

Expi293™ PRO Expression System

USER GUIDE

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C	2 February 2026	Manufacturing site has changed to "Carlsbad".
B	27 June 2025	The value change has been adjusted from 30 mL to 3 mL in topic "Guidelines for addition of Expi293™ PRO Feed and Expi293™ PRO Enhancer at the time of transfection".
A	11 August 2025	Initial release.

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

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Product information

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

Product description

The Gibco™ Expi293™ PRO Expression System is an advanced HEK293-based transient expression platform delivering exceptional protein yields across a broad range of proteins including challenging and low-expressing targets. The Expi293™ PRO Expression System features an enhanced clonal cell line paired with an optimized transfection reagent, enhancer, feed, and high-performance culture medium designed to maximize cell density, transfection efficiency, and protein output. The Expi293™ PRO Expression System enables scalable, rapid, cost-effective protein production while maintaining native folding and post-translational modifications. It is easily adaptable to small-scale and automated workflows, making it ideal for both discovery and production applications.

Contents and storage

Table 1 Expi293™ PRO Expression System, (Cat. No. [A40003643](#))

Component	Amount	Storage
Expi293™ PRO Cells ^[1]	1 × 10 ⁷ cells/mL	Vapor phase liquid nitrogen ^[2]
Expi293™ PRO Expression Medium ^[3, 4]	1 L bottle	2°C to 8°C. Protect from light.
Expi293™ PRO Transfection Kit: • Expi293™ PRO Transfection Reagent^[1] • Expi293™ PRO Enhancer^[1] • Expi293™ PRO Feed^[1, 4]	For 1 L transfection	
Antibody-Expressing Positive Control Vector (at 1 mg/mL in TE Buffer, pH 8.0 ^[1])	150 µg	≤20°C
Opti-Plex™ Complexation Buffer ^[1]	100 mL	2°C to 8°C. Protect from light.

^[1] For Research Use Only. Not for use in diagnostic procedures.

^[2] Store the frozen cells in vapor phase liquid nitrogen until ready to use. Do not store cells at -80°C.

^[3] For Research Use or Further Manufacturing. Not for diagnostic use or direct administration into humans or animals.

^[4] Contains CADMIUM CHLORIDE 2.5 H₂O, NICKELOUS SULFATE 6H₂O. Prop 65: WARNING: Cancer and Reproductive Harm -www.p65warnings.ca.gov.

System components

Expi293™ PRO Cells

Expi293™ PRO cells are a clonal derivative of the HEK293F cell line that have been adapted to high-density suspension culture in Expi293™ PRO Expression Medium. These cells should be thawed and maintained directly in Expi293™ PRO Expression Medium.

Cell line characteristics:

- Expi293™ PRO cells are a robust cell line adapted to high-density growth conditions with a doubling time of approximately 23–24 hours during log phase growth.
- Expi293™ PRO cells may be sub-cultured when they reach a density of approximately 4×10^6 to 6×10^6 viable cells/mL (i.e., early log-phase growth), typically every 3–4 days.

Note: Cells that are passaged 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 4×10^6 to 6×10^6 viable cells/mL at the time of passaging.

- When maintained according to the recommended culture conditions, Expi293™ PRO cells retain a non-clumpy morphology supporting scalable protein production for over 30 passages.

Expi293™ PRO Expression Medium

Expi293™ PRO Expression Medium is a chemically-defined, serum-free, protein-free, animal origin-free, phenol red-free formulation for growth and transfection of Expi293™ PRO cells. The medium is formulated with GlutaMAX™ Supplement and does not require further supplementation.

Expi293™ PRO Expression Medium exhibits the following features:

- Supports growth of suspension Expi293™ PRO cells to densities $\geq 15 \times 10^6$ cells/mL
- Enables sustained, high-level protein expression from high-density transfected cultures across a broad range of passages
- Designed for scalable growth and transfection across volumes and vessels
- Eliminates the need for subculturing cells on the day prior to transfection.

Expi293™ PRO Transfection Reagent

Expi293™ PRO Transfection Reagent is optimized for the transfection of plasmid or linear DNA into high-density Expi293™ PRO cell cultures. Use of transfection reagents other than Expi293™ PRO Transfection Reagent to transfect high density Expi293™ PRO cells will lead to substantially reduced performance. Expi293™ PRO Transfection Reagent is a component of the Expi293™ PRO Transfection Kit.

