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Expi293™ Expression System

USER GUIDE

For scalable transfection of Expi293F™ cells in a chemically defined, serum-free medium, using ExpiFectamine™ 293 Transfection Kit

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ThermoFisher SCIENTIFIC

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For descriptions of symbols on product labels or product documents, go to **thermofisher.com/symbols-definition**.

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

Revision history: Pub. No. MAN0019402

Revision	Date	Description
B.0	02 September 2020	Technical changes in recommended pH settings for growth.
A.0	29 May 2020	New user guide.

Contents

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. The Expi293™ Expression System Kit provides cells, culture medium, and reagents to transfect a total of 1 liter production volume.

Contents and storage

For a detailed description of each component of the Expi293™ Expression System, see "Components of the Expi293™ Expression System" on page 6. For additional ordering information, see Appendix C, "Ordering information".

Table 1 Expi293™ Expression System (Cat. No. A14635, A14635CN)

Contents	Amount	Storage
Expi293F™ Cells (1×10^7 cells/mL)	2 × 1 mL	Liquid nitrogen[1]
Expi293™ Expression Medium	1 L	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	— 2°C to 8°C 2°C to 8°C. Protect from light. 2°C to 8°C
Antibody-Expressing Positive Control Vector (at 1 mg/mL in TE Buffer, pH 8.0)[2]	150 µg	–20°C
Opti-MEM™ I Reduced Serum Medium[3]	100 mL	2°C to 8°C. Protect from light.

[1] Store the frozen cells in liquid nitrogen until ready to use. Do not store the cells at –80°C.

[2] TE buffer, pH 8.0: 10 mM Tris-HCl, 1 mM EDTA, pH 8.0.

[3] Opti-MEM™ I Reduced Serum Medium is a serum-free reagent.

Components of the Expi293™ Expression System

Expi293™ Expression System

The Expi293™ Expression System is a major advance in transient expression technology for rapid and high-yield protein production from mammalian cells. It is based on high density suspension culture of Expi293F™ Cells in Expi293™ Expression Medium. Transient expression is powered by the cationic lipid-based ExpiFectamine™ 293 Reagent in combination with specialized transfection enhancers. All components work in concert to generate 2- to 10-fold higher protein yields than previous generation transient expression systems such as the FreeStyle™ 293 Expression System. Expression yields of up to 1 gram per liter of transfected culture have been demonstrated for some antibody and non-antibody proteins. (Visit **www.thermofisher.com/expi293** for protein expression data.)

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 C, “Ordering information”.

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

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.

Note: ExpiFectamine™ 293 Transfection Enhancer 1 may occasionally exhibit a slightly yellowish tint. However, internal studies show this has no impact on system performance and protein titers.

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.

Opti-MEM™ I Reduced Serum Medium

Opti-MEM™ I Reduced Serum Medium is a serum-free medium used to complex plasmid DNA with ExpiFectamine™ 293 Reagent, providing superior protein expression through efficient transfection.

Antibody-Expressing Positive Control Vector

Antibody-Expressing Positive Control Vector is provided for transfection and expression in Expi293F™ cells. The rabbit IgG that is produced in Expi293F™ cells after transfection with the control vector is secreted into the Expi293™ Expression Medium, with optimal yields occurring 5–7 days post-transfection. For more information on using the Antibody-Expressing Positive Control Vector, see Appendix B, “Positive control for transfection and expression”.

Procedural guidelines for Expi293F™ cell culture

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; avoid vigorous shaking/pipetting. Cell health is critical for optimal performance.
- Expi293F™ cells are a robust cell line adapted to high-density growth conditions with a doubling time of approximately 24 hours during log phase growth.
- 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 may show longer doubling times and lower 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.
- 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.

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™ cells

Guidelines to thaw and establish Expi293F™ cells

- **IMPORTANT!** 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 prior to thaw.

- Expi293F™ cell lines are supplied in a vial containing 1 mL of cells at 1×10^7 viable cells/mL in 90% Expi293™ Expression Medium and 10% DMSO.
- Thaw the cells directly into Expi293™ Expression Medium, pre-warmed to 37°C.
- Before starting experiments, ensure that cells are established and frozen stocks have been prepared. On receipt, grow and freeze multiple vials of Expi293F™ cells to ensure that you have an adequate supply of early-passage cells.
- Allow freshly thawed cells to recover in culture for three or more passages post-thaw before transfecting.

