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Freedom™ ExpiCHO-S™ Kit

For transfection of ExpiCHO-S™ Cells (cGMP-banked) and development of stable cell lines for protein production

Catalog Number A46847

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Revision 1.0

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. MAN0025765

Revision	Date	Description
1.0	11 November 2021	New document for the use of Freedom™ ExpiCHO-S™ Kit

Contents

1 Product information

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

Product description

Gibco™ Freedom™ ExpiCHO-S™ Kit, co-developed with ProBioGen AG, is an easy-to-use, beginning-to-end product, for cloning and expression of recombinant proteins in Chinese Hamster Ovary (CHO)-derived suspension culture. The Gibco™ Freedom™ ExpiCHO-S™ Kit includes components for the creation of stable producing cell lines, all in a conveniently packaged kit. The Gibco™ Freedom™ ExpiCHO-S™ Kit also delivers high titers and offers simplified commercial licensing options.

- Seamless transition from transient to stable clones – all in one cell line
- Regulatory-friendly cGMP-banked cells and dual vector system provides ability to control heavy and light chain ratios
- Ability to achieve titers of 3 g/L or higher in fed batch cultures
- Short development timeline with less than 6 months needed to get from gene of interest to clone
- Access to technical support and consultation

Contents and storage

Table 1 Freedom™ ExpiCHO-S™ Kit, Cat. No. A46847

Item	Quantity	Shipping	Storage
ExpiCHO-S™ Cells (cGMP-banked), 1×10^7 cells/mL	1 mL	Dry ice	Upon receipt, immediately place in liquid nitrogen vapor phase.
ExpiCHO™ Expression Medium	1000 mL	Ambient temperature	2°C to 8°C, protect from light
One Shot™ TOP10 Chemically Competent *E. coli*	21 × 50 µL	Dry ice	-80°C
Freedom™ pCHOm 3.1 and Freedom™ pCHOp 3.2 expression vectors	1 kit	Dry ice	-10°C to -30°C

Culture conditions

Media:

- ExpiCHO™ Expression Medium (This medium is already supplemented with 4 mM GlutaMAX™ Supplement)
- ExpiCHO™ Stable Production Medium (Supplement with 4 mM GlutaMAX™ Supplement except when otherwise stated)

Incubator parameters: 37°C and 5–8% CO_2

Orbital shakers shake speed: 125 ± 5 rpm to be used with a 19 mm shaking diameter. Calculate adjusted rpm if using a different sized orbit.

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
Cells	
ExpiCHO-S™ Cells (cGMP Banked)	A29127
Transfection reagent	
ExpiFectamine™ CHO Transfection Kit	A29129
Selective pressures	
Methotrexate	Sigma-Aldrich™ A6770
Puromycin (10 mg/mL)	A1113803
Kanamycin	11815024 or 15160054
Media and components	
GlutaMAX™ Supplement	35050061
L-Glutamine	25030081
Anti-Clumping Agent	0010057AE
OptiPRO™ Serum Free Medium (SFM)	12309050
Efficient-Pro™ AGT™ Feed 2	A5221601
Efficient-Pro™ Feed 2	A5221401
EfficientFeed™ C+ 2X Supplement	A3937601
EfficientFeed™ C+ AGT™ Supplement	A2503104
ExpiCHO™ Stable Production Medium	A3711001
ExpiCHO™ Stable Production AGT™ Medium	A3711101
ClonaCell™-CHO AOF Supplement	Stemcell Technologies100-0382
DMSO	BP231-1
NaOH 1M	SS266-1
PBS, pH 7.4	10010-031
Glucose, 200 g/L	A2494001
Water For Injection (WFI) for Cell Culture	A1287301

(continued)

Item	Source
Flasks and vessels (Corning™)	
125 mL	431143
250 mL	431144
500 mL	431146
1000 mL	431147
25 cm^2	3056
75 cm^2	3276
150 cm^2	430825
225 cm^2	3001
Nalgene™ filters (0.2 micron)	
150 mL	565-0020
500 mL	569-0020
1 L	567-0020
Cell strainer	
40 µm nylon mesh	22363547
Multi-well plates (Greiner)	
6-well plates	657185
96-well plates	655185
24-well plates	662102
Cloning reagents (Molecular Devices)	
CloneMatrix	K8500
CloneDetect	K8202

Workflow overview

The diagram on the following page schematically depicts the steps necessary to express your one or two-subunit protein(s) of interest using the Freedom™ ExpiCHO-S™ Kit, and our recommended experimental path from stable transfectants to clone scale-up.

Note: The times shown for various experimental steps are approximations, and the actual times depend on your protein(s) of interest and the specific workflow you choose.

Workflow

Workflow	
	Clone your gene(s) of interest into Freedom™ pCHOm 3.1 & pCHOp 3.2
1 Day	**Transfect (≥ 2X) ExpiCHO-S™ cells (cGMP-banked)**
2–3 Weeks	**Selection Phase 1: [10P/100M] or [20P/200M][1]**
1–3 Weeks	**Selection Phase 2: [30P/500M] or [50P/1000M][1]**
5–14 Days	**Assess productivity of pools; Choose 1–4 pools for clone isolation**
17–18 Days	**Clone isolation by limiting dilution cloning (or other method) [8–10 × 96-well plates/pool]**
4–5 Weeks	**1st screen [identify ≥ 100 clones]** ▼ **2nd screen [identify ≥ 30 clones]** ▼ **3rd screen [identify top 10 clones]**

[1]**Legend**: P = µg/mL Puromycin; M = nM Methotrexate

2 Methods

Prepare cells for transfection

Required materials:

- ExpiCHO-S™ Cells (cGMP Banked)
- ExpiCHO™ Expression Medium

1. Remove the vial of ExpiCHO-S™ cells from liquid nitrogen and place in a 37°C water bath for 2 to 3 minutes to initiate thawing.
2. Just before the cells are completely thawed, decontaminate the vial by wiping it with 70% alcohol, prior to transferring to a biosafety cabinet. Ensure cells have been completely thawed prior to opening the vial.
3. Transfer the entire contents of the cryovial into a 125-mL polycarbonate, disposable, sterile, vent-cap Erlenmeyer shaker flask containing 30 mL of ExpiCHO™ Expression Medium pre-warmed to 37°C.
4. Take a sample for viable cell density (VCD) and viability. Cell viability should be ≥90%.
5. Incubate the cells in an incubator with ≥80% relative humidity, 37°C, and 5–8% CO_2 on an orbital shaker platform of 125 ±5 rpm.
6. Passage cells at 1.5 × 10^5–2 × 10^5 seeding density every 3–4 days, respectively, for 3–5 passages before transfection. Cell viability should be ≥90% and cell density between 2–8 × 10^6 viable cells/mL before each passage.

