, and A14526)

Contents	Amount	Cat. No.	Storage
ExpiFectamine™ 293 Transfection Kit: • ExpiFectamine™ 293 Reagent • ExpiFectamine™ 293 Transfection Enhancer 1 • ExpiFectamine™ 293 Transfection Enhancer 2	• 1 kit for 1 L of culture • 1 kit for 10 L of culture • 1 kit for 50 L of culture	• A14524 • A14525 • A14526	2°C to 8°C Protect from light.

Table 6 ExpiFectamine™ 293 Met (-) Transfection Kit (Cat. No. A39249)

Contents	Amount	Cat. No.	Storage
ExpiFectamine™ 293 Met (-) Transfection Kit • ExpiFectamine™ 293 Reagent • ExpiFectamine™ 293 Transfection Enhancer 1 • ExpiFectamine™ 293 Met (-) Transfection Enhancer 2 • Opti-Plex™ Complexation Buffer	1 kit for 1 L culture	A39249	2°C to 8°C Protect from light.

Components of the Expi293™ Expression System: structural biology and inducible expression modules

The Expi293™ Expression System is based on high density culture of Expi293F™ Cells in Expi293™ Expression Medium. Transient expression is by the cationic lipid-based ExpiFectamine™ 293 Reagent with specialized transfection enhancers. The components work together to generate 2- to 10-fold higher protein yields than previous generation transient expression systems.

Expi293F™ Cells

Expi293F™ Cells are human cells derived from the 293F cell line, and are a core component of the Expi293™ Expression System. They are maintained in suspension culture and optimized to grow to high density in Expi293™ Expression Medium. Expi293F™ Cells are highly transfectable and generate superior transient protein yields compared to standard 293 cell lines.

- **Growth conditions**: Suspension at 37°C, 8% CO_2.
- **Doubling time**: 24–25 hours during log phase growth. Doubling times may vary based on cell health, handling, and passage number.
- **Viability**: Cell viability should be greater than 90% by 7 days post-thaw.
- **Subculture conditions**: Grow cells to 3–5 × 10^6 cells/mL; then, split cells to 0.3–0.5 × 10^6 cells/mL every 3 days or 0.2–0.4 × 10^6 cells/mL every four days. Do not grow above 5 × 10^6 cells/mL for best performance. Discard cells after passage number 30.

Note: We also offer fully-documented cGMP-banked Expi293F™ Cells. For ordering information, see Appendix D, “Ordering information”.

Expi293F™ GnTI- Cells

Expi293F™ GnTI- Cells are derived from Expi293F™ Cells and have been engineered to lack N-acetylglucosaminyl-transferase I (GnTI) enzyme activity leading to the production of glycoproteins with a uniform $(GlcNAc)_2Man5$ glycopattern.

Expi293F™ Inducible Cells

Expi293F™ Inducible Cells are derived from Expi293F™ Cells and stably express high levels of the tetracycline repressor protein (TetR) from the pcDNA™6/TR plasmid. When used with compatible inducible vectors (e.g. pcDNA™5/TO Mammalian Expression Vector), the gene of interest is repressed with very low levels of basal expression until induction with tetracycline. Expression can be modulated by addition of different amounts of tetracycline. For best results, Expi293F™ Inducible Cells should be maintained under selective pressure of blasticidin. For further details, see “Guidelines for inducible cells” on page 15.

Expi293F™ Inducible GnTI- Cells

Expi293F™ Inducible GnTI- Cells are derived from Expi293F™ GnTI- Cells and stably express high levels of the tetracycline repressor protein (TetR) from the pcDNA™6/TR plasmid in combination with knockout of the GnTI gene. When used with compatible inducible vectors (e.g. pcDNA™5/TO Mammalian Expression Vector), the gene of interest is repressed with very low levels of basal expression until induction with tetracycline. Expression can be modulated by addition of different amounts of tetracycline. Resultant glycan patterns of the expressed glycoprotein are consistent with those expressed by Expi293F™ GnTI- Cells. For best results, Expi293F™ Inducible GnTI- Cells should be maintained under selective pressure of blasticidin. For further details, see “Guidelines for inducible cells” on page 15.

Expi293™ Expression Medium

Expi293™ Expression Medium is a chemically defined, serum-free, protein-free, animal origin-free medium for growth and transfection of suspension-adapted HEK 293 cells. It is a core component of the Expi293™ Expression System and supports high density culture of Expi293F™ cell lines for scalable transient protein expression. Expi293™ Expression Medium is formulated with GlutaMAX™ Supplement, is ready to use without additional supplementation. Expi293™ Expression Medium contains no human or animal-origin components. The chemically defined formulation results in high lot-to-lot reproducibility and reliability. The medium is not recommended for adherent cell cultures.

Expi293™ Expression Medium exhibits the following features:

- Supports growth and transfection of Expi293F™ cells in culture formats of less than 1 mL in multi-well plates to greater than 10 L in disposable bioreactors
- Supports growth of suspension Expi293F™ cultures to densities over 15×10^6 cells/mL.
- Transfection compatible medium enables transfection efficiencies of approximately 80% using ExpiFectamine™ 293 Transfection Reagent
- Enables sustained, high-level expression of high-density transiently transfected cultures, achieving yields of up to 1 gram per liter of recombinant protein
- Does not contain phenol red

Expi293™ Met (-) Expression Medium

Expi293™ Met (-) Expression Medium is an optimized, chemically defined formulation designed to support methionine labeling of proteins and transfection of 293 cells (e.g., Expi293F™ cells) in suspension. The medium does not contain methionine, protein, undefined lysates, or components of animal origin. Expi293™ Met (-) Expression Medium is a complete, ready-to-use medium formulated with GlutaMAX™ Supplement, but requires methionine supplementation. The medium is not recommended for adherent 293 cell culture.

ExpiFectamine™ 293 Reagent

ExpiFectamine™ 293 Reagent is optimized for the transfection of plasmid DNA into high-density Expi293™ cell cultures.

ExpiFectamine™ 293 Reagent has the following features:

- Designed specifically for transfection of high-density suspension cell culture, with matching transfection enhancers that boost transfection performance and protein expression
- Achieves protein yields 2- to 10-fold higher than other transfection reagents used on high-density 293 cell cultures
- Employs the same transient expression protocols typically used in current low-density 293 suspension culture systems to easily switch from low-density systems to the high yield, high-density Expi293™ Expression System
- Provides robust and reproducible transfection results
- Enables scalable transfections for culture volumes of less than 1 mL to greater than 10 liters, while maintaining equivalent volumetric protein yields

ExpiFectamine™ 293 Transfection Enhancer 1

ExpiFectamine™ 293 Transfection Enhancer 1 is an optimized, chemically defined, serum-free, protein-free, animal origin-free formulation designed to work in conjunction with Expi293™ Expression Medium to support, high-density transient transfections. ExpiFectamine™ 293 Transfection Enhancer 1 is a component of the ExpiFectamine™ 293 Transfection Kit.