Expi293™ PRO Enhancer

Expi293™ PRO Enhancer is a proprietary, animal origin-free formulation developed to be used in conjunction with Expi293™ PRO Transfection Reagent and Expi293™ PRO Feed to enhance protein production, resulting in maximal protein yields. Expi293™ PRO Enhancer is a component of the Expi293™ PRO Transfection Kit.

Expi293™ PRO Feed

Expi293™ PRO Feed is an optimized, chemically-defined, serum-free, protein-free, animal origin-free formulation designed to work in conjunction with Expi293™ PRO Expression Medium to support high-density transient transfections up to 7 days post-transfection. Expi293™ PRO Feed is a component of the Expi293™ PRO Transfection Kit.

Antibody-Expressing Positive Control Vector

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

The rabbit IgG produced in Expi293™ PRO cells after transfection with the control vector is secreted into the Expi293™ PRO Medium, with optimal yields obtained at 7 days post-transfection with titers ranging from 1.0–1.5 g/L when expressed at 37°C.

Note: The titer ranges referenced above were determined in crude cell culture supernatant using a Sartorius ForteBio™ Octet™ instrument equipped with a protein A sensor. Due to differences in measurement systems bias, a range of titers is provided for guidance purposes only.

Required materials not supplied

Unless otherwise indicated, all materials are available through [thermofisher.com](https://www.thermofisher.com). "MLS" indicates that the material is available from [fisherscientific.com](https://www.fisherscientific.com) or another major laboratory supplier.

Item	Amount	Source
Disposable, sterile Erlenmeyer flasks (for example, Nalgene™ Single-Use PETG Erlenmeyer Flasks with Plain Bottom: Sterile)	125 mL	4115-0125
	250 mL	4115-0250
	500 mL	4115-0500
	1 L	4115-1000
	2 L	4115-2000
	5 L	4115-5001
Reagents and equipment to determine viable cell density and percent viability	—	MLS
Temperature, Humidity, and CO ₂ controlled incubator (for example Large-Capacity Reach-In CO ₂ Incubator with reinforced shelves (ETO option))	—	3950 , 3951
25 mm Orbital shaker (for example MaxQ™ HP Tabletop Orbital Shaker)	—	SHKE416HP
3 mm Orbital shaker (for example CO ₂ Resistant Shaker)	—	88881103B
Fisherbrand™ 96-Well DeepWell™ Polypropylene Microplates	96-well	12-566-612

(continued)

Item	Amount	Source
Thomas Scientific™ AeraSeal™ Film, Sterile	—	NC0884352
Axygen™ Square Bottom Storage Microplates	24-well	14-222-350

Related products

Unless otherwise indicated, all materials are available through [thermofisher.com](#). "MLS" indicates that the material is available from [fisherscientific.com](#) or another major laboratory supplier.

Item	Amount	Source
Expi293™ PRO Cells	1 vial	A40001140
	6 vials	A40001978
Expi293™ PRO Expression Medium	1 L	A4000135501
	6 × 1 L	A4000135505
	10 L	A4000135503
	20 L	A4000135504
Expi293™ PRO Transfection Kit	1 L kit	A40002926
	6 × 1 L kit	A40002929
	10 L kit	A40002927
	5 × 10 L kit	A40002930
Opti-Plex™ Complexation Buffer	100 mL	A4096801
	500 mL	A4096803
Antibody-Expressing Positive Control Vector	1 vial	A14662



Culture Expi293™ PRO cells

Thaw and establish Expi293™ PRO cells

IMPORTANT! Store the frozen cells in vapor phase liquid nitrogen until ready to use. Do not store 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.
- For all cell manipulations, mix cells by gentle swirling and avoid vigorous shaking/pipetting that can damage the cells.
- Allow freshly thawed cells to recover until they reach their normal doubling time of 23–24 hours during log phase growth, typically 2–3 passages post-thaw.
- Expi293™ PRO cells are supplied in a vial containing 1 mL of cells at 1×10^7 viable cells/mL in 50% conditioned medium, 42.5% fresh Expi293™ PRO Expression Medium, and 7.5% DMSO.
- Thaw the cells directly into Expi293™ PRO Expression Medium pre-warmed to 37°C.