Prepare vector

Required materials:

- Freedom™ pCHOm 3.1 and Freedom™ pCHOp 3.2 vectors
- One Shot™ TOP10 Chemically Competent *E. coli*
- Kanamycin
- Restriction enzymes

1. Prior to cloning your gene(s) of interest into the Freedom™ pCHOm 3.1 and Freedom™ pCHOp 3.2 vectors, it is highly recommend that you have the nucleotide sequences optimized, not only for codon usage, but also for cryptic splice sites, RNA secondary structure, killer motifs, etc. GeneArt™ GeneOptimizer™ software is an example of service that is available to assist with this (**https://www.thermofisher.com/genesynthesis**). Also, it is recommended that your gene of interest does not have the *NruI* site as this is the restriction site used to linearize the plasmid for transfection. If you experience challenges, please contact your Thermo Fisher Scientific Field Application Scientist for additional support.

2. Insert your gene of interest into the vector/s by using the *EcoRV* (blunt) restriction enzyme and check for orientation. See Appendix A, “Vector maps”.

3. For best ligation results, ensure your insert is in molar excess of the plasmid in the ligation reaction. We recommend trying a few insert:vector ratios to ensure the isolation of the desired product (for example, 3:1 and 5:1 [insert:vector])

   IMPORTANT! Your inserts must contain an ATG initiation codon in the context of a Kozak translation initiation sequence for proper initiation of translation in mammalian cells. An example of a Kozak consensus sequence is provided below. Other sequences are possible, but the G or A at position –3 and the G at position 4 (shown in bold in the example below) illustrates the most commonly occurring sequence with strong consensus. Replacing one of the two bases at these positions provides moderate consensus, while having neither results in weak consensus. The ATG initiation codon is shown underlined.

   Example Kozak consensus sequence: (G/A)NNA<u>TG</u>**G**G

   Also, if you will be expressing secreted proteins, be sure to include a signal peptide sequence for the secretion of your protein of interest.

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4. Freedom™ pCHOm 3.1 and Freedom™ pCHOp 3.2 vectors contain the bacterial selection marker aph, which allows for selection of transformed cells by generating resistance to the antibiotic Kanamycin. When transforming One Shot™ TOP10 Chemically Competent *E. coli* with the Freedom™ pCHOm 3.1 and Freedom™ pCHOp 3.2 vectors, use 50 µg/mL of Kanamycin.

5. Prepare the resulting vector construct for transfection of the ExpiCHO™ cells by linearizing of the plasmid using the *NruI* restriction enzyme. Confirm linearization by gel electrophoresis (0.8% agarose gel).

6. Isolate linearized plasmid DNA using method of choice (Options include: PureLink™ PCR Purification Kit or to do a phenol-chloroform-isoamyl alcohol extraction followed by DNA precipitation for larger amount of DNA).

Transfect cells

Required materials:

- ExpiFectamine™ CHO Reagent
- OptiPRO™ Serum Free Medium
- ExpiCHO™ Expression Medium

The transfection method described in this section is lipid-based transfection using ExpiFectamine™ CHO Reagent. Other transfection methods, such as electroporation can also be used.

For more details and examples, see Appendix B, “Possible variations and examples”.

1. On the day prior to transfection (Day -1), split the ExpiCHO-S™ culture to a final density of 4–5 × 10^5 viable cells/mL and strain them. Allow the cells to grow overnight.

2. On the day of transfection (Day 0), determine viable cell density and viability. The cells should have reached a density of approximately 1 × 10^6–1.5 × 10^6 viable cells/mL. Viability should be 95–99% to proceed with transfection.

3. Dilute the cells to a final density of 1 × 10^6 viable cells/mL (in total 30 mL) with fresh ExpiCHO™ Expression Medium, pre-warmed to 37°C. Mix the cells by gently swirling the flask.

4. Prepare ExpiFectamine™ CHO/linearized plasmid DNA complexes using cold reagents (4°C). Total plasmid DNA in the range of 1–2 µg per mL of culture volume to be transfected is appropriate.
   a. Gently invert the ExpiFectamine™ CHO Reagent bottle 4–5 times to mix thoroughly.
   b. Dilute plasmid DNA with cold OptiPRO™ Serum Free Medium to a total volume of 1 mL. Mix by inversion.
   c. Dilute 40 µL of ExpiFectamine™ CHO Reagent with 0.96 mL OptiPRO™ Serum Free Medium. Mix by inversion. Do not exceed 5 minutes before complexation with diluted DNA.
   d. Add the diluted ExpiFectamine™ CHO Reagent to diluted DNA. Mix by swirling the tube or by inversion.

5. Incubate ExpiFectamine™ CHO/plasmid DNA complexes (from substep 4d) at room temperature for 1–5 minutes as after such time the efficacy decreases, and then slowly transfer the solution to the shaker flask from step 3, swirling the flask gently during addition.

6. Incubate the cells in a 37° C incubator with a humidified atmosphere of 5–8% CO_2 on an orbital shaker (19 mm throw at 125 ±5 rpm).