ExpiFectamine™ 293 Transfection Enhancer 2

ExpiFectamine™ 293 Transfection Enhancer 2 is a proprietary, animal origin-free formulation developed to be used in conjunction with ExpiFectamine™ 293 Reagent and ExpiFectamine™ 293 Transfection Enhancer 1 to enhance protein production, resulting in maximal protein yields. ExpiFectamine™ 293 Transfection Enhancer 2 is a component of the ExpiFectamine™ 293 Transfection Kit.

ExpiFectamine™ 293 Met (-) Transfection Enhancer 2

ExpiFectamine™ 293 Met (-) Transfection Enhancer 2 is a proprietary, animal origin-free, methionine-free formulation developed to be used in conjunction with ExpiFectamine™ 293 Reagent and ExpiFectamine™ 293 Transfection Enhancer 1 to enhance protein production during metabolic protein labeling experiments. ExpiFectamine™ 293 Met (-) Transfection Enhancer 2 is a component of the ExpiFectamine™ 293 Met (-) Transfection Kit.

Opti-Plex™ Complexation Buffer

Opti-Plex™ Complexation Buffer is a chemically defined, animal origin-free, serum-free, phenol red-free, methionine-free, and biotin-free buffer used to complex plasmid DNA with ExpiFectamine™ 293 Reagent.

Note: Opti-Plex™ Complexation Buffer must be used when expressing proteins using the ExpiFectamine™ 293 Met (-) Transfection Kit to ensure maximal incorporation of labeled methionine into the expressed proteins. For routine transient transfections, the absence of methionine in Opti-Plex™ will not impact transfection efficiency and protein expression levels.

pRABBIT IgG IRES-EmGFP Positive Control Vector

pRABBIT IgG IRES-EmGFP Positive Control Vector is provided as a positive control for transfection, expression, and evaluation of transfection efficiency in Expi293F™ cell lines. The rabbit IgG that is produced in Expi293F™ Cells after transfection with the control vector is secreted into the culture medium, with the optimal yields occurring 5–7 days posttransfection (typical yield range: 450–500 mg/L). Emerald Green Florescent Protein (EmGFP) is an intracellular protein that can be used to qualitatively assess Expi293F cell transfection by fluorescence microscopy, fluoresescent plate reader, or flow cytometry, 24–96 hours posttransfection. For more information on using the pRABBIT IgG IRES-EmGFP Positive Control Vector, see Appendix B, “Positive control for transfection and expression”.

pcDNA™5/TO Mammalian Expression Vector

pcDNA™5/TO Mammalian Expression Vector is a 5.7 kb expression vector that utilizes the complete CMV promoter and adds control elements from the bacterial tetracycline resistance operon to effectively repress and derepress transcription from one of the strongest mammalian promoter sequences known. The pcDNA™5/TO vector allows tetracycline-regulated expression of the gene of interest in mammalian host cells expressing the tetracycline repressor protein (TetR).

PNGase F Glycan Cleavage Kit

The PNGase F Glycan Cleavage Kit includes all components required to perform the enzymatic removal of almost all N-linked oligosaccharides from glycoproteins. The kit includes recombinant PNGase F enzyme, which cleaves N-glycan chains at the innermost GlcNAc and asparagine residues of high mannose, hybrid, and complex oligosaccharides.

2 Methods

Procedural guidelines for Expi293F™ cell culture

Note: The cell handling instructions below are applicable to all Expi293F™ cell lines including Expi293F™ Cells, Expi293F™ GnTI- Cells, Expi293F™ Inducible Cells, and Expi293F™ Inducible GnTI- Cells.

General cell handling

- All solutions and equipment that come in contact with the cells must be sterile. Always use proper aseptic technique and work in a laminar flow hood.
- For all cell manipulations, mix the cells by gentle swirling and avoid vigorous shaking and pipetting. Cell health is critical for maximal performance.
- Expi293F™ cell lines are robust cell lines adapted to high-density growth conditions with a doubling time of approximately 24 hours during log phase growth.

Guidelines for thawing and storing cells

- On receipt, either thaw the cells immediately into pre-warmed Expi293 Expression Medium or immediately place the frozen cells 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 following receipt on dry ice, allow the cells to remain in liquid nitrogen for 3–4 days before thaw.
- Before starting experiments, ensure to have cells that are established and have frozen stocks on hand. On receipt, grow and freeze multiple vials of Expi293F™ cells to ensure that you have an adequate supply of early-passage cells.

Guidelines for cell maintenance and subculturing

- Allow freshly thawed cells to recover in culture for three or more passages post-thaw before transfecting.
- Use an automated cell counter or a hemocytometer with the trypan blue exclusion method to determine cell viability. Log phase cultures should be ≥95% viable.
- When thawing or subculturing cells, transfer cells into pre-warmed medium.
- Cell viability should be ≥90% within 4–7 days post-thaw with viable cell density typically > 1×10^6 viable cells/mL at this time; if viability is not ≥90%, cells should be incubated for up to an additional 3 days in order to reach this criterion.
- At the time of first subculture, cells should be subcultured when the viable cell density reaches $1–3 \times 10^6$ viable cells/mL.

- For general maintenance of cells, passage Expi293F™ cells when they reach a density of approximately 3–5 × 10^6 viable cells/mL (i.e., early log-phase growth), typically every 3–4 days.

Note: Cells that are subcultured at densities outside of this early log-phase growth window can show longer doubling times and lower protein titers over time. Modify the initial seeding density to attain the target cell density of 3–5 × 10^6 viable cells/mL at the time of subculturing.

Guidelines for inducible cells

For Expi293F™ Inducible Cells or Expi293F™ Inducible GnTI- Cells:

- For routine culture maintenance, add blasticidin to a final concentration of 20 µg/mL to culture medium.
- Blasticidin can be present in the media during cryopreservation without impacting cell health.
- An inducible expression vector must be utilized in conjunction with the Expi293F™ Inducible Cells and Expi293F™ Inducible GnTI- Cells. pcDNA™5/TO expression vector is recommended for lowest levels of basal expression and highest levels of expression upon induction with tetracycline.
- We recommend making a 1 mg/mL tetracycline stock solution in water.

