gibco

LV-MAX™ Lentiviral Production System

USER GUIDE

For suspension format lentiviral production in a chemically defined, serum-free medium

Catalog Number A35684

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Revision G.0

ThermoFisher SCIENTIFIC

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Revision	Date	Description
G.0	8 February 2023	Updated thaw protocol and cryopreserve protocol.
F.0	11 October 2021	The recommended volume of total plasmid DNA (LPM + LTV) was corrected from 2.5 mg to 2.5 µg per mL of culture volume (final production culture volume). See Table 6.
E.0	5 January 2021	The reagent volumes were updated in the protocol.
D.0	22 March 2019	The user guide was overhauled to bring it up to current style and standards.
C.0	24 August 2018	A related product was removed.
B.0	13 June 2018	The flask type was added.
A.0	14 July 2017	New document for LV-MAX™ Lentiviral Production System.

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™ LV-MAX™ Lentiviral Production System is a lentiviral vector production system based on transient transfection of high-density, suspension HEK293F cells adapted to a chemically defined, serum-free and protein free medium. The system is available in both Research Use and Cell Therapy Systems (CTS) options to enable a seamless transition from discovery to production.

The Gibco™ LV-MAX™ Lentiviral Production System provides cells, production medium, supplement, transfection reagent, and enhancer to produce high titer lentiviral vectors.

Contents and storage

Reagents provided in the kit are sufficient for 300 mL of lentiviral production volume.

Table 1 LV-MAX™Lentiviral Production System Starter Kit (Cat No. A35684)

Component	Cat. No.	Amount	Storage
Viral Production Cells[1] (1 X 10^7 cells/mL)	A35347	2 × 1 mL	Liquid nitrogen[2]
LV-MAX™ Production Medium	A3583401	1 L	• 2°C to 8°C • Protected from light
LV-MAX™ Transfection Kit • LV-MAX™ Supplement • LV-MAX™ Transfection Reagent • LV-MAX™ Enhancer	A35346	1 Kit • 15 mL • 2 × 0.9 mL • 12 mL	

[1] In 90% LV-MAX™ Production Medium and 10% DMSO

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

System components

The following section provides descriptions of the components in the Gibco™ LV-MAX™ Lentiviral Production System.

Viral production cells

Gibco™ Viral Production Cells are derived from the HEK293F cell line, and are adapted to suspension culture in LV-MAX™ Production Medium. These cells can be thawed directly into LV-MAX™ Production Medium.

Cell line characteristics:

- Transformed via culture with sheared human adenovirus 5 DNA
- Expresses E1A adenoviral gene
- Lacks the SV40 large T antigen
- Cell doubling time of ~26 hours
- Achieves maximum cell densities of ~1 × 10^7 cells/mL in shaker flask cultures
- High lentiviral production capabilities between cell passages 5–20

Growth medium

LV-MAX™ Production Medium is a complete, chemically defined, animal origin-free, serum-free, protein-free formulation, developed for growth and transfection of Gibco™ Viral Production Cells. This medium is ready-to-use and does not require the addition of supplements.

Transfection supplement

LV-MAX™ Supplement is a chemically defined, animal origin-free, serum-free, protein-free formulation designed to control cell growth during transfection and increase lentiviral vector production without compromising cell viability.

Transfection reagent

LV-MAX™ Transfection Reagent is uniquely designed for high efficiency co-transfection of multiple plasmids into Gibco™ Viral Production Cells, with low toxicity.

Production enhancer

LV-MAX™ Enhancer is a chemically defined, animal origin-free, serum-free, protein-free formulation that is designed to boost lentiviral vector production in Gibco™ Viral Production Cells.

Required materials not supplied

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.

Catalog numbers that appear as links open the web pages for those products.

Table 2 Materials required for lentiviral vector production

Item	Source
CO_2 resistant orbital shaker	88881101
Adjustable micropipettors	**MLS**
Laboratory mixer (Vortex mixer or equivalent)	**MLS**
3 mm orbital shaker	**MLS**
Equipment and reagents to determine cell density and viability	**MLS**
LV-MAX™ Lentiviral Packaging Mix	A43237
Opti-MEM™ I Reduced Serum Medium	A4124801, A4124802
Material for cryopreservation	
DMSO	**MLS**
Cryovials	**MLS**
Plastics	
2-mL sterile 96-deep well block	**MLS**
2-mL sterile 96-deep well block (v-bottom)	**MLS**
96-well round bottom plate	**MLS**
Nunc™ 50-mL conical tube	339653
Nalgene™ Single-Use PETG Erlenmeyer Flasks with Plain Bottom: Sterile	
125 mL	4115-0125
250 mL	4115-0250
1 L	4115-1000
2 L	4115-2000
3 L	4115-2800