7. On day 2 after transfection check flasks for viability, viable cell counts, and titer (above 4 mg/L ideally).

8. Passage flasks into selection.

Selection

To achieve high protein producing cell lines, a two-phase selection scheme generates up to 4 pools of stably-transfected cells in which the linearized Freedom™ pCHOm 3.1 and pCHOp 3.2 vectors have integrated into the host cell genome. Perform the selection using complete ExpiCHO™ Stable Production Medium + 4mM GlutaMAX™ Supplement containing a combination of Puromycin and Methotrexate. Note that only cells that have been transfected with the Freedom™ pCHOm 3.1 and pCHOp 3.2 construct can be propagated in ExpiCHO™ Stable Production Medium containing Puromycin and Methotrexate, because untransfected ExpiCHO-S™ Cells (cGMP Banked) lack pac activity and only have basal DHFR activity.

Workflow

The workflow depicts the major steps of the two-phase selection scheme to generate a pool of stably-transfected cells. The two-phase selection scheme takes approximately 3–7 weeks to complete.

Workflow

Selection Phase 1: [10P/100M] or [20P/200M][1]

VCD ≥ 1× 10^6; V ≥85%

Selection Phase 2: [30P/500M] or [50P/1000M][1]

V ≥90%

Proceed to stable pool assessment

[1]**Legend**: P = µg/mL Puromycin, M = nM Methotrexate, VCD = viable cell density (cells/mL), V=viability

Selection Phase 1

Required materials for Selection Phase 1 and Phase 2:

- ExpiCHO™ Stable Production Medium
- GlutaMAX™ Supplement
- Methotrexate
- Puromycin

1. Passage cells into two shake flasks, each with 30 mL each of ExpiCHO™ Stable Production Medium at a density of 5 × 10^5 viable cells/mL.

   This procedure is all in shake flasks, for optional methods in T-flasks see "T-flask: Selection Phase 1" on page 29.

2. To the shake flasks containing the transfected cells, add 10P to one and 20P to the other. See "Workflow" on page 14.

   Note: 10P = 100 nM of Methotrexate and 10 µg/mL of Puromycin; 20P = 200 nM of Methotrexate and 20 µg/mL of Puromycin.

3. Place the shake flasks in the 37°C incubator, 5–8% CO_2, and 125 rpm.

4. Sample flasks on day 7 post-selection for a viable cell count only. This will serve as a reference data point; if selection is occurring, you can expect a drop in viability and cell density relative to day 0 of selection. If viability is >30%, proceed directly to step 7. Otherwise, return the flask to incubator and proceed to step 5.

5. Sample flasks on day 10 or 11 post-selection for a viable cell count. If cell viability is still below the last measured value, perform a complete media exchange by centrifuging (200 × *g*, 5 min) the cells and re-suspending them in fresh ExpiCHO™ Stable Production Medium with pressure. Reduce the volume as needed to keep the viable cell density above 3 × 10^5 viable cells/mL. If viability has increased since day 7, perform a complete media exchange as described, and transfer cells to a new flask as described in step 6.

6. Sample the flasks every 3–4 days and perform a complete medium exchange once a week until the cells show signs of recovery.

7. As soon as the cells show signs of recovery, passage them to a new shake flask at a seeding density of 3 × 10^5 viable cells/mL. Typically, this transition occurs when viability is between 30–50%.

8. Incubate the cells on a shaking platform at a 37°C, 80% relative humidity, and 5–8% CO_2.

9. Passage the cells in shake flasks every 3–4 days, seeding them at 3 × 10^5 viable cells/mL at each passage. Maintain selective pressure appropriate for the volume of fresh medium added. Centrifugation for full medium exchange is only required when the dilution factor is <2. Use a cell strainer whenever clumping is observed.

10. Selection Phase 1 is complete when viability exceeds 85% and the viable cell density exceeds 1×10^6 viable cells/mL.

11. Cryopreserve (see "Cryopreservation" on page 23) at least 3 vials of cells from each Selection Phase 1 pool as a back-up and proceed directly to Selection Phase 2.

Selection Phase 2

1. For each Selection Phase 1 pool, determine the viable and total cell counts.

2. Seed two new 125-mL shake flask per Selection Phase 1 pool flask at 5×10^5 viable cells/mL in 30 mL of ExpiCHO™ Stable Production Medium and add Selection Phase 2 pressures (30P and 50P).

   Note: 30P = 500 nM of Methotrexate and 30 µg/mL of Puromycin; 50P = 1000 nM of Methotrexate and 50 µg/mL of Puromycin.

3. Incubate the cells. Sample the flasks every 3–4 days; if cells do not show signs of recovery (i.e., cell densities above the last measured value), leave as-is and perform a complete medium exchange once a week. Once cells show signs of recovery, proceed to step 4.

4. Passage the cells in shake flasks every 3–4 days, seeding them at 3×10^5 viable cells/mL at each passage. Maintain selective pressure appropriate for the volume of fresh media added. Cell pelleting and full media exchange is only required when the dilution factor is <2.

5. Selection is complete when viability meets or exceeds 90%.

6. Cryopreserve (see "Cryopreservation" on page 23) at least 5 vials of cells from each stable pool as a back-up and proceed to "Productivity assessment of the pools" on page 16.

Productivity assessment of the pools

1. Seed fully recovered cell pools (viability >90%) at 3×10^5 viable cells/mL using 30 mL ExpiCHO™ Stable Production Medium without selective pressure in 125-mL shake flasks.

2. Incubate the cells on a shaking platform at 37°C, 80% relative humidity, 5–8% CO_2.

3. Sample cultures on days 0, 4, 7, 10, 12, and 14 to determine the cell density, viability, and productivity until culture viability drops below 50% or day 14 of culture is reached.