Guidelines for media

Expi293™ Expression Medium is formulated with GlutaMAX™ Supplement. For suspension growth and transfection applications, use the Expi293™ Expression Medium without any supplementation.

IMPORTANT! Expi293™ Expression Medium is sensitive to light. For optimal results, use and store media protected from light.

Note: ExpiFectamine™ 293 Transfection Enhancer 1 can exhibit a slightly yellowish tint. Internal studies show this has no impact on system performance or protein titer.

Thaw and establish Expi293F™ cell lines

Note: The thawing and cell line establishment instructions below are applicable to all Expi293F™ cell lines including Expi293F™ Cells, Expi293F™ GnTI- Cells, Expi293F™ Inducible Cells, and Expi293F™ Inducible GnTI- Cells.

Required materials

- One of the following Expi293F™ cell lines:
  - Expi293F™ Cells
  - Expi293F™ GnTI- Cells
  - Expi293F™ Inducible Cells
  - Expi293F™ Inducible GnTI- Cells
- 125-mL polycarbonate or PETG, non-baffled, disposable, sterile, vented shaker flask for culturing suspension cells, such as Nalgene™ Single-Use PETG Erlenmeyer Flasks with Plain Bottom: Sterile

- Expi293™ Expression Medium, pre-warmed to 37°C
- Orbital shaker in a 37°C incubator with ≥80% relative humidity and 8% CO_2
- Reagents and equipment to determine cell viability (e.g., hemocytometer with trypan blue or cell counter)

Thaw Expi293F™ cells

1. Add 30 mL of pre-warmed Expi293™ Expression Medium to a 125-mL Erlenmeyer shaker flask.
2. Remove a vial of Expi293F™ cells from liquid nitrogen and swirl gently in a 37°C water bath for 1 to 2 minutes to thaw the cells rapidly until only a small amount of ice remains.

   Note: Do not submerge the vial in the water.
3. Just before the cells are completely thawed, decontaminate the vial with 70% ethanol before opening it in a laminar flow hood.
4. Gently invert the cell vial to mix the contents. Uncap the cell vial and transfer 50 µL of cells into 450 µL of Ca^{2+}/Mg^{2+} free PBS for viability and viable cell density determination by trypan blue dye exclusion assay.

   Note: Trypan blue may interact with components in cell culture media leading to aggregation that can be misinterpreted as dead cells using typical cell counting instruments and algorithms. Dilution with PBS is not required during routine cell culture maintenance.
5. Using a 1-, 2-, or 5-mL pipette, gently transfer the remaining cell volume drop wise to the shake flask containing the pre-warmed culture medium prepared in step 1.
6. Incubate the cells in a 37°C incubator with ≥80% relative humidity and 8% CO_2 on an orbital shaker platform. The use of non-humidified incubators is not recommended due to the significant loss of volume in the culture flasks by evaporation.

   Note: Set the shake speed to 125 ±5 rpm for shakers with a 19-mm shaking diameter, 120 ±5 rpm for shakers with a 25-mm shaking diameter and 95 ±5 rpm for shakers with a 50-mm shaking diameter.
7. Three to four days post-thaw, determine the viable cell density and percent viability. Cell viability should be ≥90% with a viable cell density >1 × 10^6 viable cells/mL.

   Note: If the viability is <90% on days 3 to 4 post-thaw, cells may be cultured for up to an additional 3 days in order to reach the desired viability. Cells should not be subcultured until viable cell density reaches >1 × 10^6 viable cells/mL.
8. For subsequent routine cell culture maintenance, subculture cells every 3 to 4 days when the viable cell density reaches 3 × 10^6 to 5 × 10^6 viable cells/mL.

   Note: Do not subculture cells before reaching early log phase growth of 3 × 10^6 viable cells/mL. Similarly, do not let cells overgrow above 5 × 10^6 cells/mL. Modify the initial seeding density to attain target cell density of 3 × 10^6 to 5 × 10^6 viable cells/mL at the time of subculturing.

Subculture Expi293F™ cell lines

Note: The cell handling and cell freezing instructions below are applicable to all Expi293F™ cell lines including Expi293F™ Cells, Expi293F™ GnTI- Cells, Expi293F™ Inducible Cells, and Expi293F™ Inducible GnTI- Cells.

Required materials

- Expi293F™ cell culture at 3–5 × 10^6 viable cells/mL
- Expi293™ Expression Medium, pre-warmed to 37°C
- Polycarborate or PETG, non-baffled, disposable, sterile, vented shaker flask for culturing suspension cells
- Reagents and equipment to determine viable cell density and percent viability (e.g., hemocytometer or an automated cell counter, trypan blue)
- Orbital shaker in a 37°C incubator with ≥80% relative humidity and 8% CO_2

Subculture Expi293F™ cells

1. Use the viable cell density to calculate the volume of cell suspension required to seed a new shake flask according to the recommended seeding densities in Table 7 and the recommended culture volumes in Table 8.

Table 7 Recommended seeding densities for routine cell culture maintenance

Sub-culture timing	Recommended seeding density
For cells ready 3 days post-subculture	0.4–0.5 × 10^6 viable cells/mL
For cells ready 4 days post-subculture	0.3–0.4 × 10^6 viable cells/mL

Table 8 Recommended volumes for routine cell culture maintenance in vented, non-baffled flask

Flask size	Culture volume (mL)	Shake speed
125 mL	30–35 mL	125 ± 5 rpm (19 mm shaking diameter) 120 ± 5 rpm (25 mm shaking diameter) 95 ± 5 rpm (50 mm shaking diameter)
250 mL	60–70 mL	
500 mL	100–120 mL	
1 L	220–240 mL	
2 L	440–480 mL	
3 L	800–1,000 mL	90 ± 5 rpm 85 ± 5 rpm 80 ± 5 rpm

2. Transfer the calculated volume of cells to fresh, pre-warmed Expi293™ Expression Medium in a shake flask.

3. Incubate flasks in a 37°C incubator with ≥80% relative humidity and 8% CO_2 on an orbital shaker platform until cultures reach a density of 3–5 × 10^6 viable cells/mL.

Note: Do not let cells grow above 5 × 10^6 viable cells/mL during routine culture.

Note: Cells that are subcultured at densities outside of this early log-phase growth window can show longer doubling times and lower protein titers over time. Modify the initial seeding density to attain the target cell density of 3–5 × 10^6 viable cells/mL at the time of subculturing.

