Standard Operating Procedure **SOP031**

Title: Cryopreservation And Storage of Mammalian Cells

Location: CBE laboratories.

1. PURPOSE

This SOP describes how to preserve mammalian cells using slow-freezing cryopreservation methods.

2. SCOPE

This SOP provides a generic slow-freezing process suitable for the long-term preservation of mammalian cells in liquid nitrogen cryostorage units.

The SOP describes procedures for:
- Harvesting of adherent cells, adherent cell colonies or suspension cells (**Section 5.1a – 5.1c**)
- Equilibrating cells with freezing medium (**Section 5.2**)
- Slow-freezing cells (**Section 5.3**)
- Transfer of frozen cells to liquid nitrogen cryostorage units (**Section 5.4**)

Cryopreservation is a multi-stage process with several factors potentially influencing how well a cell population will be preserved. Mammalian cells are diverse, and will differ in their tolerance to cryopreservation. Consequently, no single method can universally preserve all cells at optimal efficiency. This SOP is written with flexibility in mind, providing a framework for the key stages of a cryopreservation process with useful ranges rather than defined set-points for critical process parameters. It is suggested that inexperienced users refer to the scientific literature, or users experienced with their culture system for advice on how to adapt this SOP for their needs.

Users may also find the following web-based technical guide useful:
(https://www.atcc.org/~/media/PDFs/Cryopreservation_Technical_Manual.ashx).

Read this SOP in conjunction with **SOP013** (Safe Use and Maintenance of Liquid Nitrogen Stores); **SOP032** (Revival of Cryopreserved Mammalian Cells); **SOP036** (Maintenance of a Quality Laboratory Environment) and **SAFMM6405 Risk Assessment for Liquid Nitrogen**

3. RESPONSIBILITES

3.1 Authorised CBE Laboratory Users

- **Shall** understand the health and physical hazards of cryogenic liquids (read **SAFMM6405** and **SOP013 (Section 5.4) before** using this SOP).

- **Shall** undertake proper training with the Laboratory Manager or an authorised deputy (as per **SOP013)**.

- **Shall** ensure their work with liquid nitrogen does not create additional hazards for other CBE laboratory users (**SOP013)**.

- **Shall** fill out cryostorage transfer slips when depositing cryopreserved cells into the CBE liquid nitrogen cryostores and hand these to the RP/LM as soon as possible.

- **Shall** input the vial information into Procuro (sample tracking software) and keep it up to date with Cell line, passage number, vial location, date and any other relevant information.

3.2 Responsible Persons (RP)/Laboratory Manager (LM)

- **Shall** designate and organise training for CBE laboratory users who are required to use liquid nitrogen. Training **must** be recorded using the form (FSOP013.1) and placed in the individuals training folder as proof of authorisation. It is the RP/LM's responsibility to ensure that **only** authorized CBE users handle liquid nitrogen.

- **Shall** ensure necessary controls (cryogenic personal protective equipment) are available and implemented to minimize the quantity and likelihood of liquid nitrogen spills and exposure to liquid nitrogen or its gaseous form.

- **Monitor** the handling of cryogenic liquids in accordance with good work practices (outlined in **SOP013 (Section 5.4)**).

- **Shall** monitor the release and collection of cryostorage unit keys.

- **Shall** monitor the recording of cryopreserved cell transfer into cryostorage units.

- **Shall** maintain an electronic database of cryopreserved cells stored within CBE cryostorage units

- **Shall** monitor sample recording within Pro-curo at regular intervals

4. EQUIPMENT AND MATERIALS

4.1 Equipment

a. Biological Safety cabinet.
b. Humidified Incubator (set to 37°C with a 5 % CO_2 in air atmosphere).
c. Water bath (set to 37°C).
d. Bench-top centrifuge.
e. Light microscope (inverted) with a 10 x objective.
f. –80°C Freezer.
g. Biorack 750 cryostorage units (7 units) including Roller bases and padlocks/keys.
h. Cryogenic PPE (Face visor or safety goggles, covered footwear, laboratory coat). i. Metal spill tray (2 trays).
j. (If passive cooling) Cryopreservation container (e.g. "Mr Frosty" or Coolcell™).
k. (If active cooling) Controlled-rate freezer (e.g. EF600).
l. There are Cryolabels, which can be used on frozen and unfrozen vials, see Jen Bowdrey (CBE Technician)