4. After sampling, feed the cultures with glucose as follows (adjust if necessary):

Day	Glucose only	Glucose + feed
Day 4	4 g/L glucose	2 g/L glucose and 5% feed
Day 7	4 g/L glucose	2 g/L glucose and 7.5% feed
Day 10	4 g/L glucose	2 g/L glucose and 10% feed

Limiting dilution cloning

Workflow

The flowchart depicts the major steps to obtain a clonal cell line (i.e., derived from a single cell) by diluting the pool(s) of stably transfected cells to ½ a cell per well in a 96-well plate containing ExpiCHO™ Stable Production Medium + 6 mM L-glutamine. In most cases, a single cell will form a distinct colony that can be scaled up using the procedures described in this section. You may also statistically calculate the desired number of cells per well to help ensure monoclonality, or subclone the top-producing clones.

Workflow

2 Hours	**Prepare cloning medium and determine cell number and viability**
4 Hours	**Serially dilute cells and seed 96-well plates (0.5–1 cells/well)**
12–18 Days	**Grow single-cell clones undisturbed and without media change until 60–100% confluent**
	Proceed to clone scale up

Limiting dilution cloning

Limiting dilution cloning is used to isolate and scale up single clones from the pools. For this section we recommend 6mM of L-glutamine instead of GlutaMAX™ Supplement to supplement the media.

Required materials:

- Cloning medium: ExpiCHO™ Stable Production Medium + 6 mM L-glutamine + cloning supplement
- Cell strainer
- Multi-channel pipettor
- 96-well plates

1. To create cloning medium, add 6 mM L-glutamine to ExpiCHO™ Stable Production Medium and pre-warm it to 37°C. It is recommended to use a cloning supplement (such as ClonaCell™-CHO AOF Supplement from Stemcell Technologies) or conditioned media to increase cloning efficiency.

2. Thaw frozen stable pool(s) for limiting dilution cloning 2–5 days in advance without selection pressure; no more than one passage should be needed to reach >90% viability before seeding limiting dilution cloning. However, two to three passages are recommended for the cells to adapt to L-glutamine instead of GlutaMAX™ Supplement.

3. For each pool, label five 50-mL conical tubes “1” through “5”.

4. Use a cell strainer such as Fisherbrand™ 40-µm nylon mesh cell strainer to obtain a uniform single-cell suspension into the 50-mL tube labeled "1".

5. Accurately determine the viable cells/mL of the strained pool.

6. Serially dilute the cells to a final concentration of 1,000 viable cells/mL using cloning medium as in the example pictured.

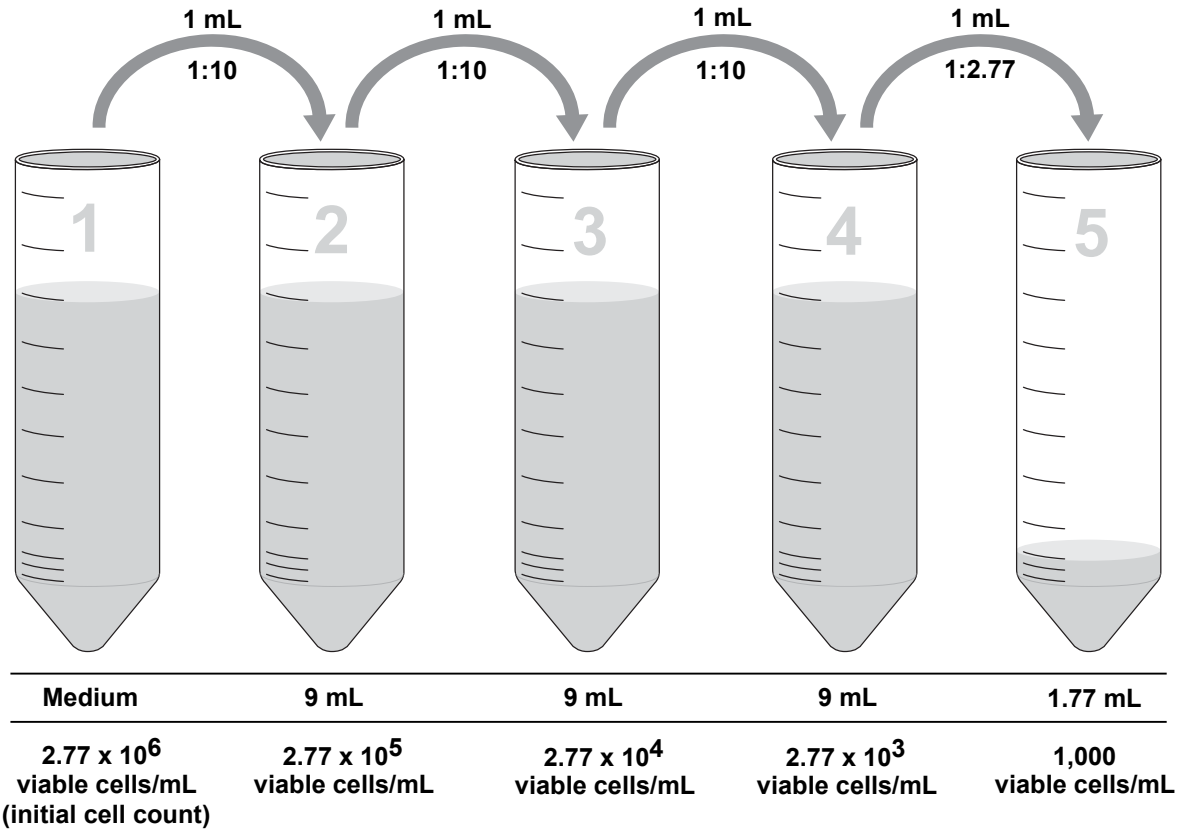

Figure 1 Serial dilution of viable cells

7. Manually count the last dilution (Tube 5) and adjust the volume accordingly. For example, pipette 8 µL of the cell suspension into 12 wells of a 96-well plate (96 µL total volume). Allow the cells to settle and examine them under the microscope. Counting is facilitated by maintaining the 8 µL as a droplet in each well. If the cells in tube 5 are at 1,000 cells/mL, then you will count 96 cells.