4. Repeat step 1 to step 3 to maintain or expand the cells for transfection.

Cryopreserve Expi293F™ cells

Expi293F™ cells can be frozen directly in Expi293™ Expression Medium with 7.5% DMSO. Alternatively, conditioned cryopreservation medium consisting of 42.5% fresh Expi293™ Expression Medium, 50.0% conditioned medium and 7.5% DMSO can be used.

Note: It is critical that chemical compatibility be maintained throughout the freezing process to eliminate the potential for plastics leachables/extractables to negatively impact cell health. For all steps where DMSO is present (with exception only to the pipet tips used for aliquoting the final cell solution into cryo-vials), glass serological pipettes are suggested to be used, as polystyrene is generally not compatible with concentrated DMSO. Similarly, DMSO containing freeze medium must be prepared in polypropylene or other known DMSO compatible bottles and the final cell suspension in freeze medium must be prepared in polypropylene or other known DMSO compatible bottles prior to aliquoting into polypropylene freeze tubes. Additionally, all pipets and bottles may be rinsed with sterile water, PBS or culture medium before use as desired. Refer to **https://tools.thermofisher.com/content/sfs/brochures/D20480.pdf** for guidance on chemical compatibility.

1. Determine the density and volume of cells required for banking cells at a final density of 1 × 10^7 viable cells/mL in 1.1 mL total volume. Expand the cells, maintaining a viable cell density of 3 × 10^6 – 5 × 10^6 viable cells/mL, until the desired volume of cells to be banked is ready. Do not use shake flasks larger than 2 L to culture the cells, as these flasks differ in shape and require altered shaking speeds and be sure to adhere to the shaking speed, orbital diameter and maximum flask volume recommendations.

2. While expanding the cells for banking, prepare an additional flask which will be used to generate conditioned medium. This flask should be prepared and expanded in the same manner as the flasks used for cell banking.

Note: Based on the total volume of the bank, prepare at least ½ volume conditioned medium (i.e., If total volume for cell banking is 100 mL, at least 50 mL of conditioned medium will be required).

3. Prepare labels and label the appropriate number of vials. If vials are labeled on a day other than that of the harvest, store vials in a biosafety cabinet.

Note: To reduce the risk of damaging the cells during freezing procedures, cells pellets will be resuspended in cold, 100% conditioned medium followed by 1:1 addition of cold freeze medium with 15% DMSO to reach a final concentration of 7.5% DMSO, 50% conditioned medium, and 42.5% fresh medium. It is critical to resuspend the cell pellets in cold medium free of DMSO.

Note: If the use of 100% fresh culture medium for banking cells is desired, cells pellets will be resuspended in cold, 100% fresh medium followed by a 1:1 addition of cold, 15% DMSO in fresh cell culture medium to reach a final concentration of 7.5% DMSO and 92.5% fresh medium.

Prepare conditioned medium

All conditioned medium is to be pre-chilled before use in cell banking.

1. Remove conditioned medium flask from incubator and transfer the entire volume of cell suspension into a sterile polypropylene centrifuge tube or bottle.
2. Centrifuge the cells at 300 × *g* for 10 minutes at 2–8°C.
3. Carefully decant the supernatant into a sterile polypropylene bottle without disturbing the cell pellet; the decanted supernatant will be used as the conditioned medium.
4. Store the conditioned medium in a 2–8°C refrigerator or on ice for a minimum of 2 hours.
5. Discard the cell pellets.

Prepare freeze medium (2X)

1. In a sterile polypropylene bottle, prepare the required amount of fresh culture medium supplemented with 15% DMSO. This represents a 2X freeze medium. It is recommended to use glass serological pipettes for transferring the concentrated DMSO to the culture medium. Keep 2X DMSO freeze medium cold at 2–8°C or on ice until use.
2. Remove calculated volume of cells from incubation and transfer into sterile polypropylene centrifuge bottle/tube. Centrifuge the cells at 200 × *g* for 10 minutes at 2–8°C. Carefully decant the supernatant without disturbing the cell pellets. After removing the supernatant, gently flick the bottom of the centrifuge tube to loosen the cell pellet.
3. Resuspend the cell pellet by gently pipetting with ~10% volume of conditioned medium using a wide bore pipet (i.e., If total bank volume is 200 mL, use 20 mL to resuspend cell pellet).
4. Add additional conditioned medium to the centrifuge bottle to obtain a 2X cell stock. For example, if banking at 1×10^7 cells/mL prepare a 2X cell stock at 2×10^7 cells/mL. Gently swirl the bottle to ensure a homogenous mixture and keep cold on ice or cold blocks.

   Note: It is critical that the next steps are performed as quickly as possible to limit the DMSO exposure time.

5. Using a glass serological pipette, add the calculated volume of cold, 2X freeze medium to the bottle containing 2X cell stock in conditioned medium from step 4.
6. Gently swirl the bottle to ensure a homogenous mixture and keep cold on ice or cold blocks.

   At this point the volume should be equal to the total bank volume at a final concentration of 7.5% DMSO, 50% conditioned medium, and 42.5% fresh medium with a cell density of 1×10^7 cells/mL.

7. Immediately dispense a 1.1 mL aliquot of the final cell suspension from step 6 into labeled cryo-vials using a repeater pipette or sterile serological pipettes.

8. Gently swirl the cell suspension to mix before each refill of the multi-dispenser pipette or serological pipette. Make sure to keep the cell suspension cold during the entire aliquoting process.

   Note: The DMSO in the 2X freeze medium is harmful to the cells so it is important to limit the amount of DMSO exposure prior to freezing. We recommend keeping the DMSO exposure time ≤ 60 minutes as possible and keeping all reagents cold during this time.

9. Transfer the cryo-vials to isopropanol containing cryo-freezing chambers and store at -80°C for 24–48 hours. Do not store cells at -80°C for more than 48 hours. After 24–48 hours transfer cells to final storage in vapor phase liquid nitrogen.

   This freezing regimen approximates a 1°C per minute cooling in the isopropanol containers to -80°C followed by a 2°C per minute cooling in vapor phase liquid nitrogen to final storage temperature. These cooling conditions may be utilized as a basis for controlled rate freezing protocol design.

Transfect Expi293F™ cell lines using ExpiFectamine™ 293 Transfection Kit

Note: The cell transfection instructions below are applicable to all Expi293F™ cell lines including Expi293F™ Cells, Expi293F™ GnTI- Cells, Expi293F™ Inducible Cells, and Expi293F™ Inducible GnTI- Cells.

For optimal transfection of high-density suspension Expi293F™ cell cultures, use the ExpiFectamine™ 293 Reagent included in the transfection kits.