4.2 Supplies

a. Aspirating pump with connected aspiration line.
b. Pre-labelled 2ml Cryovials/ampoules.
c. Plastic Serological Pipettes (5 ml – 25 ml).
d. Aspirating pipettes (2 ml).
e. Polypropylene centrifuge tubes (15 ml and/or 50 ml).
f. Polypropylene microcentrifuge tubes (0.5 ml).

4.3 Media and Chemicals

a. 70% IMS
b. Detachment solution (e.g. 0.25 %wt Trypsin + 0.91 mM EDTA).
c. Growth medium (e.g. DMEM + 10 % FBS + 2 mM Ultraglutamine).
d. Washing solution (e.g. PBS without Calcium or Magnesium ions).
e. Freezing medium (e.g. 90% Foetal Bovine Serum (FBS) + 10% Dimethylsulfoxide (DMSO)).

5. PROCEDURE

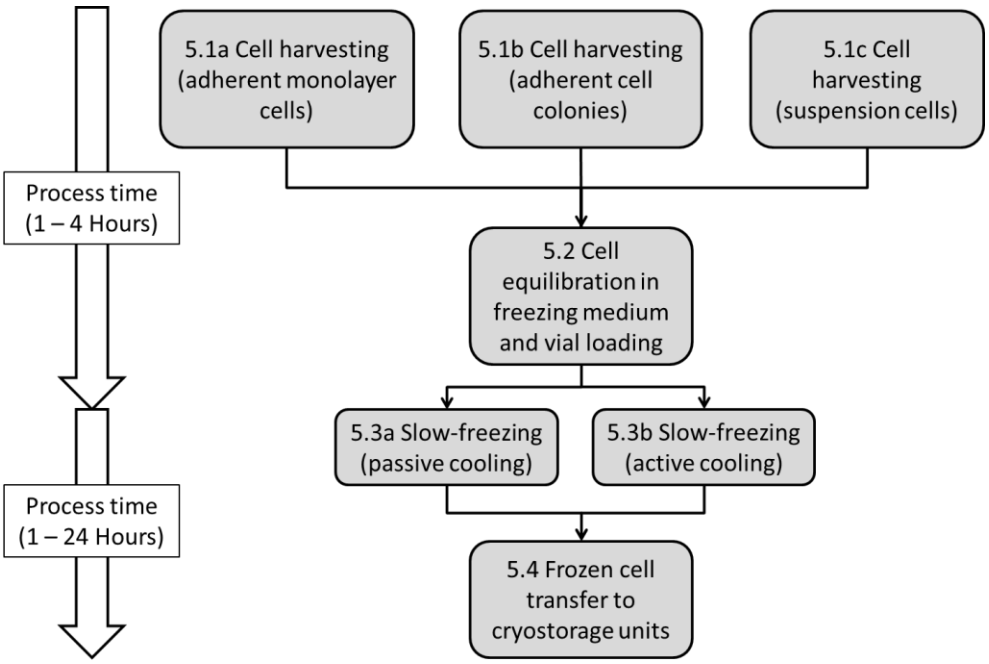


Figure 1: SOP031 overview. A process map defining process stages (including variants for cell harvesting and slow-freezing process stages) with suggested ranges for process timing before freezing (**Sections 5.1 – 5.2**) and after freezing (**Sections 5.3 – 5.4**).

5.1a Cell harvesting (adherent monolayer cells*)*

This procedure describes a basic manual cell harvesting protocol for use with adherent mammalian cells cultured in planar culture vessels (e.g. T-flasks).

Note: Harvesting procedures for cells cultured on 3D scaffolds (e.g. microcarriers) at larger scales can require a more involved process than the one outlined here. To preserve cells harvested from 3D culture systems, define a suitable harvesting procedure to efficiently collect suspended cells then proceed with this SOP from **Section 5.2**.