Required materials

- Expi293F™ cells
- 125-mL non-baffled, disposable, sterile, vent-cap shaker flask for culturing suspension cells
- Expi293™ Expression Medium, pre-warmed to 37°C
- Orbital shaker in temperature and CO_2 controlled incubator
- Reagents and equipment to determine cell viability (for example, hemocytometer with trypan blue or cell counter)

Thaw Expi293F™ cells

1. Add 30 mL of pre-warmed (37°C) Expi293™ Expression Medium into a 125-mL polycarbonate or PETG, disposable, sterile, vented Erlenmeyer shaker flask.
2. Remove one vial of cells from liquid nitrogen, then swirl in a 37°C water bath for 1 to 2 minutes to thaw the cells rapidly until only a small amount of ice remains.

   IMPORTANT! Do not submerge the vial in the water.
3. Just before the cells are completely thawed, decontaminate the vial by wiping it with 70% ethanol before opening it in a laminar flow hood.
4. Use a 2-mL or 5-mL pipette to transfer the entire contents to the flask with 30 mL of pre-warmed (37°C) Expi293™ Expression Medium.
5. Incubate the cells in a 37°C incubator with ≥80% relative humidity and 8% CO_2 on an orbital shaker platform according to Table 2.

   Note: Other CO_2 settings (5%) can be used if necessary but may compromise cell health or protein production.

   Table 2

Shaker diameter	Shake speed (rpm)
19 mm	125 ± 5
25 mm	120 ± 5
50 mm	95 ± 5

6. Allow cells to culture for 3–4 days post-thaw, then determine viable cell density and percent viability.

   Note: Cell viability should be ≥90% 3–4 days post-thaw with viable cell density typically >1 × 10^6 viable cells/mL. Cells may be incubated for up to an additional 3 days in order to reach ≥90% viability post-thaw.
7. Perform the first subculture when the viable cell density reaches 1–3 × 10^6 viable cells/mL (typically 4–7 days post-thaw).
8. For routine cell culture maintenance, subculture cells every 3–4 days when cells reach 3–5 × 10^6 cells/mL. Do not subculture cells before reaching early log phase growth of ≥3 × 10^6 cells/mL.

Subculture Expi293F™ cells

Expi293F™ cells are capable of achieving high cell densities; therefore, we recommend that the cells attain a minimum density of 3–5 × 10^6 viable cells/mL at the time of subculturing.

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

Passage 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 and the recommended culture volumes in .

Table 3 Recommended seeding densities for routine cell culture maintenance

Sub-culture timing	Recommended seeding density
For cells ready 3 days post-subculture	0.4–0.6 × 10^6 viable cells/mL
For cells ready 4 days post-subculture	0.2–0.4 × 10^6 viable cells/mL

Table 4 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	120–140 mL	
1 L	240–280 mL	
2 L	480–560 mL	
3 L	720–840 mL	90± 5 rpm (19 mm shaking diameter) 85± 5 rpm (25 mm shaking diameter) 80± 5 rpm (50 mm shaking diameter)

Note: If using volumes outside of the recommended ranges, it is critical to ensure that all cell growth (i.e., doubling times), health (i.e., cell diameter, viability) and expression levels remain consistent with control conditions. Cell performance will be decreased if cell health is compromised. Typically, when using higher volumes shaking speed will need to be increased to the upper limit of the ranges provided above.

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: Cells that are subcultured at densities outside of this early log-phase growth window may show longer doubling times and lower 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 through step 3 to maintain or expand the cells for transfection.

Cryopreserve Expi293F™ cells

Expi293F™ cells can be frozen directly in Expi293™ Expression Medium plus DMSO or a mixture of conditioned culture medium plus DMSO. When freezing Expi293F™ cells, follow these recommendations.

1. Centrifuge cells that have attained a viable cell density of 3–5 x 10^6 viable cells/mL at 300 × *g* for 5 minutes to pellet the cells.