Unlike other serum-free media formulations, Expi293™ Expression Medium does not inhibit transfection. Expi293™ Expression Medium is specifically formulated to enable transfection without the need to change or add media pre- and posttransfection.

Note: For metabolic labeling of proteins using the ExpiFectamine™ 293 Met (-) Transfection Kit, proceed to “Transfect Expi293F™ cell lines using ExpiFectamine™ 293 Met (-) Transfection Kit for metabolic protein labeling” on page 23.

Required materials

- Expi293F™ cell culture in Expi293™ Expression Medium
- Plasmid DNA, sterile, free from phenol and sodium chloride, and containing mostly supercoiled DNA

Note: We recommend isolating plasmid DNA using the PureLink™ Plasmid Isolation Kits (For ordering information, see Appendix D, "Ordering information"). To ensure sterility, DNA can be filtered through a 0.22-µm filter before use.

- Antibody-Expressing Positive Control Vector
- ExpiFectamine™ 293 Transfection Kit
- Opti-MEM™ I Reduced Serum Medium
- Expi293™ Expression Medium, pre-warmed to 37°C

Note: Do not add antibiotics to culture media during transfection as this will decrease transfection efficiency. If necessary, antibiotics can be added to cultures approximately 24 hours posttransfection.

- Disposable, sterile Erlenmeyer flasks
- Orbital shaker in a 37°C incubator with ≥80% relative humidity and 8% CO_2
- Reagents and equipment to determine viable cell density and percent viability

Guidelines for transfection

- Use of transfection reagents other than the ExpiFectamine™ 293 Reagent to transfect Expi293F™ cell cultures can substantially reduce performance.
- Gently invert the ExpiFectamine™ 293 Reagent 4–5 times before use to ensure thorough mixing.
- Complexation of plasmid DNA and ExpiFectamine™ 293 Reagent takes place at room temperature.
- Incubate the ExpiFectamine™ 293/DNA complexes for 10–20 minutes before adding to the cells. Longer incubation times can lead to slight reduction in performance. Incubation times over 20 minutes are not recommended.
- For Expi293F™ Inducible Cells or Expi293F™ Inducible GnTI- Cells only, the presence of blasticidin will not impact transfection, thus, there is no need to remove blasticidin before transfection.
- For scaling up transfections, see Appendix C, "Scaling up transfections".

Day –2: Subculture cells

Subculture and expand Expi293F™ cells until cell density reaches approximately 3–5 × 10^6 viable cells/mL.

Day –1: Seed cells

Seed Expi293F™ cells to a final density of 2.5–3 × 10^6 viable cells/mL, then allow cells to grow overnight.

Day 0: Transfect cells

1. Determine viable cell density and percent viability.

   Viable cell density and percent viability should be 4.5–5.5 × 10^6 viable cells/mL and ≥95%, respectively, to proceed with transfection.

2. Dilute the cells to a final density of 3 × 10^6 viable cells/mL with fresh, pre-warmed Expi293™ Expression Medium, then swirl the culture flasks gently to mix the cells.

   Note: Discard the remaining cells. Do not re-use high-density cells for routine subculturing.

3. Gently invert the ExpiFectamine™ 293 Reagent bottle 4–5 times before use to ensure thorough mixing.

4. Dilute plasmid DNA with Opti-Plex™ Complexation Buffer (or Opti-MEM™ I Medium), then mix by swirling or inversion.

   Note: Total plasmid DNA concentration of 1.0 µg/mL of culture volume to be transfected is appropriate for most proteins.

5. Dilute ExpiFectamine™ 293 Reagent with Opti-Plex™ Complexation Buffer (or Opti-MEM™ I Medium, if used in step 4), then mix by swirling or inversion.

6. Incubate at room temperature for 5 minutes.

7. Add the diluted ExpiFectamine™ 293 Reagent to the diluted plasmid DNA, then mix by swirling or inversion.

8. Incubate the ExpiFectamine™ 293/plasmid DNA complexes at room temperature for 10–20 minutes.

9. Slowly transfer the complexes to the cells, swirling the culture flask gently during addition, then incubate the cells in a 37°C incubator with ≥80% relative humidity and 8% CO_2 on an orbital shaker (for suggested shake speeds, see Table 9).

Day +1: Add Enhancers

1. 18–22 hours posttransfection, add ExpiFectamine™ 293 Transfection Enhancer 1 and ExpiFectamine™ 293 Transfection Enhancer 2 to the transfection flask, gently swirling the flask during addition.

   Note: It is not necessary to pre-warm ExpiFectamine™ 293 Transfection Enhancer 1 and ExpiFectamine™ 293 Transfection Enhancer 2 before addition.

   ExpiFectamine™ 293 Transfection Enhancer 1 and ExpiFectamine™ 293 Transfection Enhancer 2 can be mixed together just before addition.

2. *(Optional)* If transfecting Expi293F™ Inducible Cells or Expi293F™ Inducible GnTI- Cells, add 1 mg/mL tetracycline to a final concentration of 1 µg/mL to induce protein expression.

   Note: Lower concentrations of tetracycline (ranging from 10 ng/mL to 1 µg/mL) can be added to modulate the expression of the protein of interest in a dose-dependent manner.

3. Immediately return the flask to the 37°C incubator with ≥80% relative humidity and 8% CO_2 on an orbital shaker platform.

Day +5: Harvest protein

Optimal time to harvest protein depends on the specific properties of the protein being expressed.

- For many secreted proteins, 5–7 days posttransfection is a typical harvest time to reach maximum titer.
- For membrane proteins and intracellular proteins, 3–4 days is a typical harvest time to reach maximum titer.

Transfect Expi293F™ cell lines using ExpiFectamine™ 293 Met (-) Transfection Kit for metabolic protein labeling

Note: The cell transfection instructions below are applicable to all Expi293F™ cell lines including Expi293F™ Cells, Expi293F™ GnTI- Cells, Expi293F™ Inducible Cells, and Expi293F™ Inducible GnTI- Cells.

For optimal transfection of high-density suspension Expi293F™ cell cultures, use the ExpiFectamine™ 293 Reagent included in the transfection kit.

Unlike other serum-free media formulations, Expi293™ Expression Medium does not inhibit transfection. Expi293™ Expression Medium is specifically formulated to enable transfection without the need to change or add media pre- and posttransfection.

Note: For routine transfections, proceed to “Transfect Expi293F™ cell lines using ExpiFectamine™ 293 Transfection Kit” on page 20.