Thaw Expi293™ PRO cells

1. Add 30 mL of pre-warmed Expi293™ PRO Expression Medium to a 125-mL Erlenmeyer shake flask.
2. Remove the vial of Expi293™ PRO 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 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 initial 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 suggested for the initial cell count when cells are at low density. However, dilution is not needed during routine cell culture maintenance once the cells have attained typical growth densities.

5. Use a serological pipette to 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% CO₂ on an orbital shaker platform. The use of humidified incubators is recommended to prevent evaporation, particularly in smaller volume cultures.

Note: Set the shake speed to 125 ± 5 rpm for shakers with a 19-mm shaking diameter, 125 ± 5 rpm for shakers with a 25-mm shaking diameter and 110 ± 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. Cells should be growing as well dispersed singlet cells with viability ≥90% and viable cell density ≥1 × 10⁶ 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 passaged until viable cell density reaches ≥1 × 10⁶ viable cells/mL.

IMPORTANT! Do not proceed with the passaging of cells unless cell viability is ≥90% with a viable cell density ≥1 × 10⁶ viable cells/mL

IMPORTANT! For subsequent routine cell culture maintenance, passage cells every 3 to 4 days when the viable cell density reaches 4 × 10⁶ to 6 × 10⁶ viable cells/mL according to Table 2.

IMPORTANT! Do not subculture cells before reaching early log phase growth of ≥4 × 10⁶ cells/mL. Modify the initial seeding density to attain target cell density of 4 × 10⁶ to 6 × 10⁶ viable cells/mL at the time of passaging.

Note: Optimal cell health is critical for maximum system performance.

8. See Appendix B, “Guidelines for cryopreserving Expi293 PRO cells”.

Passage Expi293™ PRO cells

Expi293™ PRO cells are capable of achieving high cell densities; therefore, we recommend cells attain a minimum density of 4 × 10⁶ to 6 × 10⁶ viable cells/mL at the time of passaging.

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 2 and the recommended culture volumes in Table 3.

Table 2 Recommended seeding densities for routine cell culture maintenance

Passage timing	Recommended seeding density
For cells ready 3 days post-passaging	Target 0.6×10^6 cells/mL (0.55×10^6 to 0.65×10^6 cells/mL)
For cells ready 4 days post-passaging	Target 0.3×10^6 cells/mL (0.25×10^6 to 0.35×10^6 cells/mL)

Note: Adjust seeding densities within the ranges provided in Table 2 to attain target cell densities of 4×10^6 to 6×10^6 viable cells/mL at the time of passaging.

Table 3 Recommended volumes for routine cell culture maintenance

Flask size	Culture volume	Shake speed
125 mL	30 mL	125 ± 5 rpm (19 mm shaking diameter) 125 ± 5 rpm (25 mm shaking diameter) 110 ± 5 rpm (50 mm shaking diameter)
250 mL	60 mL	
500 mL	120 mL	
1 L	240 mL	
2 L	480 mL	
2.8 L	600–800 mL	110 ± 5 rpm (19 mm shaking diameter) 110 ± 5 rpm (25 mm shaking diameter) 110 ± 5 rpm (50 mm shaking diameter)
5 L	1–3 L	115 ± 5 rpm (19 mm shaking diameter) 110 ± 5 rpm (25 mm shaking diameter) 110 ± 5 rpm (50 mm shaking diameter)

Note: If culturing cells in volumes outside of the ranges above, adjust shake speeds to ensure that cell growth (i.e. doubling time), health (i.e. cell diameter, viability) and expression levels remain consistent with control conditions; cell performance will be decreased if cell health is compromised.

Table 4 Recommended Vi-CELL™ XR cell counting settings

Parameter	Value	Parameter	Value
Minimum diameter	5	Cell brightness (%)	85
Maximum diameter	50	Cell sharpness	100
Number of images	50	Viable cell spot brightness	65
Aspirate cycles	3	Viable cell spot area (%)	5

Table 4 Recommended Vi-CELL XR cell counting settings (*continued*)

Parameter	Value	Parameter	Value
Trypan Blue mixing cycles	3	Minimum circularity	0
Decluster degree	Medium		

Table 5 Recommended Vi-CELL™ BLU cell counting settings

Parameter	Value	Parameter	Value
Minimum diameter	5	Cell sharpness	7.0
Maximum diameter	50	Viable cell spot brightness (%)	40
Number of images	100	Viable cell spot area (%)	5
Aspirate cycles	6	Minimum circularity	0.10
Trypan Blue mixing cycles	6	—	—
Decluster degree	High		

- Transfer the calculated volume of cells to fresh, pre-warmed Expi293™ PRO Expression Medium in a shake flask.
- Incubate flasks in a 37°C incubator with ≥80% relative humidity and 8% CO₂ on an orbital shaker platform until cultures reach a density of 4×10^6 to 6×10^6 viable cells/mL.