2 Thaw and establish Gibco™ Viral Production Cells

Guidelines for handling cells

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

- Avoid subjecting cells to short-term, extreme temperature changes.
- After storing cells in liquid nitrogen following receipt on dry ice, allow the cells to remain in liquid nitrogen for 3–4 days before thawing.
- For all cell manipulations, mix cells by gentle swirling and avoid vigorous shaking/pipetting.
- For routine cell culture maintenance, subculture cells every 3–4 days when they reach 3.5–5.5 × 10^6 cells/mL (see “Subculture Gibco™ Viral Production Cells” on page 10). Do not subculture cells that have not reached early log phase growth of ≥3.5 × 10^6 cells/mL.

Required materials not supplied

- Gibco™ Viral Production Cells
- 125-mL Erlenmeyer Flask (e.g., Nalgene™ Single-Use PETG Erlenmeyer Flasks with Plain Bottom: Sterile for culturing Viral Production Cells, Cat. No. 4115-0125)
- Orbital shaker (e.g., MaxQ™ HP Tabletop Orbital Shaker, Cat. No. SHKE416HP)
- Temperature and CO_2 controlled incubator (e.g., Large-Capacity Reach-In CO_2 Incubator, Cat. No. 3950)
- Reagents and equipment to determine cell viability (e.g., hemocytometer with trypan blue or cell counter)

Thaw Gibco™ Viral Production Cells

1. Add 30 mL of pre-warmed LV-MAX™ Production Medium to a 125-mL Erlenmeyer shaker flask.
2. Remove the vial of Gibco™ Viral Production Cells from liquid nitrogen and 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.

   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. Use a 1-mL, 2-mL or 5-mL pipette, to gently transfer the remaing 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 viable cell density typically >1 × 10^6 viable cells/mL.

   Note: If 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 3.5 - 5.5 × 10^6 viable cells/mL according to Table 3.

   Note: Do not subculture cells before reaching early log phase growth of ≥3.5 × 10^6 cells/mL. Similarly, do not let cells overgrow above ≥5.5 × 10^6 cells/mL. Modify the initial seeding density to attain target cell density of 3.5 – 5.5 × 10^6 viable cells/mL at the time of subculturing.

Subculture Gibco™ Viral Production Cells

Gibco™ Viral Production Cells are capable of achieving high cell densities; therefore, it is important that cells attain a minimum density of 3.5–5.5 × 10^6 viable cells/mL at the time of subculturing.

Required materials

- Gibco™ Viral Production Cells cultured in LV-MAX™ Production Medium
- LV-MAX™ Production Medium, pre-warmed to 37°C
- Opti-MEM™ I Reduced Serum Medium
- Disposable, sterile Erlenmeyer flasks
- Orbital shaker (e.g., MaxQ™ HP Tabletop Orbital Shaker, Cat. No. SHKE416HP)
- Temperature and CO_2 controlled incubator (e.g., Large-Capacity Reach-In CO_2 Incubator, Cat. No. 3950)
- Reagents and equipment to determine cell viability (e.g., hemocytometer with trypan blue or cell counter)

Passage Gibco™ Viral Production 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 3 and the recommended culture volumes in Table 5.

Table 3 Recommended seeding densities for routine cell culture maintenance

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

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 volumes for routine cell culture maintenance in vented, non-baffled flasks

Flask size	Culture volume [1]	Parameter
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	
2.8–3 L	720–840 mL	90±5 rpm 85±5 rpm 80±5 rpm

[1] If using volumes outside of the recommended range, 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 is decreased if cell health is compromised.

2. Transfer the calculated volume of cells to fresh, pre-warmed LV-MAX™ Production 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 3.5–5.5 × 10^6 viable cells/mL.

Note: Cells subcultured at densities outside of the 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 3.5–5.5 × 10^6 viable cells/mL at the time of subculturing.

4. Repeat Steps 1–3 to maintain or expand cells for transfection.

Cryopreserve Gibco™ Viral Production Cells

Gibco™ Viral Production Cells can be frozen directly in LV-MAX™ Production Medium with 7.5% DMSO. Alternatively, conditioned cryopreservation medium consisting of 42.5% fresh LV-MAX™ Production Medium, 50% 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.

Cryopreserve 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 $3.5 – 5.5 \times 10^6$ 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 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 condition 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. 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 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 cryovials 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.