2. Discard the spent medium, replace the spent medium with ice-cold Expi293™ Expression Medium with 10% DMSO, then gently resuspend the cell pellet by pipetting.

3. Dilute the cells to a final density of 1 × 10^7 viable cells/mL in 1 mL total volume of 90% fresh Expi293™ Expression Medium and 10% DMSO.

   Note: Alternatively, conditioned medium obtained following centrifugation of the cells prior to freeze down can be added to fresh Expi293™ Expression Medium in the following ratios: 45% fresh Expi293™ Expression Medium, 45% conditioned medium and 10% DMSO to generate a conditioned freeze medium.

4. Freeze the cells in an automated or manual controlled-rate freezing apparatus following standard procedures.

   Note: For ideal cryopreservation, the freezing rate should be a decrease of 1°C per minute.

5. Transfer frozen vials to liquid nitrogen for long-term storage.

Transfect Expi293F™ cells

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

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 C, "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

- Allow freshly thawed cells to recover in culture for three or more passages post-thaw before transfecting.
- During all cell manipulations, mix the cells by gentle swirling; avoid vigorous mixing/pipetting. Cell health is critical to maximal performance.
- Use of transfection reagents other than the ExpiFectamine™ 293 Reagent to transfect Expi293F™ cultures can lead to substantially reduced 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.
- Once combined, you should add the ExpiFectamine™ 293/DNA complexes to the cells after a 10–20 min incubation period. Longer hold times may lead to slight losses in performance. Hold times over 20 minutes are not recommended.

Scale up transfections

You can scale up the Expi293F™ 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. For more information on protein expression in 3L bioreactors, see page 18. 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 5 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 (3mm obital shaking diameter	225±5 250±5 235±5	240±5 250±5 245±5	125±5 (19mm orbital shaking diameter) 120±5 (25mm orbital shaking diameter) 95±5 (55mm orbital shaking diameter)				90±5 90±5 55±5
Amount of plasmid DNA	1.0 µg total plasmid DNA per mL of culture volume to transfect							
Volume of plasmid DNA[2]	0.8 µL	2.5 µL	15 µL	25 µL	50 µL	200 µL	400 µL	800 µL
Opti-MEM™ I Reduced Serum 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-MEM™ I Reduced Serum Medium[4]	50 µL	140 µL	850 µL	1.4 mL	2.8 mL	11.2 mL	22.5 mL	45 mL
ExpiFectamine™ 293 Transfection Enhancer 1	5 µL	15 µL	90 µL	150 µL	300 µL	1.2 mL	2.4 mL	4.8 mL

Table 5 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 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 Reduced Serum Medium used to dilute plasmid DNA
[4] Volume of Opti-MEM™ I Reduced Serum Medium used to dilute ExpiFectamine™ 293 Reagent

Transfect Expi293F™ cells

During all cell manipulations, mix the cells by gentle swirling; avoid vigorous mixing/pipetting. Cell health is critical to maximal performance.

Refer to Table 3 for suggested volumes for transfection at various scales.

1. Subculture and expand Expi293F™ cells until the cells reach a density of approximately 3–5 × 10^6 viable cells/mL.

Day –1: Split cells

2. On the day prior to transfection (Day –1), split the Expi293F™ culture from Step 1 to a final density of 2.5 –3 × 10^6 viable cells/mL and allow the cells to grow overnight.

Day 0: Transfect cells

3. On the next day (Day 0), determine viable cell density and percent viability.

   The cells should have reached a density of approximately 4.5–5.5 × 10^6 viable cells/mL. Viability should be 95–99% to proceed with transfection.

4. Dilute the cells from Step 2 to a final density of 3 × 10^6 viable cells/mL with fresh Expi293™ Expression Medium, pre-warmed to 37°C. Swirl the flasks gently to mix the cells.