8. Pipette 0.1 mL of the cell suspension from "Tube 5" (1,000 cells/mL) into tubes containing 39.9 mL of cloning medium. This brings the final volume in each tube to 40 mL with a cell density of 2.5 cells per mL, allowing a seeding density of 0.5 cell per well when 200 µL of diluted cells is added into each well.

9. Mix the cell suspension gently by inverting the tube 5 or 6 times and transfer it into a sterile reagent reservoir or trough.

10. Using a multi-channel pipettor, aseptically dispense 200 µL of the diluted cells into each of the wells of the 96-well plate.

Note: To make it easier to later see and focus the cells under the microscope, add 20 µL (about 20 cells) of the 1000 cell/mL (Tube 5) into the first well (A1) of each 96-well plate.

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11. Incubate the plates undisturbed for 14 days at 37°C and 5–8% CO_2 in humidified air in a static (non-shaking) incubator. Stack no more than 5 plates together.

12. After day 14 of incubation, examine the wells visually using a microscope for growth of monoclonal colonies.

Note: If the note of step 10 was followed, A1 wells can be used to focus the microscope as they will have a lot of cells.

Optional: On Day 13–14, feed wells with 25–50 µL of ExpiCHO™ Stable Production Medium supplemented with 6 mM L-glutamine.

13. Perform your primary screen on Day 14 to 16 to identify clones of interest.

14. Calculate the percent cloning efficiency:

$$\text{Cloning efficiency} = 100\% \times \frac{\text{Number of wells showing growth}}{\text{Number of wells seeded x cells per well seeded}}$$

For example, the cloning efficiency with 120 colonies growing out of total 600 seeded wells (10 plates with 60 seeded wells/plate) at a seeding density of 0.5 cell per well is 40%.

Clone scale up and screening

Workflow

	Workflow
	Single-cell protein-producing clones in 96-well plate
2–5 days	**Transfer each clone to a well of a 24-well plate containing 0.5–1 mL of pre-warmed medium[1]**
2–5 days	**Transfer each clone to a well of a 6-well plate containing 2–2.5 mL pre-warmed medium[1]**
2–5 days	**Passage each clone in 6-well plate (static or shaking) (e.g., 1 mL of each clone into 2 mL of pre-warmed medium)[1]**
5 days	**Secondary screen: Seed each clone to a well of a 6-well plate (static or shaking) at 3 × 10^5 in 3 mL of medium; sample for protein production on day 5.**
2–5 days	**Passage top 30 clones at 2 × 10^5–3 × 10^5 cells/mL in a 125-mL shake flask with 15–30 mL of pre-warmed medium[1]**
14 days	**Tertiary screen: Seed top 30 clones at 3 × 10^5 cells/mL, in a 125-mL shake flask, at 125 rpm. Perform 14-Day fed-batch to identify the top 10 clones.**
	Proceed to optimize protein production using different culture conditions, or continue the scale-up procedure to meet your bioproduction needs.

[1]**Legend**: ExpiCHO™ Stable Production Medium + 4 mM GlutaMAX™ Supplement + 1% Anti-Clumping Agent

Scale up of the individual clones and basic screening

Required materials:

- ExpiCHO™ Stable Production Medium + 4 mM GlutaMAX™ Supplement + 1% Anti-Clumping Agent
- 24-well plates
- 6-well plates
- 125-mL shake flask
- Glucose
- Feed

1. When individual clones in a 96-well plate are >60% confluent (day 17 or 18 post-seeding), aseptically harvest the desired clones by pipetting the cells up and down gently. Transfer the entire contents of each well into a separate well of 24-well tissue culture plates containing 0.3–0.5 mL of fresh ExpiCHO™ Stable Production Medium + 4 mM GlutaMAX™ Supplement with 1% Anti-Clumping Agent.

2. After 2–5 days, transfer the desired clones into 6-well plates using the same procedure. The final culture volume in a 6-well plate is 2–3 mL.

3. Perform a second passage in a 6-well plate shaking at 125 rpm to ensure that the vast majority of the clones are >90% viable before performing the secondary screen.

Secondary Screen: 5-Day Productivity Assessment:

4. Set up the secondary screen in shaking 6-well plates, consistent with the conditions used in the preceding step. Seed the cells at 3×10^5 viable cells/mL in 3 mL of ExpiCHO™ Stable Production Medium + 3 g/L glucose. Incubate for 5 days before sampling for productivity.

5. Expand the top 30 clones into 125-mL shaker flasks in 30 mL of ExpiCHO™ Stable Production Medium. Incubate the cells at 37°C and 5–8% CO_2, with shaking at 125 rpm.

6. Passage clones two times in shaker flasks.

7. Cryopreserve 3–5 vials of cells from each clone as a back-up and proceed to the tertiary screening. See “Cryopreservation” on page 23.

Tertiary Screen: 14-Day Simple Fed-Batch Assessment:

8. Seed the 30 clones at 3×10^5 cell/mL in 125-mL shaker flasks in 30 mL of ExpiCHO™ Stable Production Medium.

9. Sample cultures on days 0, 4, 7, 10, 12, and 14 to determine cell density, viability, and productivity until culture viability drops below 50% or day 14 of culture is reached.

10. After sampling, feed the cultures with glucose, or glucose and feed, such as EfficientFeed™ C+ 2X Supplement or Efficient-Pro™ Feed 2 as follows (adjust if necessary):

Day	Glucose only	Glucose + feed
Day 4	4 g/L glucose	2 g/L glucose and 5% feed
Day 7	4 g/L glucose	2 g/L glucose and 7.5% feed
Day 10	4 g/L glucose	2 g/L glucose and 10% feed

Stability assessment

Select the most stable clones of the top 16 identified during "Scale up of the individual clones and basic screening" on page 21. Sub-culture clones for 60 generations or 12 weeks, whichever comes first with a single lot of medium.

Note: 60 generations is only a recommendation, 30–40 generations may be acceptable depending on the intended use.