3 Produce lentiviral vector

Procedural guidelines

- Allow freshly thawed cells to recover in culture for three or more passages post-thaw before transfection.
- During all cell manipulations, mix the cells by gentle swirling; avoid vigorous mixing/pipetting. Cell health is critical to maximal performance.
- Gently invert the Transfection Reagent 4–5 times before use to ensure thorough mixing.
- Complexation of plasmid DNA and Transfection Reagent takes place at room temperature.
- To ensure sterility, DNA can be filtered through a 0.22-µm filter before use.
- See Table 6 for transfection at various scales.

Equipment guidelines

- It is critical that shaking diameter, shaking speed, flask size/type, and volume of culture to be transfected matches the recommendations in this protocol for both routine subculture and viral production runs for optimal performance.
- Humidified incubators (≥80% relative humidity) are recommended to reduce evaporation during expression runs. When using multi-well plates, use high-humidity settings if available, as evaporation is of greater concern.
- 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%.

Required materials

- Gibco™ Viral Production Cells cultured in LV-MAX™ Production Medium
- LV-MAX™ Production Medium, pre-warmed to 37°C
- Opti-MEM™ I Reduced Serum Medium
- Disposable, sterile Erlenmeyer flasks
- Orbital shaker (e.g., MaxQ™ HP Tabletop Orbital Shaker, Cat. No. SHKE416HP)

- Temperature and CO_2 controlled incubator (e.g., Large-Capacity Reach-In CO_2 Incubator, Cat. No. 3950)
- Reagents and equipment to determine cell viability (e.g., hemocytometer with trypan blue or cell counter)

Optimized transfection conditions

Condition	Amount
Total Opti-MEM™ I Reduced Serum Medium	2 × 5% of lentivirus production volume
Total DNA per mL of lentivirus production	2.5 µg/mL (LPM + LTV)
Ratio of lentiviral packaging mix (LPM) to lentiviral transfer vector (LTV)	3 to 2
LV-MAX™ Transfection Reagent	6 µL/mL of lentivirus production volume
LV-MAX™ Enhancer	4% of the lentivirus production volume
Gibco™ Viral Production Cells	4×10^6 cells/mL production volume
Total LV-MAX™ Supplement	5% of lentivirus production volume

Transfect Gibco™ Viral Production Cells

Use 2.5 µg total plasmid DNA per mL of culture volume to be transfected. A 3:2 ratio of lentiviral packaging plasmid (LPM) to lentiviral transfer plasmid (LTV), this translates to 1.5 µg of packaging plasmid to 1 µg of transfer plasmid.

Table 6 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
No. of cells required	3.84×10^6	12×10^6	72×10^6	1.2×10^8	2.4×10^8	9.6×10^8	19.2×10^8	38.4×10^8
Starting culture volume	816 µL	2.55 mL	15.3 mL	25.5 mL	51 mL	204 mL	408 mL	816 mL
Shake speed [1]	900±50 rpm (3 mm orbital shaking diameter)	225±5 rpm 250±5 rpm 235±5 rpm	240±5 rpm 250±5 rpm 245±5 rpm	125±5 rpm (19 mm orbital shaking diameter) 120±5 rpm (25 mm orbital shaking diameter) 95±5 rpm (55 mm orbital shaking diameter)				90±5 rpm 90±5 rpm 55±5 rpm
Total amount plasmid DNA (LPM + LTV) [2]	2.5 µg total plasmid DNA (LPM + LTV) per mL of culture volume for transfection (final production culture volume)							
Volume of LPM DNA [3]	1.44 µL	4.5 µL	27 µL	45 µL	90 µL	360 µL	720 µL	1.44 mL
Volume of LTV DNA [3]	0.96 µL	3 µL	18 µL	30 µL	60 µL	240 µL	480 µL	960 µL
Volume of OptiMEM™ I to dilute plasmid DNA [4]	48 µL	150 µL	900 µL	1.5 mL	3 mL	12 mL	24 mL	48 mL
LV-MAX™ Transfection Reagent	5.76 µL	18 µL	108 µL	180 µL	360 µL	1.44 mL	2.88 mL	5.76 mL
Volume of OptiMEM™ I to dilute transfection reagent [5]	48 µL	150 µL	900 µL	1.5 mL	3 mL	12 mL	24 mL	48 mL
LV-MAX™ Supplement	48 µL	150 µL	900 µL	1.5 mL	3 mL	12 mL	24 mL	48 mL
LV-MAX™ Enhancer	38.4 µL	120 µL	720 µL	1.2 mL	2.4 mL	9.6 mL	19.2 mL	38.4 mL
Final production volume [6]	960 µL	3.0 mL	18 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] As an option, the amount of packaging plasmid and transfer plasmid can be halved, but the change should be tested to determine the effect on transfection efficiency.