Required materials

- Expi293F™ cell culture in Expi293™ Expression Medium
- Plasmid DNA preparation, sterile, free from phenol and sodium chloride, and containing mostly supercoiled DNA

Note: We recommend isolating plasmid DNA using the PureLink™ HiPure Plasmid Kit (For ordering information, see Appendix D, "Ordering information"). To ensure sterility, DNA can be filtered through a 0.22-µm filter before use.

Note: Expi293F™ Inducible Cells and Expi293F™ Inducible GnTI- Cells require an inducible expression vector (e.g., pcDNA™5/TO Mammalian Expression Vector) for inducible expression of protein of interest. For more information, see Appendix D, "Ordering information".

- pRABBIT IgG IRES-EmGFP Positive Control Vector
- ExpiFectamine™ 293 Met (-) Transfection Kit
- Expi293™ Met (-) Expression Medium, pre-warmed to 37°C

Note: Do not add antibiotics to culture media during transfection as this will decrease transfection efficiency. If necessary, antibiotics can be added to cultures approximately 24 hours posttransfection.

- Polycarborate or PETG, non-baffled, disposable, sterile, vented shaker flask for culturing suspension cells, such as Nalgene™ Single-Use PETG Erlenmeyer Flasks with Plain Bottom: Sterile
- Orbital shaker in a 37°C incubator with ≥80% relative humidity and 8% CO_2
- Reagents and equipment to determine viable cell density and percent viability

Guidelines for transfection

- Use of transfection reagents other than the ExpiFectamine™ 293 Reagent to transfect Expi293F™ cell cultures can substantially reduce performance.
- Gently invert the ExpiFectamine™ 293 Reagent 4–5 times before use to ensure thorough mixing.
- Complexation of plasmid DNA and ExpiFectamine™ 293 Reagent takes place at room temperature.
- Incubate the ExpiFectamine™ 293/DNA complexes for 10–20 minutes before adding to the cells. Longer incubation times can lead to slight reduction in performance. Incubation times over 20 minutes are not recommended.
- For Expi293F™ Inducible Cells or Expi293F™ Inducible GnTI- Cells only, the presence of blasticidin will not impact transfection, thus, there is no need to remove blasticidin before transfection.
- For scaling up transfections, see Appendix C, "Scaling up transfections".

Day –2: Subculture cells

Subculture and expand Expi293F™ cells until cell density reaches approximately 3–5 × 10^6 viable cells/mL.

Day –1: Seed cells

Seed Expi293F™ cells to a final density of 2.5–3 × 10^6 viable cells/mL, then allow cells to grow overnight.

Day 0: Perform metabolic labeling

1. Determine the number of cells required for transfection.

   Nominally, 75 × 10^6 total cells are required for every 25 mL of culture to be transfected (3 × 10^6 cells/mL). We recommend including at least 10% overage of cells to account for any loss of cells on centrifugation.

2. Centrifuge the required number of cells at 300 × *g* for 5 minutes.

3. Discard the supernatant, then resuspend cells in fresh Expi293™ Met (-) Expression Medium, pre-warmed to 37°C.

4. Incubate the cells in a 37°C incubator with ≥80% relative humidity and 8% CO_2 on an orbital shaker platform for 5–7 hours to allow the cells to exhaust their cellular supplies of methionine.

5. Determine cell density and cell viability using an automated cell counter or the trypan blue dye exclusion method.

   Note: Viable cell density should be 3 × 10^6 cells/mL at ≥95% viability to proceed with transfection. If necessary, dilute cell culture by adding fresh, pre-warmed Expi293™ Met (-) Expression Medium to achieve 3 × 10^6 cells/mL.

6. Immediately add labeled methionine to the culture flasks according to the following table:

Methionine source	Final concentration
L-Methionine (Methyl-^{13}C)	225 mg/L
L-Selenomethionine	50 mg/L

   Note: Empirically optimize the concentration of labeled methionine for your experiments.

   Note: L-Selenomethionine is toxic to cells. Therefore, use the lowest concentration required to achieve labeling of expressed protein.

   Note: For metabolic labeling with L-Methionine (Methyl-^{13}C), cells can be cultured in Expi293™ Met (-) Expression Medium containing labeling reagent for up to 3 passages before transfection. This strategy increases the efficiency of L-Methionine (Methyl-^{13}C) incorporation into the protein of interest.

Day 0: Transfect cells

1. Determine viable cell density and percent viability.
2. If necessary, dilute the cells to a final density of 3×10^6 viable cells/mL with fresh, pre-warmed Expi293™ Met (-) Expression Medium, then swirl the flask gently to mix the cells.

   Note: Discard the remaining cells. Do not re-use high-density cells for routine subculturing.
3. Gently invert the ExpiFectamine™ 293 Reagent bottle 4–5 times before use to ensure thorough mixing.
4. Dilute plasmid DNA with Opti-Plex™ Complexation Buffer, then mix by swirling or inversion.

   Note: Total plasmid DNA concentration of 1.0 µg/mL of culture volume to be transfected is appropriate for most proteins.
5. Dilute ExpiFectamine™ 293 Reagent with Opti-Plex™ Complexation Buffer, then mix by swirling or inversion.
6. Incubate at room temperature for 5 minutes.
7. Add the diluted ExpiFectamine™ 293 Reagent to the diluted plasmid DNA, then mix by swirling or inversion.
8. Incubate the ExpiFectamine™ 293/plasmid DNA complexes at room temperature for 10–20 minutes.
9. Slowly transfer the complexes to the cells, swirling the flask gently during addition, then incubate the cells in a 37°C incubator with ≥80% relative humidity and 8% CO_2 on an orbital shaker (for suggested shake speeds, see Table 9).

Day +1: Add Enhancers

1. 18–22 hours post-transfection, add ExpiFectamine™ 293 Transfection Enhancer 1 and ExpiFectamine™ 293 Met (-) Transfection Enhancer 2 to the flask, gently swirling the flask during addition.

   Note: It is not necessary to pre-warm ExpiFectamine™ 293 Transfection Enhancer 1 and ExpiFectamine™ 293 Met (-) Transfection Enhancer 2 before addition.

   ExpiFectamine™ 293 Transfection Enhancer 1 and ExpiFectamine™ 293 Met (-) Transfection Enhancer 2 can be mixed together just before addition.

2. *(Optional)* If transfecting Expi293F™ Inducible Cells or Expi293F™ Inducible GnTI- Cells, add 1 mg/mL tetracycline to a final concentration of 1 µg/mL to induce protein expression.

   Note: Lower concentrations of tetracycline (ranging from 10 ng/mL to 1 µg/mL) can be added to modulate the expression of the protein of interest in a dose-dependent manner.