1. Thaw each clone in a 125 mL shake flask with 30 mL pre-warmed ExpiCHO™ Stable Production Medium.
2. Passage clones at a seeding density of 2×10^5 cells/mL twice a week (3–4 day passages) for 60 generations or 12 weeks, whichever comes first.
3. Calculate generations by the following simple formula:

$$\text{Previous generation} + \frac{\ln(\text{VCD}/\text{seeding density})}{\ln(2)}$$

4. Set up a 7-Day productivity assessment for each clone one week after thaw in a different shake flask. These assessments will be seeded at 2×10^5 cells/mL once a week, fed 5 g/L glucose on day 3 or 4, and assayed for viable cell density (VCD) and productivity on day 7. This will be done throughout the entire study once a week.
5. At generation ~30, we recommend doing a safety freeze of 3 vials of each clone to mitigate loss.
6. Using the data from the productivity assessments, plot a regression line and establish a slope. Calculate the change in titer (or growth or both) over the 60 generations to determine clone stability.
7. Establish a criterion for clone stability. We have used a decrease in titer of 30% or less to indicate that the specific clone is stable.

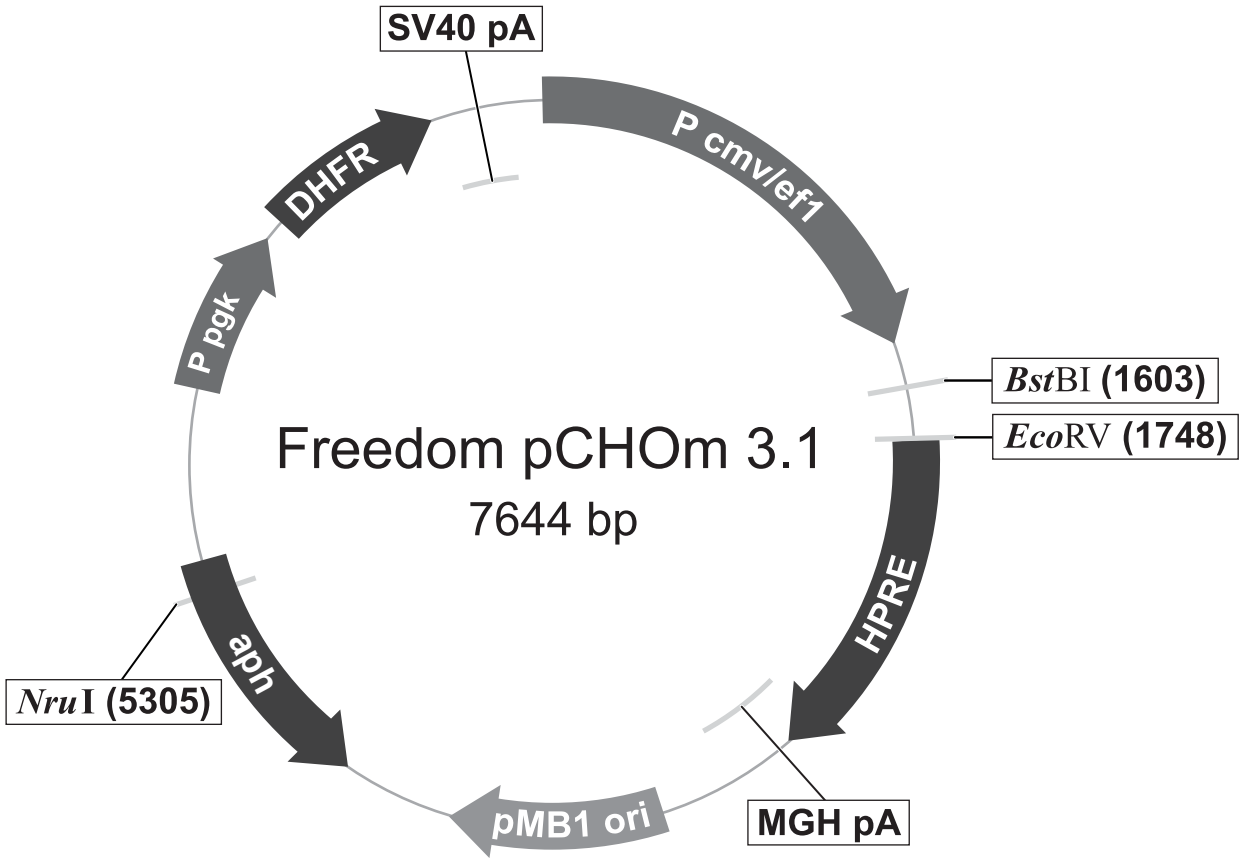
Cryopreservation

ExpiCHO-S™ cells can be frozen directly in ExpiCHO™ Stable Production Medium:

1. Freeze ExpiCHO-S™ cells at a final density of 1×10^7 viable cells/mL in 1 mL total volume of 90% fresh ExpiCHO™ Stable Production Medium and 10% DMSO.
2. Allow cells to attain a viable cell density of 4×10^6–6×10^6 cells/mL and > 95% viability before harvest.
3. Centrifuge the cells at 200 × *g* for 5 minutes to pellet, discard the spent medium, and replace it with cold ExpiCHO™ Stable Production Medium with 10% DMSO. Gently re-suspend the cell pellet by pipetting.
4. Dilute the cells to a final density of 1×10^7 viable cells/mL and aliquot 1 mL per cryovial.
5. Freeze the cells in an automated or manual controlled-rate freezing apparatus following standard procedures. For ideal cryopreservation, the freezing rate should be a decrease of 1°C per minute.
6. Transfer frozen vials to liquid nitrogen for long-term storage.