[3] Assuming a plasmid DNA stock concentration of 1 mg/mL and a final concentration of 2.5 µg plasmid DNA (LPM + LTV) per mL of culture to transfect.

[4] Volume used to dilute plasmid DNA (LPM + LTV).

[5] Volume used to dilute LV-MAX™ Transfection Reagent.

[6] Production volume minus LV-MAX™ Enhancer

Transfect cells

See Table 6 for recommended volumes for transfection at various scales.

1. Subculture and expand cells until they reach a density of 3.5–5.5 × 10^6 viable cells/mL.

Split cells (Day –1)

2. One day before performing transfection, dilute the cells from Step 1 to a final density of 3.5 × 10^6 viable cells/mL and allow the cells to grow overnight.

Transfect cells (Day 0)

3. Determine viable cell density and percent viability. Cells should have reached a density of approximately 5.0–6.0 × 10^6 viable cells/mL. Viability should be ≥95% to proceed with transfection.

4. Dilute the cells from Step 3 to a final density of 4.7 × 10^6 viable cells/mL with fresh LV-MAX™ Production Medium followed by addition of LV-MAX™ Supplement. Swirl the flasks gently to mix the cells.

Note: After the addition of all reagent's cells are transfected at a final density of 4.0 × 10^6 viable cells/mL.

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

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5. Prepare LV-MAX™ Transfection Reagent/plasmid DNA complexes.
   a. Gently invert the bottle of LV-MAX™ Transfection Reagent 4–5 times to mix.
   b. Dilute plasmid DNA with Opti-MEM™ I Medium. Mix by swirling the tube and/or by inversion.
   c. Dilute LV-MAX™ Transfection Reagent with Opti-MEM™ I Medium. Mix by swirling the tube and/or by inversion or gentle pipetting 2–3 times.
   d. Add the diluted plasmid DNA (from 5b) to the diluted LV-MAX™ Transfection Reagent (from 5c). Mix by swirling the tube and/or by inversion or gentle pipetting 2–3 times.

6. Incubate LV-MAX™ Transfection Reagent/plasmid DNA (from Step 5d) at room temperature for 10 minutes, and then slowly transfer 10% of the final production volume (e.g., 3 mL for a 30 mL final production volume) to the shaker flask, swirling the flask gently during addition.

Note: The LV-MAX™ Transfection Reagent/plasmid DNA is stable for up to 1.5 hours.

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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 5).

8. Five to six hours post transfection, add LV-MAX™ Enhancer (e.g., 1.2 mL LV-MAX™ Enhancer if transfecting 30 mL of cells).

Note: LV-MAX™ Enhancer can be added up to a maximum of14 hours post transfection.

Harvest the lentiviral vector

1. Harvest the lentiviral vector 48–55 hours post-transfection.
   a. Collect the culture medium, then centrifuge the cells at 1,300 × *g* in a swinging bucket centrifuge for 15 minutes.

      Note: For smaller volumes, use a 1.5 mL microcentrifuge tube and centrifuge in a benchtop centrifuge at 13,000 rpm for 5 minutes.

   b. Harvest the lentiviral vector by transferring the supernatant to a fresh tube.

   c. Further remove cell debris by filtration through a 0.45 µm low protein binding filter.

2. Immediately following harvest, store the lentiviral vector at -80°C.

   Note: Prepare aliquots of the supernatant to avoid multiple freeze thaw cycles.

Titer the lentiviral vector

Thaw, then titer the lentiviral vector using your method of choice (see Chapter 5, “Titer lentiviral vector”).

Note: Thaw the virus on ice before use.

4 Produce recombinant lentiviral vector in a 3-L stirred tank bioreactor

Procedural 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 5 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 for scaling up reactions

- When scaling up to vessels with a >2 L working volume, the power input per volume (P/V), tip speed, mixing time, and addition of the transfection complex must be taken into consideration. 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. It is recommended to scale up using P/V when possible.
- Ensure proper attention is given to hold time and mixing of transfection reagents, DNA, and the transfection complex. Plan ahead to ensure each step is thoroughly mixed and timing of incubation/addition is not prolonged unnecessarily.
- 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 helps to minimize decreases in titer.