Note: Cells that are passaged 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 4×10^6 to 6×10^6 viable cells/mL at the time of passaging.

- Repeat step 1–step 3 to maintain or expand cells for transfection.



Transfect Expi293™ PRO cells

Important procedural guidelines

IMPORTANT!

- **Optimize protein expression:** Total plasmid DNA of 1.0 µg per mL of culture to be transfected is appropriate for most proteins. If using more or less plasmid DNA, alter the volume of transfection reagent proportionally.
 - **Optimize antibody expression:** When expressing antibody molecules with the heavy and light chains encoded on two separate plasmids, it is critical to start with a heavy chain:light chain ratio of 1:2 to 1:3 unless alternative ratios have been determined empirically. Insufficient light chain expression will reduce titers significantly and negatively impact cell viability.
-

Procedural guidelines

Transfection guidelines

- Allow freshly thawed cells to recover in culture until cells reach a doubling time of 23–24 hours during mid-log phase growth, typically 2–3 passages post-thaw, before transfecting cells.
- During all cell manipulations, mix the cells by gentle swirling; avoid vigorous mixing/pipetting that can damage the cells. Cell health is critical to maximal performance.
- See Table 6 for suggested volumes for transfection at various scales.

Equipment guidelines

- For optimal performance, it is important to follow the recommended shaking diameter, shaking speed, flask size/type and volume of culture to be transfected provided in this protocol.
- Humidified incubators (≥80% relative humidity) are recommended to reduce evaporation during transfections. When using multi-well plates, use high-humidity settings if available, as evaporation can introduce variation and impact results.
- Ensure that the 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 the equipment is calibrated for CO₂. The levels of CO₂ should be 8 ± 0.5%.

Optimized transfection conditions

Condition	Amount
Expi293™ PRO cells	5×10^6 viable cells/mL density at transfection
Opti-Plex™ Complexation Buffer	100 μ L per mL of culture volume to be transfected (10%)
Plasmid DNA	1.0 μ g plasmid DNA per mL of culture volume to be transfected
Expi293™ PRO Transfection Reagent	4.5 μ L per mL of culture volume to be transfected (0.45%)
Expi293™ PRO Feed	80 μ L per mL of culture volume to be transfected (8%)
Expi293™ PRO Enhancer	6 μ L per mL of culture volume to be transfected (0.6%)

Prepare cells for transfection

Expi293™ PRO cells may be cultured for 1, 2, or 3 days prior to transfection.

Note: Cells should reach a density of approximately 6×10^6 to 8×10^6 viable cells/mL on the day of transfection. Adjust the seeding density as necessary to attain a cell density in this range.

1. One day prior to transfection (Day –1), split the Expi293™ PRO cell culture to a final density of 3.2×10^6 to 3.6×10^6 viable cells/mL.
or
2. Two days prior to transfection (Day –2), split the Expi293™ PRO cell culture to a final density of 1.6×10^6 to 1.8×10^6 viable cells/mL.
or
3. Three days prior to transfection (Day –3), split the Expi293™ PRO cell culture to a final density of 0.8×10^6 to 0.9×10^6 viable cells/mL.

Day 0: Transfect cells

1. Determine viable cell density and percent viability. Cells should have reached a density of approximately 6×10^6 to 8×10^6 viable cells/mL with viability $\geq 95\%$ to proceed with transfection.

Note: Using cells outside of these densities may negatively impact system performance.

2. Dilute the cells from step 1, 2, or 3 to a final density of 5×10^6 viable cells/mL with fresh Expi293™ PRO Expression Medium, pre-warmed to 37°C. Swirl the flasks gently to mix the cells. Incubate the cells in a 37°C incubator with a humidified atmosphere of 8% CO₂ in air on an orbital shaker. For suggested shake speeds, see Table 3).