Vector maps

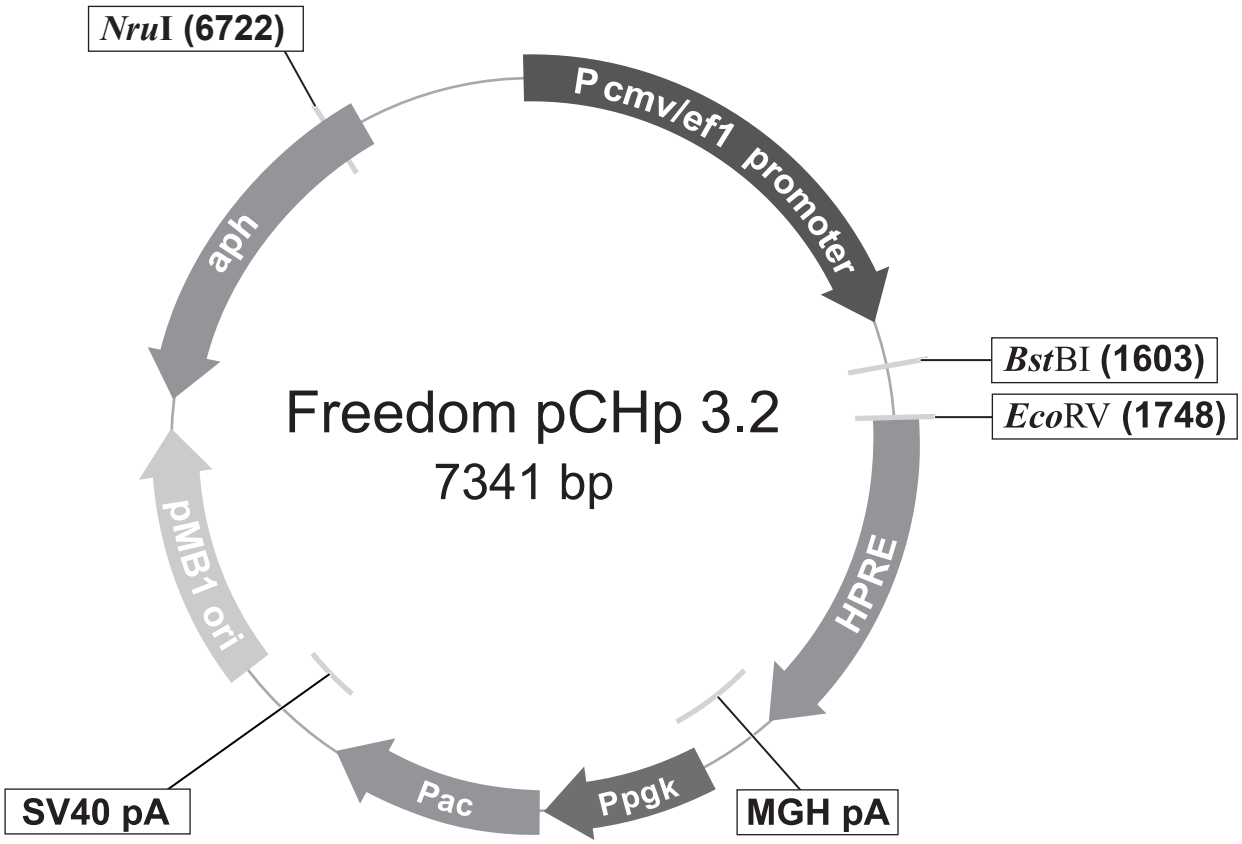
Freedom™ pCHOm 3.1 vector



Label	Description	Position
P cmv/ef1	CMV/EF1 hybrid promoter	1–1473
HPRE	HBV (hepatits B virus) post transcriptional regulatory element	1751–2818
mGH pA	mGH pA (mouse growth hormone) polyadenylation signal	2830–3196
pMB1 ori	pMB1 origin of replication	3448–4036
aph[1]	Kanamycin resistance gene (aph)	4578–5395
P pgk	PGK (phosphoglycerate kinase) promoter	5999–6505
DHFR	DHFR (dihydrofolate reductase) gene	6612–7175
SV40 pA	SV40 polyadenylation signal:	7286–7513

[1] complimentary strand

Freedom™ pCHOp 3.2 vector



Label	Description	Position
P cmv/ef1	CMV/EF1 hybrid promoter	1–1473
HPRE	HBV (hepatits B virus) post transcriptional regulatory element	1751–2818
mGH pA	mGH pA (mouse growth hormone) polyadenylation signal	2830–3196
P pgk	PGK (phosphoglycerate kinase) promoter	3248–3690
pac	Puromycin resistance gene (pac)	3711–4310
SV40 pA	SV40 polyadenylation signal	4434–4674
pMB1 ori	pMB1 origin of replication	4820–5453
aph[1]	Kanamycin resistance gene (aph)	5955–6810

[1] complimentary strand

B Possible variations and examples

Transfection procedural guidelines

- Monitor if there is a precipitate after complexation and before you add the complex to the cell culture.
- Measure titer 2 days post transfection if possible.
- In the following example we transfect both vectors at a ratio of 1:1 with different amounts of DNA and ExpiFectamine™ CHO Reagent. We recommend not exceed the 60 µg of DNA per vector. The best values and ratios should be empirically determined by the user.