Required materials

- LV-MAX™ Transfection Kit
- Gibco™ Viral Production Cells cultured in LV-MAX™ Production Medium
- Plasmid DNA preparation, sterile, free from phenol and sodium chloride, and containing mostly supercoiled DNA

  Note: Isolate plasmid DNA using the PureLink™ HiPure Plasmid Kit(see “Related products” on page 30 for ordering information)

- Opti-MEM™ I Reduced Serum Medium

- LV-MAX™ Production Medium, pre-warmed to 37°C
- Disposable, sterile Erlenmeyer flasks
- Orbital shaker (e.g., MaxQ™ HP Tabletop Orbital Shaker, Cat. No. SHKE416HP)
- Temperature and CO_2 controlled incubator (e.g., Large-Capacity Reach-In CO_2 Incubator, Cat. No. 3950)
- Reagents and equipment to determine cell viability (e.g., hemocytometer with trypan blue or cell counter)
- 3-L HyPerforma™ Glass Stir Tank 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 gas concentrations, pH, and metabolites

Transfect Gibco™ Viral Production Cells

Use 2.5 mg total plasmid DNA per liter of culture volume to be transfected. A 3:2 ratio of lentiviral packaging plasmid (LPM) to lentiviral transfer plasmid (LTV).

Table 7 Recommended volumes for 3 L stirred tank bioreactor

Reagent	Volume
Culture volume	1.6 L
Plasmid DNA	2.5 mg total plasmid DNA per liter of culture volume
Volume of plasmid DNA (LPM + LTV) [1]	4 mL
Volume OptiMEM™ I required to dilute plasmid DNA	80 mL
LV-MAX™ Transfection Reagent	9.6 mL
Volume OptiMEM™ I required to dilute transfection reagent	80 mL
LV-MAX™ Supplement	64 mL
LV-MAX™ Enhancer	80 mL
Final volume	~1.9 L

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

Table 8 Recommended bioreactor settings

Reagent	Volume
Temperature	37°C ± 0.5
Working volume	2 L
Sparger	L-shaped drilled hole macrosparge
Impellers	1 × Rushton (bottom), 1 × three pitched blade (top)
Impeller diameter	55 mm
Impeller power number	1.4
Agitation	140 rpm, P/V 4.5 W/m^3, 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 cells

1. Subculture and expand cells until they reach a density of 3.5–5.5 × 10^6 viable cells/mL in a culture volume that will yield at least 1 × 10^9 viable cells to inoculate one bioreactor.
2. Allow cells to recover for at least three passages following thaw before transfecting.
3. Calibrate DO and pH probes and add to assembled bioreactor.
4. Sterilize bioreactor and allow to cool to room temperature.
5. Add 700 mL LV-MAX™ Production Medium to the bioreactor. Turn on agitation, headspace gassing, and temperature control. Allow medium to equilibrate to temperature set point before seeding cells.
6. Seed bioreactor by adding cells from Step 1 to a final cell density of 1 × 10^6 viable cells/mL in a total volume of 1 L.
7. Check calibration of pH and perform a 1 point calibration offset if required.

Transfect cells (Day 0)

8. On the day of transfection, determine viable cell density and percent viability. Cells should have reached a density of approximately 3.5–5.0 × 10^6 cells/mL. Viability should be ≥99% to proceed with transfection.
9. Add pre-warmed LV-MAX™ Production Medium to the bioreactor to dilute the cells to a final density of 4.0 × 10^6 viable cells/mL.
10. Shift pH set point down to 6.8 ± 0.05 controlled by CO_2. Allow bioreactor to equilibrate.

11. Prepare LV-MAX™ Transfection Reagent/plasmid DNA complexes.

Note: The final concentration of 2.5 mg plasmid DNA per liter of culture to transfect contains both packaging plasmid mixture and transfer plasmid. At a 3:2 ratio, this equates to 1.5 mg packaging plasmid mixture and 1.0 mg transfer plasmid.
As an option, the amount of packaging plasmid and transfer plasmid can be halved, but the change should be tested to determine the effect on transfection efficiency.

   a. To a 250-mL PETG Nalgene bottle, add CTS™ Opti-MEM™ I Medium at 5% of the cell culture volume to be transfected (i.e., 80 mL CTS™ Opti-MEM™ I Medium for transfecting 1.6 L of culture).

   b. Gently invert the bottle of LV-MAX™ Transfection Reagent 4–5 times to mix.

   c. Add LV-MAX™ Transfection Reagent to a final concentration of 6 mL/L of culture to be transfected to the bottle in step 11a (i.e., 9.6 mL LV-MAX™ Transfection Reagent to transfect 1.6 L of cells).

   d. To a second 250 mL PETG Nalgene™ bottle, add CTS™ Opti-MEM™ I Medium at 5% of the cell culture volume to be transfected (i.e., 80 mL CTS™ Opti-MEM™ I Medium for transfecting 1.6 L of culture).

   e. Add plasmid DNA to a final concentration of 2.5 mg/L of culture to be transfected to the second 250 mL PETG Nalgene™ bottle in step 11d (i.e., 4 mg DNA to transfect 1.6 L of cells).

   f. Combine the two bottles by adding the diluted plasmid DNA (from 11e) to the diluted LV-MAX™ Transfection Reagent (from 11c).