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

5. Prepare ExpiFectamine™ 293/plasmid DNA complexes as described (see Table 5 for recommended volumes).

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

   a. Gently invert the ExpiFectamine™ 293 Reagent bottle 4–5 times to mix.

   b. Dilute plasmid DNA with Opti-MEM™ I Reduced Serum Medium. Mix by swirling the tube and/or by inversion.

c. Dilute ExpiFectamine™ 293 Reagent with Opti-MEM™ I Reduced Serum Medium. Mix by swirling the tube and/or by inversion or gentle pipetting 2–3 times and allow to incubate at room temperature for 5 minutes prior to initiating the plasmid DNA complexation reaction.

d. Add the diluted ExpiFectamine™ 293 Reagent to diluted plasmid DNA. Mix by swirling the tube and/or by inversion or gentle pipetting 2–3 times.

6. Incubate ExpiFectamine™ 293/plasmid DNA complexes (from Step 5d) at room temperature for 10–20 minutes, and then slowly transfer the solution to the shaker flask from Step 4, swirling the flask gently during addition.

7. Incubate the cells in a 37°C incubator with a humidified atmosphere of 8% CO_2 in air on an orbital shaker (for suggested shake speeds, see Table 4).

Day 1: Add ExpiFectamine™ 293 Transfection Enhancer 1 and ExpiFectamine™ 293 Transfection Enhancer 2

8. On the day after transfection (Day 1, 18–22 hours post-transfection), add ExpiFectamine™ 293 Transfection Enhancer 1 and ExpiFectamine™ 293 Transfection Enhancer 2 to the flask (see Table 5), gently swirling the flask during addition. Return the flask to the 37°C incubator with a humidified atmosphere of 8% CO_2 with shaking.

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

   Note: ExpiFectamine™ 293 Transfection Enhancer 1 and ExpiFectamine™ 293 Transfection Enhancer 2 may be pre-mixed together just prior to adding to flasks for convenience.

Day 5:

9. Optimal time to harvest protein will depend on the specific properties of the protein being expressed. 5–7 days post-transfection is a typical harvest time to reach maximum titers for many secreted proteins. For membrane proteins, 3–4 days is a typical harvest time.

Protein expression in 3L Bioreactors

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™ kits (For ordering information, see Appendix C, "Ordering information"). To ensure sterility, DNA can be filtered through a 0.22-µm filter before use.
- Opti-MEM™ I Reduced Serum Medium

  Note: If transfection under AOF (animal origin free) conditions is desired, Opti-MEM™ I can be replaced with Opti-Plex™ Complexation Buffer.
- 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 post-transfection.
- Polycarborate or PETG, non-baffled, disposable, sterile, vented shaker flask for culturing suspension cells
- Orbital shaker in a 37°C incubator with ≥80% relative humidity and 8% CO_2
- 3L HyPerforma™ Glass Bioreactor with HyPerforma™ G3Lab Controller or comparable
- Nalgene™ PETG bottles with transfer caps or other transfer bottles
- Sterile Tube Welder
- Reagents and equipment to determine viable cell density and percent viability
- Reagents and equipment to determine gas concentrations, pH, and metabolites

Recommended volumes for 3L Bioreactor transfection

Table 6

Reagent	Volume
Culture volume to transfect	1.8 L
Amount of plasmid DNA required	1.0 mg total plasmid DNA per liter of culture to transfect
Volume of plasmid DNA[1] required	1.8 mL
Volume of Opti-MEM™ I Reduced Serum Medium or Opti-Plex™ Complexation Buffer required to dilute plasmid DNA	90 mL
Volume of ExpiFectamine™ 293 Reagent	5.8 mL

Table 6 *(continued)*

Reagent	Volume
Volume of Opti-MEM™ I Reduced Serum Medium or Opti-Plex™ Complexation Buffer required to dilute ExpiFectamine™ 293 Reagent	90 mL
ExpiFectamine™ 293 Transfection Enhancer 1 Volume	10.8 mL
ExpiFectamine™ 293 Transfection Enhancer 2 Volume	108 mL
Final culture volume	~2 L

[1] Assuming a plasmid DNA stock concentration of 1mg/mL.