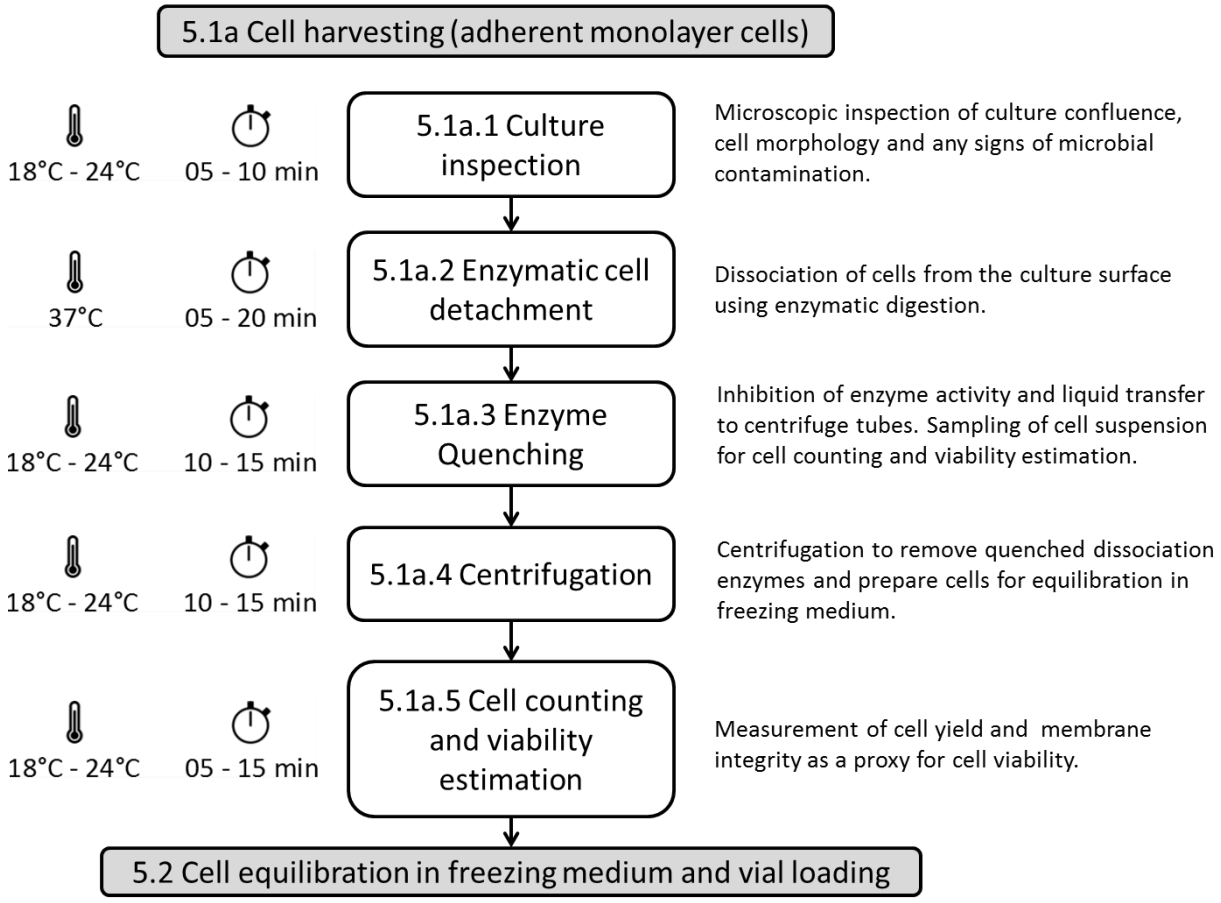


Figure 2: Cell harvesting (adherent monolayer cells) overview. A Process map describing the cell harvesting (adherent monolayer cells) stage of SOP031. Operating ranges for temperature and time are shown with broad descriptions of each task.