12. Incubate LV-MAX™ Transfection Reagent/plasmid DNA (from step 11f) at room temperature for 10 minutes, then attach a transfer cap to the bottle, sterile tube weld the bottle to the bioreactor and use a peristaltic pump to transfer the solution into the bioreactor.

Note: For optimal performance, the LV-MAX™ Transfection Reagent/plasmid DNA complex should be fully added to the bioreactor within 30 minutes of initiating complexation.

13. Four hours post transfection, add LV-MAX™ Enhancer and LV-MAX™ Supplement.
   a. Add LV-MAX™ Enhancer at a final volume of 4% v/v (40 mL/L) of culture volume transfected (i.e., 64 mL LV-MAX™ Enhancer if transfecting 1.6 L of cells).

   b. Add LV-MAX™ Supplement at a final volume of 5% (50 mL/L) of culture volume transfected (i.e., 80 mL LV-MAX™ Supplement if transfecting 1.6 L of cells).

14. Sample Bioreactor daily.
   a. Count cells on a cell counter.

   b. Check pH, O_2, and CO_2 on a gas analyzer.

   c. Check metabolites on a bioanalyzer.

   d. Aseptically remove samples for analysis to determine optimal harvest time, approximately 48 hours post transfection.

5 Titer lentiviral vector

IMPORTANT! Handling of lentivirus must be performed as per institutional guidelines. All materials should be treated with a 10% bleach solution prior to disposal.

Titer using GFP expression

Procedural guidelines

- Thaw the virus on ice before use. Do not use any acceleration method because it can decrease the virus titer.
- Mix the virus by tapping or inverting the tube. Do not vortex and avoid mixing vigorously.

Required materials not supplied

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.

Catalog numbers that appear as links open the web pages for those products.

Item	Source
HT1080 cell line	ATCC, 50-188-307FP
DMEM, high glucose, GlutaMAX™ Supplement, pyruvate[1]	10569010
Polybrene [2]	Fisher Scientific™, NC0663391
96-well culture plates	**MLS**
96-well round bottom plates	**MLS**
Large swinging bucket centrifuge	**MLS**
TrypLE™ Express Enzyme (1X), no phenol red	12604013
DPBS, no calcium, no magnesium	14190250

[1] Add 10% FBS to make culture medium for HT1080

[2] Stock solution: 10 mg/mL in sterile H_2O

Perform lentiviral vector titration

Day 1 (morning) - infect HT1080 cells

1. Four hours before infection, seed a 96-well culture plate according to the following table:

Cells	Density	Volume of media
HT1080	7,000 cells/well	100 µL/well

Note: Cells are ~30% confluent at time of infection.

2. Two hours before infection, thaw the virus on ice.

Note: Do not accelerate the lentiviral vector thawing process because it can decrease the titer of the virus.

3. Prepare dilution medium by combining 15 mL of fresh culture medium with 12 µL of 10 mg/mL Polybrene (final concentration of 8 µg/mL), then vortex to mix.

4. Prepare a 4-log serial dilution (10^{-1} to 10^{-4}) by sequentially diluting 15 µL of virus into 135 µL of dilution medium per well of a round bottom 96-well plate.

Note: We recommend performing each dilution in quadruplicate.

Note: If your virus was concentrated, you may need more dilutions.

5. Remove the culture media from the HT1080 cells, then infect by transferring 100 µL of the diluted virus to each corresponding well.

6. Centrifuge the infected cell plate at 900 × *g* for 30 minutes at room temperature.

7. Incubate the infected cell plate overnight.

Day 2 (morning) – change medium, then incubate cells

1. Remove the medium from each well, then add 100 µL/well of fresh HT1080 culture medium (without Polybrene).

Note: When replacing medium, always start from the Negative Control and work backwards to minimize the amount of virus carried over.

2. Incubate the infected plate for 72 hours.

Day 5 - prepare cells, then run through a flow cytometer

1. Remove the medium from each well, then add 150 µL of trypsinization medium (75% TrypLE™ + 25% DPBS mixture) to each well.
2. Incubate the infected plate for 15 minutes in an incubator at 37°C.
3. Use a microscope to check the cells ands ensure that the cells are rounded up.
4. Detach the cells from the culture plate bottom by gently pipetting up and down.
5. Process the cells through a flow cytometer.