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

3. Prepare Expi293™ PRO Transfection Reagent/plasmid DNA complexes. For example volumes, see Table 6.

Note: The volume of Opti-Plex™ Complexation Buffer used for plasmid DNA complexation equals 10% of the volume of culture to be transfected.

Note: Total plasmid DNA of 1.0 µg per mL of culture to be transfected is appropriate for most proteins. If using more or less plasmid DNA, alter the volume of transfection reagent proportionally.

Note: 4.5 µL of Expi293™ PRO Transfection Reagent is utilized per mL of culture volume to be transfected.

- a. Gently invert the Expi293™ PRO Transfection Reagent bottle 2–3 times to mix.
 - b. Dilute plasmid DNA with cold Opti-Plex™ Complexation Buffer. Mix by swirling the tube and/or by inversion.
 - c. Add un-diluted Expi293™ PRO Transfection Reagent to diluted plasmid DNA. Mix immediately by swirling the tube and/or by inversion to establish homogeneity.
4. Incubate Expi293™ PRO Transfection Reagent/plasmid DNA complexes from substep 3c at room temperature for 2–5 minutes without agitation. After 2–5 minutes, slowly transfer the entire complex reaction to the shaker flask from step 2, swirling the flask gently during the addition.
 5. Incubate the cells in a 37°C incubator with a humidified atmosphere of 8% CO₂ in air on an orbital shaker. For suggested shake speeds, see Table 6.

Post-transfection

Add Expi293™ PRO Enhancer and Expi293™ PRO Feed

Tip: It is not necessary to pre-warm Expi293™ PRO Enhancer and Expi293™ PRO Feed prior to addition to flasks.

Tip: For convenience Expi293™ PRO Enhancer and Expi293™ PRO Feed may be pre-mixed together at the ratios in Table 6 prior to adding to flasks. This mixture is stable for one month when stored 2°C to 8°C and protected from light.

1. 18–22 hours post-transfection, add Expi293™ PRO Enhancer and Expi293™ PRO Feed to the flask, swirling the flask gently during addition. See Table 6 for example volumes.
Example: Add 80 µL of Expi293™ PRO Feed per mL of culture transfected (8%).

Example: Add 6 µL of Expi293™ PRO Enhancer per mL of culture transfected (0.6%).

2. Return the flask to the 37°C incubator with a humidified atmosphere of 8% CO₂ with shaking.

Optional: After the addition of Expi293™ PRO Enhancer and Expi293™ PRO Feed, culture temperature may be shifted to 32°C with 5–8% CO₂ for proteins that may benefit from expression at reduced temperatures. General guidance for proteins that may respond to temperature shift is shown in the following table; however, empirical testing should be performed to confirm impact of temperature shift for specific proteins.

Class of protein	Potential benefit from temperature shift to 32°C
Monoclonal Antibody	No
Bi-Specific Antibody	No
FC Fusion	No
Secreted Non-Antibody	Yes
Membrane	Yes
Intracellular	Yes
Low/Non-Expressing Proteins	Determine Empirically

3. The optimal time to harvest protein will depend on the specific properties of the protein being expressed. Typical harvest times range from 5–7 days post-transfection for secreted proteins and 2–3 days post-transfection for membrane and intracellular proteins.