3. Immediately return the flask to the 37°C incubator with ≥80% relative humidity and 8% CO_2 on an orbital shaker platform.

Day +5: Harvest protein

Optimal time to harvest protein depends on the specific properties of the protein being expressed.

- For many secreted proteins, 5–7 days posttransfection is a typical harvest time to reach maximum titer.
- For membrane proteins and intracellular proteins, 3–4 days is a typical harvest time to reach maximum titer.

A Additional guidelines

Guidelines to optimize protein expression

- Expression levels will vary depending on the specific recombinant protein expressed and the vector used; however, the Expi293™ Expression System will exhibit consistent expression level for any particular protein from one transfection to the next.
- When expressing a protein for the first time, you may want to perform a time course (e.g., harvest cells or media at several time points posttransfection) to optimize the length of the expression run.
- When expressing antibody molecules with the heavy and light chains encoded on two separate plasmids, we also recommend optimizing the ratio of heavy chain to light chain for each individual antibody. We recommend initial testing of heavy chain:light chain ratio at 1:2.

Equipment

- For optimal performance, it is critical that the shaking diameter, shaking speed, flask size/type, and volume of culture to be transfected match the recommendations in this protocol for both routine subculture and protein expression runs.
- Humidified incubators (≥80% relative humidity) are recommended to reduce evaporation during expression runs. When using multi-well plates, high-humidity settings should be used if available, as evaporation will be greater.
- Ensure equipment is calibrated for temperature. In some instances, the total heat from the incubator and the shaker can cause cell culture temperatures to exceed the recommended ranges and lead to decreased cell growth, clumping or cell death. In such instances, reduce the temperature setting of the incubator to compensate for heat generated by the shaker.
- Ensure that equipment is calibrated for CO_2. Levels of CO_2 should not exceed 8%.

Cells

- Cells should exhibit growth profiles within the guidelines of the protocol during routine cell culture maintenance (see "Expi293F™ Cells" on page 10).

Note: At 24 hours post-thaw, viability can drop to 80%, but should not get below 70%. It can take up to 7 days for cells to recover and reach ≥90% viability post-thaw.

- Expi293F™ Cells are high-density cell lines: subculture cells when density has reached log phase growth at 3–5 × 10^6 viable cells/mL. Subculturing cells before they have reached log phase growth can negatively impact cell performance.
- During all cell manipulations, mix the cells by gentle swirling; avoid vigorous mixing/pipetting, especially immediately before transfection. Cell health prior to transfection is critical to maximal performance.
- Always keep dedicated cell culture maintenance flasks: do not re-purpose remaining high-density cells from a transfection run for routine subculturing.

Plasmid DNA complexation

- Plasmid DNA is highly stable in Opti-Plex™ Complexation Buffer (or Opti-MEM™ I Medium). After ExpiFectamine™ 293 Reagent is diluted with Opti-Plex™ or Opti-MEM™ I Medium, mix by swirling the tube and/or inversion or gentle pipetting 2–3 times. Do not vortex.
- For optimal performance, once the diluted ExpiFectamine™ 293 Reagent is added to diluted plasmid DNA, mix by swirling the tube and/or inversion or gentle pipetting 2–3 times; do not vortex. Incubate 10–20 minutes post-complexation before drop-wise addition to the flasks with swirling.
- When using Expi293F™ Inducible Cells or Expi293F™ Inducible GnTI- Cells, an inducible vector such as pcDNA™5/TO Mammalian Expression Vector must be used to enable inducible expression of the protein of interest after treatment with tetracycline.

Harvest

Proteins should be harvested when cells are at ~60% or greater viable cell density posttransfection. Optimal time to harvest protein will depend on the specific properties of the protein being expressed. 5-7 days posttransfection is a typical harvest time to reach maximum titer for many secreted proteins. For membrane proteins and intracellular proteins, 3-4 days is a typical harvest time.

Cell culture supernatant clarification

- Following harvest, centrifuge the supernatant at 3,000–5,000 x g for 20–30 minutes in a refrigerated centrifuge.
- Filter supernatant through a 0.22-µm filter.

B Positive control for transfection and expression

Two different antibody expressing positive control vectors (with or without GFP) are available for assessing expression conditions in the Expi293™ Expression System.

pRABBIT IgG IRES-EmGFP Positive Control Vector

pRABBIT IgG IRES-EmGFP Positive Control Vector is provided with the various System Kits as a positive control for transfection and expression in Expi293F™ cell lines. This control vector contains a mixture of pcDNA™3.4 plasmid clones expressing the heavy and light chains of a rabbit IgG as well as Emerald Green Fluorescent Protein (EmGFP). The control is provided as a ready-to-use transfection-grade plasmid mix at a concentration of 1 mg/mL with a 1:2 heavy chain:light chain IgG ratio and is sufficient to transfect up to 150 mL of Expi293F™ cells.

Transfection and expression

Note: The transfection conditions below are identical regardless of which Positive Control Vector is to be used.

Transfect 25 mL of suspension Expi293™ cells using 25 µL of either of the positive control vectors (i.e., 1 µg of positive control per 1 mL of Expi293™ culture) following the protocol provided in "Transfect Expi293F™ cell lines using ExpiFectamine™ 293 Transfection Kit" on page 20.

The rabbit IgG that is produced in Expi293™ cells after transfection with either control vector is secreted into the culture medium, with optimal yields obtained between 5–7 days (typical yield range: 450–500 mg/L).

When using pRABBIT IgG IRES-EmGFP Positive Control Vector, EmGFP accumulates within the cells 24–96 hours post-transfection. GFP fluorescence can be used to qualitatively assess cellular transfection by fluorescence microscopy, fluorescent plate reader, or flow cytometry using standard GFP settings of 488 nm excitation and 510 nm emission.

C

Scaling up transfections

Scale up transfections

You can scale up the Expi293F™ cell cultures in spinner flasks or bioreactors. Determine the optimal spinner or impeller speed and seeding density for your culture system. We recommend that the cells be seeded at 0.3 × 10^6 to 0.5 × 10^6 viable cells/mL. Optimum spinner speed is approximately 100–130 rpm, and optimum impeller speed in Celligen™ stirred tank bioreactors is 70–100 rpm. If the split ratio of cells to fresh media is less than 1:2, centrifuge the cell suspension and re-suspend the cell pellet in fresh medium before inoculating the culture.