Transfection optimization

Table 2 Transfection optimization for Freedom™ ExpiCHO-S™ Kit

-	Ratio	1:1	1:1	1:1	1:1	1:1	1:1	1:1	1:1	No DNA	No DNA
-	Flask	1	2	3	4	5	6	7	8	Control 1	Control 2
Volume	Culture volume	30 mL	30 mL	30 mL	30 mL	30 mL	30 mL	30 mL	30 mL	30 mL	30 mL
Cells	Number of cells	30×10^6	30×10^6	30×10^6	30×10^6	30×10^6	30×10^6	30×10^6	30×10^6	30×10^6	30×10^6
Diluted DNA[1]	mLC	—	20 µg	—	30 µg	—	40 µg	—	60 µg	—	—
	pHC	—	20 µg	—	30 µg	—	40 µg	—	60 µg	—	—
	mHC	20 µg	—	30 µg	—	40 µg	—	60 µg	—	—	—
	pLC	20 µg	—	30 µg	—	40 µg	—	60 µg	—	—	—
	OptiPRO™ SFM	960 µL	960 µL	940 µL	940 µL	920 µL	920 µL	880 µL	880 µL	1000 µL	1000 µL
Diluted reagent	OptiPRO™ SFM	960 mL	960 mL	960 mL	960 mL	950 mL	950 mL	940 mL	940 mL	960 mL	940 mL
	ExpiFectamine™ CHO Reagent	40 µL	40 µL	40 µL	40 µL	50 µL	50 µL	60 µL	60 µL	40 µL	60 µL
	Incubation time	<5 min	<5 min	<5 min	<5 min	<5 min	<5 min	<5 min	<5 min	<5 min	<5 min
DNA-lipid complex	DNA-lipid incubation time	<10 min	<10 min	<10 min	<10 min	<10 min	<10 min	<10 min	<10 min	<10 min	<10 min
Shake speed	Culture shake speed	125 rpm	125 rpm	125 rpm	125 rpm	125 rpm	125 rpm	125 rpm	125 rpm	125 rpm	125 rpm
-	Shaker throw	19 mm	19 mm	19 mm	19 mm	19 mm	19 mm	19 mm	19 mm	19 mm	19 mm

[1] Add diluted DNA complex to diluted reagent complex slowly and do not mix or shake or invert.

T-flask: Selection Phase 1

An optional method during phase 1 of selection is to use T-flasks instead of directly using shake flasks as it has been observed that starting the selection phase in T-flasks resulted in gentler scale up of the pools.

1. Passage cells into T-175 flasks with 40 mL of ExpiCHO™ Stable Production Medium at a density of 5×10^5 viable cells/mL.

2. To the T-175 flask containing the transfected cells add appropriate selective pressures.

   10P = 100 nM Methotrexate and 10 µg/mL Puromycin

   20P = 200 nM Methotrexate and 20 µg/mL Puromycin

3. Incubate T-flasks at 37°C, 95% relative humidity, and 5–8% CO_2 in a static incubator (non-shaking).

4. Sample flasks on day 7 post-selection for a viable cell count only. This will serve as a reference data point; if selection is occurring, you can expect a drop in viability and cell density relative to day 0 of selection. If viability is >30%, proceed directly to step 7. Otherwise, return the flask to incubator and proceed to step 5.

5. Sample flasks on day 10 or 11 post-selection for a viable cell count. If cell viability is still below the last measured value, perform a complete media exchange by centrifuging (200 × *g*, 5 min) the cells and re-suspending them in fresh ExpiCHO™ Stable Production Medium, adding adequate selective pressure to maintain pressure. Reduce the volume and T-flask size as needed to keep the viable cell density above 3×10^5 viable cells/mL. If viability has increased since day 7, perform a complete media exchange as described, and transfer cells to shake flask as described in step 6. Typically, cells are >30% viable when this transition from T-flask to shake flask occurs.

6. Sample the flasks every 3–4 days and perform a complete medium exchange once a week until the cells show signs of recovery.

7. As soon as the cells show signs of recovery (i.e., increased viability and cell growth from the last measured values), transfer them to a shake flask at a seeding density of 3×10^5 viable cells/mL. Typically, this transition occurs when viability is between 30–50%.

   Note: To save time/materials, one could choose one pressure in between the two recommended concentrations for each phase (P15 and P45).

Standard parameters

Table 3 Shaking conditions

Parameter	Value
125 mL shake flask working volume	Up to 35 mL
250 mL shake flask working volume	25–75 mL
500 mL shake flask working volume	50–150 mL
Agitation speed for shake flasks	125–150 rpm (19-mm orbital throw)
Agitation speed for shaking 6wp	125 rpm (19-mm orbital throw)
Incubation temperature	37 ±1°C
Incubation CO_2 concentration	5–8%
Incubation relative humidity	80 ± 10% relative humidity
T-flask working volume	0.2–0.3 mL/cm^2

Table 4 Static conditions

Parameter	Value
Incubation temperature	37 ±1°C
Incubation CO_2 concentration	5–8%
Incubation relative humidity	95 ± 10% relative humidity

D

ClonePix™ 2 colony picker instructions

Single cell cloning using ClonePix™ 2 colony picker

1. After transfection and selection, seed cells from select Phase 1 and Phase 2 selection pools in semi-solid medium.
2. Transfer semi-solid medium to 6-well plates and incubate the plates under static conditions at 37°C and at 95% humidity, 5–8% CO_2.

   Do not add selection agent to the semi-solid medium.
3. After 14–16 days screen plates containing the semi-solid medium using a ClonePix™ 2 instrument (Molecular Devices) and pick colonies using the following criteria.

   The acceptance criteria for colony picking are size (> 120 microns), roundness (> 0.6), axial ratio (> 0.6), and proximity (> 1 mm).
4. Rank the acceptable colonies in each plate based on total fluorescence (FITC Sum Total Intensity) and, where possible, pick the top 96 clones from each of the plates and deposit in to a 96-well plate containing 150 µL of cell culture medium (ExpiCHO™ Stable Production Medium).
5. If there are less than 96 pick-able colonies, pick all FITC-labeled clones. Assess each picked colony or survivability using a CloneSelect™ imager (Molecular Devices).
6. After 7 to 12 days of growth in 96-well plates, transfer all clones to two new 96-well plates (final culture volume will be 200 µL).
7. After 5 to 7 days assess productivity.
8. To assess productivity, perform early titer screening in 96-well plates and rank clones according to their productivity values.
9. Use the ranking obtained from the early titer screening, select the top 24 surviving clones from each pool for expansion in to 24-well plates and then in to 6-well plates.
10. Tranfer cultures from 6-well plates directly into 125-mL shake flasks with a minimum of 15–20 mL medium. After cells recuperate, perform a 7 or 14 day growth productivity assessment (secondary screen).
11. Based on the secondary screen titers, identify a minimum of top 30 clones and create a small safety freeze of 6 vials. Additionally, generate a research cell bank of a minimum of 10 vials for each of the top 16 clones. Use the top 16 clones to perform tertiary screening.

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

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

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