Calculate the lentiviral titer

1. To calculate the titer (Transforming Units/mL), determine the appropriate dilution factor to use based on the % of GFP^+ cells.

   The optimal infection range is 1–20% of GFP^+ cells. Over 20% of GFP^+ cells results in an underestimated lentiviral vector titer.

Lentivirus dilution	$EmGFP^+$ cells
10^2	96%
10^3	65%
10^4	18%

   Using this table of results, the 10^4 dilution is selected for the calculation because the % of GFP^+ cells falls in the desired 1–20% range.

2. Use the following formula to calculate the titer:

   Titer = (F × C/V) × D

   - F = frequency of GFP^+ cells (%GFP^+ cells/100)
   - C = cell number per well at the time of transduction (7,000 cells)
   - V = volume of inoculum in mL (0.1 mL)
   - D = lentivirus dilution factor

   Example:

   - F = 18/100 (using the table in Step 1)
   - C = 7,000 (cell number at the time of infection)
   - V = 0.1 (100 µL of medium)
   - D = 10^4

   The calculation is:

   Titer = (0.18 × 7,000/0.1) × 10^4 = 1.26 × 10^8 TU/mL

Titer using antibiotic selection

Procedural guidelines

- Thaw the virus on ice before use. Do not use any acceleration method because it can decrease the virus titer.
- Mix the virus by tapping or inverting the tube. Do not vortex and avoid mixing vigorously.

Required materials not supplied

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.

Catalog numbers that appear as links open the web pages for those products.

Item	Source
HT1080 cell line	ATCC, 50-188-307FP
DMEM, high glucose, GlutaMAX™ Supplement, pyruvate[1]	10569010
Polybrene[2]	Fisher Scientific™, NC0663391
Crystal violet	Fisher Scientific™, ICN15251150
Blasticidin S HCl[3]	A1113903
Puomycin[4]	A1113802
6-well culture plates	140675
TrypLE™ Express Enzyme (1X), no phenol red	12604013

[1] Add 10% FBS to make culture medium for HT1080
[2] Stock solution: 10 mg/mL in sterile H_2O and filter through 0.2 µm filter
[3] Selection medium: culture medium with Blasticidin S HCl, 10 µg/mL final concentration (the selection medium based on pLenti transfer plasmid back bone selection marker)
[4] Select culture medium final concentration 10 µg/mL

Perform lentiviral vector titration

Day 1 (morning) – infect HT1080 cells

1. Four hours before infecting cells, seed a 6-well plate according to the following table:

Cells	Density	Volume of media
HT1080	210,000 cells/well	2 mL/well

Note: Cells are ~30% confluent at time of infection.

2. Two hours before infection, thaw the virus on ice.

Note: Do not accelerate the lentiviral vector thawing process because it can decrease the titer of the virus.

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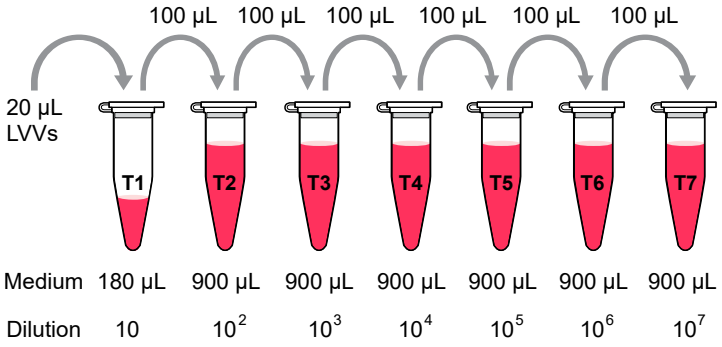
3. Prepare dilution medium by combining 20 mL of fresh culture medium with 16 µL of 10 mg/mL Polybrene (final concentration of 8 µg/mL), then vortex to mix.

4. For a virus sample, prepare seven microcentrifuge tubes and label them as T1, T2 to T7.

5. Add 180 µL of prepared dilution medium to T1, then add 900 µL to T2 through T7.

6. Add 20 µL of virus sample, crude lentivirus, or concentrated virus aliquot to T1 (10^1 dilution), then invert the tube to mix.



7. Use a new tip to transfer 100 µL of T1 dilution to T2, then invert the tube to mix (10^2 dilution).

8. Use a new tip to transfer 100 µL of T2 dilution to T3, then invert the tube to mix (10^3 dilution).

9. Repeat Step 8 to complete dilutions T4 to T7.

Note: If your virus was concentrated, you may need more dilutions.