Use the following conditions to scale up transfections:

Table 9 Recommended volumes for transfection at various scales

Vessel type	96 deep well plate	24 deep well plate	Mini Bioreactor tube	125 mL flask	250 mL flask	1 L flask	2 L flask	3 L flask
Number of cells required	2.0 × 10^6	7.5 × 10^6	45 × 10^6	75 × 10^6	150 × 10^6	600 × 10^6	1.2 × 10^9	2.25 × 10^9
Culture volume to transfect	800 µL	2.5 mL	15 mL	25 mL	50 mL	200 mL	400 mL	800 mL
Shake speed[1] (rpm)	900 ± 50 (3 mm orbital shaking diameter)	225 ± 5 250 ± 5 235 ± 5	240 ± 5 250 ± 5 245 ± 5	125 ± 5 (19 mm orbital shaking diameter) 120 ± 5 (25 mm orbital shaking diameter) 95 ± 5 (50 mm orbital shaking diameter)				90 ± 5 90 ± 5 55 ± 5
Amount of plasmid DNA[2]	1.0 µg total plasmid DNA per mL of culture volume to transfect							
Volume of plasmid DNA	0.8 µL	2.5 µL	15 µL	25 µL	50 µL	200 µL	400 µL	800 µL
Opti-Plex™ Complexation Buffer or Opti-MEM™ I Medium[3]	50 µL	150 µL	900 µL	1.5 mL	3 mL	12 mL	24 mL	48 mL
ExpiFectamine™ 293 Reagent	2.5 µL	8 µL	50 µL	80 µL	160 µL	640 µL	1.3 mL	2.6 mL
Opti-Plex™ Complexation Buffer or Opti-MEM™ I Medium[4]	1.4 mL	140 µL	850 µL	1.4 mL	2.8 mL	11.2 mL	22.5 mL	45 mL

Table 9 Recommended volumes for transfection at various scales *(continued)*

Vessel type	96 deep well plate	24 deep well plate	Mini Bioreactor tube	125 mL flask	250 mL flask	1 L flask	2 L flask	3 L flask
ExpiFectamine™ 293 Transfection Enhancer 1	5 µL	15 µL	90 µL	150 µL	300 µL	1.2 mL	2.4 mL	4.8 mL
ExpiFectamine™ 293 Transfection Enhancer 2	50 µL	150 µL	900 µL	1.5 mL	3 mL	12 mL	24 mL	48 mL
Final culture volume	~1 mL	~3 mL	~20 mL	~30 mL	~60 mL	~240 mL	~480 mL	~960 mL

[1] Recommended shake speed ranges; optimal shake speed should be determined empirically based on the specific laboratory equipment used.
[2] Assuming a plasmid DNA stock concentration of 1mg/mL and a final concentration of 1.0 µg plasmid DNA per mL.
[3] Volume of Opti-MEM™ I Medium or Opti-Plex™ Complexation Buffer used to dilute plasmid DNA.
[4] Volume of Opti-MEM™ I Medium or Opti-Plex™ Complexation Buffer used to dilute ExpiFectamine™ 293 Reagent.

D Ordering information

Unless otherwise indicated, all materials are available through **thermofisher.com**.

Additional products

Item	Amount	Source
Expi293F™ cells	1 mL	A14527 A14527CN
	6 × 1 mL	A14528
Expi293F™ Cells (cGMP-banked)	1 vial	100044202
ExpiFectamine™ 293 Transfection Kit	1 kit for 1 L of culture	A14524
Expi293™ Expression Medium	1 L	A1435101
Opti-Plex™ Complexation Buffer	100 mL	A4096801
Expi293F™ Inducible Cells	1 mL	A39241 A39241CN
Expi293F™ GnTI- Cells	1 mL	A39240 A39240CN
Expi293F™ Inducible GnTI- Cells	1 mL	A39242 A39242CN
Expi293™ Met (-) Protein Labeling Kit	1 kit	A41249
ExpiFectamine™ 293 Met (-) Transfection Kit	1 kit for 1 L of culture	A39249
Expi293™ Met (-) Expression Medium	1 L	A4096701
pRABBIT IgG IRES-EmGFP Positive Control Vector	1 kit	A39243
Antibody-Expressing Positive Control Vector	1 vial	A14662
PNGase F Glycan Cleavage Kit	1 kit (500,000 units)	A39245
pcDNA™5/TO Mammalian Expression Vector	1 kit	V103320
pcDNA™ 3.4-TOPO™ TA Cloning Kit	1 kit	A14697
L-Methionine (Methyl-^{13}C)	225 mg	A39248
L-Selenomethionine	250 mg	A39247

(continued)

Item	Amount	Source
Tetracycline Hydrochloride	500 mg	A39246
Trypan Blue Stain	100 mL	15250061

Shaker flasks for suspension culture

Item	Capacity	Source
Nalgene™ Single-Use PETG Erlenmeyer Flasks with Plain Bottom: Sterile	125 mL	4115-0125
	250 mL	4115-0250
	500 mL	4115-0500
	1,000 mL	4115-1000
	2,000 mL	4115-2000
	2,800 mL	4115-2800

Orbital shaker

Item	Source
MaxQ™ HP Tabletop Orbital Shaker	SHKE416HP

CO_2 controlled incubator

Item	Source
Large-Capacity Reach-In CO_2 Incubator	3950

Plasmid purification products

Item	Amount	Source
PureLink™ HiPure Plasmid Midiprep Kit	25 preps	K210004
PureLink™ HiPure Plasmid Filter Midiprep Kit	25 preps	K210014
PureLink™ HiPure Plasmid Maxiprep Kit	10 preps	K210006
PureLink™ HiPure Plasmid Filter Maxiprep Kit	10 preps	K210016
PureLink™ HiPure Expi Plasmid Megaprep Kit	4 preps	K210008XP

Visualization and quantitation or control antibody

Item	Amount	Source
Protein A	25 mg	101006
F(ab')2-Goat anti-Rabbit IgG (H+L) Cross-Adsorbed Secondary Antibody, HRP	500 µg	A10547
Rabbit IgG Isotype Control	10 mg	02-6102
SimplyBlue™ SafeStain	1 L	LC6060
NuPAGE™ 4–12% Bis-Tris Protein Gel, 1.0 mm, 12-well (10 gels/box)	1 box	NP0322BOX

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, visit **thermofisher.com/support**.

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)*, 6th Edition, HHS Publication No. (CDC) 300859, Revised June 2020
  www.cdc.gov/labs/pdf/CDC-BiosafetymicrobiologicalBiomedicalLaboratories-2020-P.pdf
- Laboratory biosafety manual, fourth edition. Geneva: World Health Organization; 2020 (Laboratory biosafety manual, fourth edition and associated monographs)
  www.who.int/publications/i/item/9789240011311

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

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