Recommended Bioreactor settings

Table 7

Parameter	Setting
Temperature	37°C ± 0.5
Working Volume	2 L
Sparger	L – Shaped Drilled Hole macrosparge
Impellers	1 x Rushton (bottom), 1 x three pitched blade (top)
Impeller Diameter	55 mm
Impeller Power Number	1.4
Agitation	140 RPM, P/V 4.5 W/m3, tip speed 0.4 m/s
Headspace Gassing	Air - 0.05 lpm
Dissolved Oxygen (DO)	40%
pH for Growth	≤7.25 controlled by CO_2
pH for Production	6.80 ± 0.05

Transfect Expi293F™ Cells in a 3L Bioreactor

1. Subculture and expand Expi293F™ cells until they reach a density of 3–5 × 10^6 viable cells/mL in a culture volume that will yield at least 9 × 10^8 viable cells to inoculate one bioreactor.

   Note: Allow cells to recover for at least three passages after thaw before transfecting.

2. Calibrate DO and pH probes and add to assembled bioreactor.

3. Sterilize bioreactor and allow to cool to room temperature.

4. Add 600 mL Expi293™ Expression Medium to the bioreactor. Turn on agitation, headspace gassing, and temperature control. Allow medium to equilibrate to temperature set point before seeding cells.

5. Seed bioreactor by adding Expi293F™ cells from step 1 to a final cell density of 1 × 10^6 viable cells/mL in a total volume of 900 mL.

6. Check calibration of pH and perform a 1 point calibration offset if needed.

7. Three days later, on the day of transfection, determine viable cell density and viability. Cells should have reached approximately 3.5–5.5 × 10^6 viable cells/mL. Viability should be >95% to proceed with transfection.

8. Add pre-warmed Expi293™ Expression Medium to the bioreactor to dilute the cells to a final density of 3 × 10^6 viable cells/mL.

9. Adjust pH set point to 6.8 ± 0.05 controlled by CO_2. Allow bioreactor to equilibrate.

10. Prepare ExpiFectamine™ 293 Reagent/plasmid DNA complexes.
    a. To a 250 mL Nalgene™ PETG bottle, add Opti-MEM™ I Reduced Serum Medium or Opti-Plex™ Complexation Buffer at 5% of the volume of cell culture to be transfected (for example, 90 mL for transfecting 1.8 L of culture).

    b. Gently invert the ExpiFectamine™ 293 Reagent bottle 4–5 times to mix.

    c. Add ExpiFectamine™ 293 Reagent to a final concentration of 3.2 mL/L of culture to be transfected to the bottle in substep 10a (for example, add 5.8 mL ExpiFectamine™ 293 Reagent to transfect 1.8 L of culture).

    d. To a second 250 mL Nalgene™ PETG bottle, add Opti-MEM™ I Reduced Serum Medium or Opti-Plex™ Complexation Buffer at 5% of the volume of cell culture to be transfected (for example, 90 mL for transfecting 1.8 L of culture).

    e. Add plasmid DNA to a final concentration of 1.0 mg/L of culture to be transfected to the second 250mL Nalgene™ PETG bottle (for example, add 1.8 mg of DNA to transfect 1.8 L of culture).

    f. Combine the two bottles by adding the diluted transfection reagent from substep 10c to the diluted DNA.

11. Incubate the ExpiFectamine™ 293 Reagent/plasmid DNA complex at room temperature for 10 minutes.

    Following the incubation attach a transfer cap to the bottle, sterile tube weld the bottle to the bioreactor, then use a peristaltic pump to transfer the solution into the bioreactor.

Note: For optimal performance, the ExpiFectamine™ 293 Reagent/plasmid DNA complex should be fully added to the bioreactor within 20 minutes of combining the contents of the two bottles.

12. Eighteen to 22 hours post-transfection:
    a. Add ExpiFectamine™ 293 Transfection Enhancer 1 at a final volume of 0.6% v/v (6 mL/L) of culture volume transfected (for example, add 10.8 mL ExpiFectamine™ 293 Transfection Enhancer 1 per 1.8 L of culture transfected)

    b. Add ExpiFectamine™ 293 Transfection Enhancer 2 to a final volume of 6% (60 mL/L) of culture volume transfected (for example, 108 mL ExpiFectamine™ 293 Transfection Enhancer 2 per 1.8 L of culture transfected).