Transfection volume recommendations

Table 6 Recommended volumes for transfection at various scales

	96 deep well plate	24 deep well plate	125 mL flask	2.8 L flask	5 L flask
Number of cells required	4.5×10^6 cells	15×10^6 cells	15×10^7 cells	4×10^9 cells	1.0×10^{10} cells
Culture volume to transfect	900 μ L	3.0 mL	30 mL	800 mL	2000 mL
Shake speed (orbital shaking diameter)	900–1000 rpm (3 mm)	225 $\pm$ 5 rpm (19 mm) 225 $\pm$ 5 rpm (25 mm) 235 $\pm$ 5 rpm (50 mm)	125 $\pm$ 5 rpm (19 mm) 125 $\pm$ 5 rpm (25 mm) 100 $\pm$ 5 rpm (50 mm)	110 $\pm$ 5 rpm (19 mm) 110 $\pm$ 5 rpm (25 mm) 110 $\pm$ 5 rpm (50 mm)	115 rpm (19 mm) 110 rpm (25 mm) 110 rpm (50 mm)
Opti-Plex™ Complexation Buffer (for plasmid DNA dilution)	10% of culture volume to be transfected				
	90 μ L	300 μ L	3.0 mL	80 mL	200 mL
Volume of plasmid DNA ^[1]	1.0 μ g total plasmid DNA per mL of culture volume to be transfected				
	0.9 μ L	3.0 μ L	30 μ L	800 μ L	2000 μ L
Expi293™ PRO Transfection Reagent	4.5 μ L per mL of culture volume to be transfected (0.45%)				
	4.1 μ L	13.5 μ L	135 μ L	3.6 mL	9 mL
Expi293™ PRO Enhancer	6 μ L per mL of culture volume to be transfected (0.6%)				
	5.4 μ L	18 μ L	180 μ L	4.8 mL	12 mL
Expi293™ PRO Feed	80 μ L per mL of culture volume to be transfected (8%)				
	72 μ L	240 μ L	2.4 mL	64 mL	160 mL
Final culture volume	~1.1 mL	~3.6 mL	~36 mL	~960 mL	~2.4 L

^[1] Assuming a plasmid DNA stock concentration of 1 mg/mL and a final concentration of 1.0 μ g plasmid DNA per mL of culture to be transfected.

Note: For flask sizes ranging from 250 mL to 2 L, scale reagent volumes proportionately based on 125 mL volumes in preceding table. Shaking speeds for these flask sizes will remain the same as the recommendations for the 125 mL scale.



Additional guidelines

Guidelines for automating the Expi293™ PRO Expression System on liquid handlers

When automating the Expi293™ PRO Expression System, several recommendations should be followed to ensure optimal performance. The example shown below is for production in 96 deep well plates; however, the principles should be followed for all liquid handler workflows regardless of expression volume.

Dilute plasmid DNA and Expi293™ PRO Transfection Reagent (Two tube transfection method)

Note: The two tube transfection method allows for a larger volume of diluted transfection reagent to be added by the liquid handler for increased pipetting accuracy.

- **Plasmid DNA dilution step:** In Tube 1, dilute plasmid DNA in ½ volume of Opti-Plex™ Complexation Buffer shown in Table 6. For example, for expression in a single well of a 96 deep well plate, add 0.9 µg plasmid DNA to 45 µL Opti-Plex™ Complexation Buffer.
 - **Transfection Reagent dilution step:** In Tube 2, Dilute Expi293™ PRO Transfection Reagent with the other ½ volume of Opti-Plex™ Complexation Buffer shown in Table 6. For example, for expression in a single well of a 96 deep well plate, add 4 µL Expi293™ PRO Transfection Reagent to 45 µL Opti-Plex™ Complexation Buffer.
-

Note: Expi293™ PRO Transfection Reagent is stable when diluted in Opti-Plex™ Complexation Buffer for up to 2 hours prior to plasmid DNA complexation.

Example protocol for expression in 96 deep well plates

Note: All volumes shown are nominal volumes; sufficient overage should be accounted for per specific liquid handler requirements and protocols.

1. Dilute Plasmid DNA for a single 96 deep well complexation reaction.
Add 45 µL Opti-Plex™ Complexation Buffer to each well of a 96 deep well plate followed by addition of 0.9 µg plasmid DNA to each well.
2. Dilute Expi293™ PRO Transfection Reagent for one 96 deep well plate.
Add 394 µL Expi293™ PRO Transfection Reagent to 4.32 mL Opti-Plex™ Complexation Buffer. Mix well by gentle inversion.
3. Initiate plasmid DNA/ Expi293™ PRO Transfection Reagent complexation.
 - Add 49.1 µL diluted Expi293™ PRO Transfection Reagent to each well of diluted plasmid DNA. Mix once by aspiration.
 - Allow complexes to form for 2–5 minutes and then add 900 µL of cells on top of the plasmid DNA/ Expi293™ PRO Transfection Reagent complexes.

Note: For maximal performance and reproducibility, it is critical to keep the complexation timing uniform within the 2–5 minute range. Modify liquid handler protocols to ensure adherence to this recommendation.