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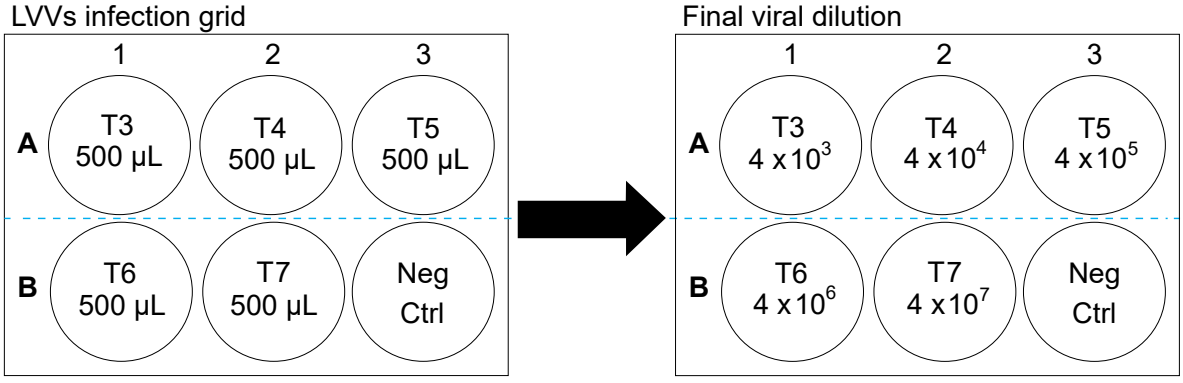
10. Replace the culture medium in A1, A2, A3, B1, and B2 of the prepared 6-well plate of HT1080 cells with 1,500 µL of prepared dilution medium, then 2,000 µL to B3 as a negative control ('Neg Ctrl')

11. Pipet 500 µL of diluted virus into each respective well, starting with T3, according to the following figure.



12. Ensure even distribution of the diluted virus across each well by moving the infected 6-well plate left, right, backward, then forward several times.

13. Centrifuge the infected cell plate at 900 × *g* (using a swinging bucket centrifuge) at room temperature for 30 minutes.

14. Incubate the infected cell plate overnight.

Day 2 (morning) – change medium, then incubate cells

1. Remove the medium from each well, then add 2 mL/well of fresh HT1080 culture medium (without Polybrene).

   Note: When replacing medium, always start from the Negative Control and work backwards to minimize the amount of virus carried over.

2. Incubate cells for 24 hours.

Day 4 – start antibiotic selection

1. Prepare HT1080 culture medium with antibiotic, such as Blasticidin with a final concentration of 10 µg/mL or Puromycin with a final concentration of 1 µg/mL.

   Note: Select antibiotic based on your pLenti-transfer back bone selection marker.

2. Replace the culture medium in each well with 2 mL of prepared selection medium, then incubate for 48 hours.

3. Repeat Step 2 until the ‘NegCtrl’ well has no cells left.

   This is an approximately 10-day process.

Day 13 – stain with Crystal Violet

1. Prepare the Crystal Violet staining solution: 1% Crystal Violet dissolved in a 10% ethanol/H_20 solution.

2. Remove the antibiotic selection medium from the 6-well plate, then wash each well with 2 mL of PBS.

3. Add 1 mL of prepared Crystal Violet staining solution to each well, then incubate the plate for 20 minutes at room temperature.

4. After 20-minutes incubation, remove the staining solution from the 6-well plate.

   Store the staining solution because it can be reused.

5. Wash the stained 6-well plate with water several times until a clear background appears.

Calculate the lentiviral titer

1. Visually count the number of stained colonies per well.

2. Use the following formula to calculate the titer (Transforming Units/mL) of the virus stock:

   Titer = (Number of discrete colonies × dilution factor) / Volume of inoculum

   Example:

   Count = 30 colonies stained in the 4×10^6 dilution well (B-1) and 5 colonies stained in the 4×10^7 dilution well (B-2)

   Volume of diluted virus = 2 mL

   The calculation is:

   Titer = $(30 \times 4 \times 10^6 + 5 \times 4 \times 10^7)/2/2 = 8 \times 10^7$ TU/mL

Related products

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

Catalog numbers that appear as links open the web pages for those products.

Item	Source
Vivid Colors™ pLenti6.3/V5-GW/EmGFP Expression Control Vector	V37006
Countess™ II Automated Cell Counter	AMQAX1000
Large capacity cell culture incubator controls 37°C, 8% CO_2 and 75–80% humidity	3950 (if applicable for scale-up)

B

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

Documentation and support

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  - 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**.