13. Sample bioreactor daily.
    - Count cells on a cell counter.
    - Check pH, O_2, and CO_2 on a gas analyzer.
    - Check metabolites on a bioanalyzer.
    - Aseptically remove samples for analysis to determine optimal harvest time, approximately 48 hours post transfection.

Additional guidelines

- For optimal performance, it is critical that the cell density, shaking diameter, shaking speed, flask size/type and volume of culture to be transfected match the recommendations in Table 3 and Table 4 above for routine maintenance of cells.
- Ensure all equipment is calibrated prior to use. Out of specification temperature, gas volumes, and pH control can negatively affect the system and lead to reduced titers, decreased cell growth, clumping or cell death.

Guidelines to scale up into larger vessels

- For scale up into larger vessels above a 2 L working volume consideration should be taken for power input per volume (P/V), tip speed, mixing time and addition of the transfection complex. The P/V for the listed protocol is 4.5 W/m^3 and the tip speed is 0.4 m/s. This provides a robust system at the 2 L working volume scale that minimizes shear from the impeller while allowing for optimal mixing. We recommend to scale up using P/V when possible.
- When larger scales are being considered attention should be given to hold time and mixing of transfection reagents, DNA, and the transfection complex. Plan ahead to ensure each step is thoroughly mixed and incubation/addition timing is not prolonged unnecessarily.
- It should also be noted that a decrease in titer is not uncommon with increased scale. Minimizing hold and addition times, ensuring proper mixing of the transfection complex, and optimization of process parameters should help minimize this decrease.

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 recover rapidly post-thaw and exhibit growth profiles within the guidelines of the protocol during routine cell culture maintenance for 3–4 days (see "Expi293F™ Cells" on page 6).
- Expi293F™ is a high-density cell line: 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-MEM™ I Reduced Serum Medium. Once ExpiFectamine™ 293 Reagent is diluted with Opti-MEM™ I Reduced Serum 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.

Harvest

- For typical proteins, high cell viability (ideally 65–75% or greater) is observed at the time of protein harvest (i.e., 5–7 days).

Cell culture supernatant clarification

- Following harvest, centrifuge the supernatant at 3000–5000 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

Antibody-Expressing Positive Control Vector

Antibody-Expressing Positive Control Vector is provided as a positive control for transfection and expression in Expi293™ cells. The control contains pcDNA3.4 plasmid clones expressing the heavy and light chains of a rabbit IgG. The control is provided as a ready-to-use transfection-grade plasmid at a concentration of 1 mg/mL with a 1:2 heavy chain: light chain ratio and is sufficient to transfect up to 150 mL of Expi293™ cells.

Transfection and expression

Transfect 25 mL of suspension Expi293™ cells using 25 µL of the Antibody-Expressing Positive Control Vector (i.e., 1 µg of positive control per 1 mL of Expi293™ culture) following the protocol provided in the Transfecting Expi293F™ cells section.

The rabbit IgG that is produced in Expi293™ cells after transfection with the control vector is secreted into the Expi293™ Expression Medium, with optimal yields obtained between 5–7 days.

Note: The titer values referenced above were determined in crude cell culture supernatants using a Pall Life Sciences FortéBio™ Octet™ instrument equipped with a protein A biosensor.

C

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
Expi293F™ GnTI- Cells	1 mL	A39240
Expi293F™ Inducible GnTI- Cells	1 mL	A39242
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

(continued)

Item	Amount	Source
L-Selenomethionine	250 mg	A39247
Tetracycline Hydrochloride	500 mg	A39246
Trypan Blue Stain	100 mL	15250-061

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	K2100-14
PureLink™ HiPure Plasmid Maxiprep Kit	10 preps	K210006
PureLink™ HiPure Plasmid Filter Maxiprep Kit	10 preps	K2100-16
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

D

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

Life Technologies Corporation and/or its affiliate(s) warrant their products as set forth in the Life Technologies' General Terms and Conditions of Sale at **www.thermofisher.com/us/en/home/global/terms-and-conditions.html**. If you have any questions, please contact Life Technologies at **www.thermofisher.com/support**.