- Cover the plate and place in a 37°C incubator with a humidified atmosphere of 8% CO₂ with shaking at 900–1000 rpm on a 3 mm orbital shaker (see Table 6).
4. Add Expi293™ PRO Feed and Expi293™ PRO Enhancer (18–22 hours post-transfection).
- Pre-mix Expi293™ PRO Feed and Expi293™ PRO Enhancer. For one 96 deep well plate, add 520 µL Expi293™ PRO Enhancer to 7 mL Expi293™ PRO Feed. Mix well by inversion.
 - Add 77 µL of pre-mixed Feed/Enhancer to each well of the 96 deep well plate.
 - Cover the plate and place in a 37°C incubator with a humidified atmosphere of 8% CO₂ with shaking at 900–1000 rpm on a 3 mm orbital shaker.
 - Harvest proteins up to 7 days post-transfection.

Guidelines for addition of Expi293™ PRO Feed and Expi293™ PRO Enhancer at the time of transfection – Streamlined Protocol

In certain instances, it may be desirable to add Expi293™ PRO Feed and Expi293™ PRO Enhancer to cultures immediately post-transfection rather than the typical addition time of 18–22 hours post-transfection; see the note in this section. The addition of Expi293™ PRO Feed and Expi293™ PRO Enhancer immediately post-transfection (or at any time on the day of transfection) may be a convenient protocol option for protein expression runs that are harvested within 72 hours (i.e. for membrane and intracellular proteins, or for short screening runs) or for convenience (i.e. when the addition of Expi293™ PRO Feed and Expi293™ PRO Enhancer the following day is not possible) as well as for automation and/or high throughput screening to reduce the number of times plates must be moved into and out of the incubator.

IMPORTANT! If adding Expi293™ PRO Feed and Expi293™ PRO Enhancer immediately post-transfection, the volume of each reagent must be reduced to 50% of the volumes shown in Table 6, respectively, to reduce cell stress. For example, for a 3 mL transfection using the Streamlined Protocol, 120 µL of Expi293™ PRO Feed and 9 µL of Expi293™ PRO Enhancer should be added immediately post-transfection instead of 240 µL of Expi293™ PRO Feed and 18 µL of Expi293™ PRO Enhancer.

Note: For protein expression runs longer than 72 hours, decreased titers will be observed when using the streamlined protocol compared to traditional addition of Expi293™ PRO Feed and Expi293™ PRO Enhancer 18–22 hours post-transfection per Table 6.



Guidelines for cryopreserving Expi293 PRO cells

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

For guidance on chemical compatibility see <https://tools.thermofisher.com/content/sfs/brochures/D20480.pdf>.

Cryopreserve Expi293™ PRO cells

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 $4\text{--}6 \times 10^6$ viable cells/mL, until the desired volume of cells to be banked is ready.
2. If using conditioned medium, prepare an additional cell flask which will be used to generate the 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 $\frac{1}{2}$ 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, cell 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 use of 100% fresh culture medium for banking cells is desired, cell 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

Pre-chill all conditioned medium before use in cell banking.

1. Remove conditioned medium flask from the incubator and transfer the entire volume of cell suspension into a sterile polypropylene centrifuge tube or bottle. Cells should be at $4\text{--}6 \times 10^6$ viable cells/mL and $\geq 95\%$ viability.
2. Centrifuge the cells at $300 \times g$ for 10 minutes at 2°C to 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°C to 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 containing 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°C to 8°C or on ice until use.
2. Remove the calculated volume of cells from incubation and transfer into a sterile polypropylene centrifuge bottle/tube. Centrifuge the cells at $200 \times g$ for 5 minutes at 2°C to 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 $\sim 10\%$ 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 homogeneous mixture and keep cold on ice or cold blocks.

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

5. Use a glass serological pipette to add the calculated volume of cold, 2X freeze medium to the bottle containing 2X cell stock in conditioned medium.

6. Gently swirl the bottle to ensure a homogeneous mixture and keep cold on ice or cold blocks. 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 throughout the 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. It is recommended to keep the DMSO exposure time ≤ 60 minutes and to keep 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.



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.



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
[cdc.gov/labs/bmbi](https://www.cdc.gov/labs/bmbi)
- Laboratory biosafety manual, fourth edition. Geneva: World Health Organization; 2020 (Laboratory biosafety manual, fourth edition and associated monographs)
[who.int/publications/i/item/9789240011311](https://www.who.int/publications/i/item/9789240011311)



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 - Safety Data Sheets (SDSs; also known as MSDSs)

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

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