5.1a.1 Culture inspection

a. Remove culture vessel(s) from the incubator (e.g. T-flask or multiwell dish).
b. Observe culture(s) using a light microscope:
   If:

I. Cells are ideally sub-confluent (e.g. 80 % - 90 %).
II. Cells display the appropriate cell-specific morphology.
III. There is no sign of microbial contamination.

Then proceed to enzymatic cell detachment (**Section 5.1a.2**)

Otherwise:

I. Continue incubating cultures until they are; sufficiently confluent; displaying the correct morphology; lacking signs of contamination, before proceeding to enzymatic cell detachment (**Section 5.1.2**).
II. Discard the culture if abnormal cell morphology is observed (See **SOP003**).
III. Implement infection control procedures and discard the culture if microbial contamination is detected (See **SOP003 Section 7.1.3.5**).

5.1a.2 Enzymatic cell detachment

a. Aseptically transfer the culture vessel(s) into a Class II BSC.
b. Aspirate the growth medium from each vessel.

Note: **Do not** let culture vessels dry out; perform **step c** within 60 seconds of aspiration.

c. Wash each vessel with 1 culture volume (See **Table 1** for culture vessel-specific definitions of culture volume) of PBS, then aspirate the PBS.

Note: **Do not** let culture vessels dry out; perform **step c** within 60 seconds of aspiration.

Table 1: **Culture vessel-specific working volumes**.

Vessel type	Flat Multiwell Plates		T-flasks		
	12	6	25	75	175
Surface area (cm^2)	4.01	9.2	25	75	175
Volume scaling factor (ml/cm^2)	0.2		0.2		
Recommended culture volume (ml)	0.8	1.8	5	15	35
Tolerable culture volume range (ml)	0.8 - 1.6	1.8 - 3.6	5 - 7.5	15 - 22.5	35 - 50

d.

Add 0.1 culture volume of detachment solution (e.g. 0.25 wt% trypsin + 0.91 mM EDTA in PBS) and incubate @ 37°C for 3 – 10 minutes.

Note: Optimal incubation time is cell-type, detachment reagent and culture system dependent. The author recommends that users consult with experienced colleagues, or experimentally determine the optimum with a time-course experiment using light microscopy to confirm cell detachment.

Tap the culture vessel(s) to dislocate the enzyme-treated cells from the culture surface and confirm the presence of suspended cells using light microscopy.

5.1a.3 Enzyme quenching

a. Aseptically transfer the culture vessel(s) into a Class II BSC.
b. Dilute the enzyme-treated cell-suspension(s) with 0.3 – 0.5 culture volumes of growth medium.
c. Gently transfer the diluted cell suspension(s) into 15 ml or 50 ml centrifuge tubes.
d. Mix the cell suspension(s) with gentle pipetting and then sample each for cell counting.

Note: Repeated sampling ($n \geq 2$) is recommended for large volumes (e.g. > 5 ml) to minimise the effect of sampling variability on the resulting cell counts.

5.1a.4 Centrifugation

a. Centrifuge the cells at 200 x g for 5 minutes.
b. While the cells are being centrifuged, begin cell counting and viability estimation (**Section 5.1a.5**)
c. Aseptically transfer the centrifuge tubes(s) into a class II BSC.
d. Aspirate the supernatant.
e. Tap the centrifuge tube(s) to dislodge the cell pellet(s).

5.1a.5 Cell counting and viability estimation

Measure cell density using one of the following protocols:

I. Manual counting with trypan blue exclusion haemocytometry (**SOP034**).
II. Cedex automated trypan blue exclusion (**SOP041**).
III. Countess automated trypan blue exclusion (**SOP102**).

IV. Nucleocounter automated cell counting (**SOP121**).
V. Or another suitable method (e.g. custom flow cytometry).

5.1b Cell harvesting (adherent cell colonies)

This procedure describes a basic manual cell harvesting protocol for use with adherent cell colonies.

Note: A range of harvesting techniques exist for colony cultures, including manual dissociation (colony cutting), or non-enzymatic detachment (e.g. EDTA). Any suitable harvesting protocol can be

substituted for the one described herein before progressing to cell equilibration in freezing medium (**Section 5.2**).

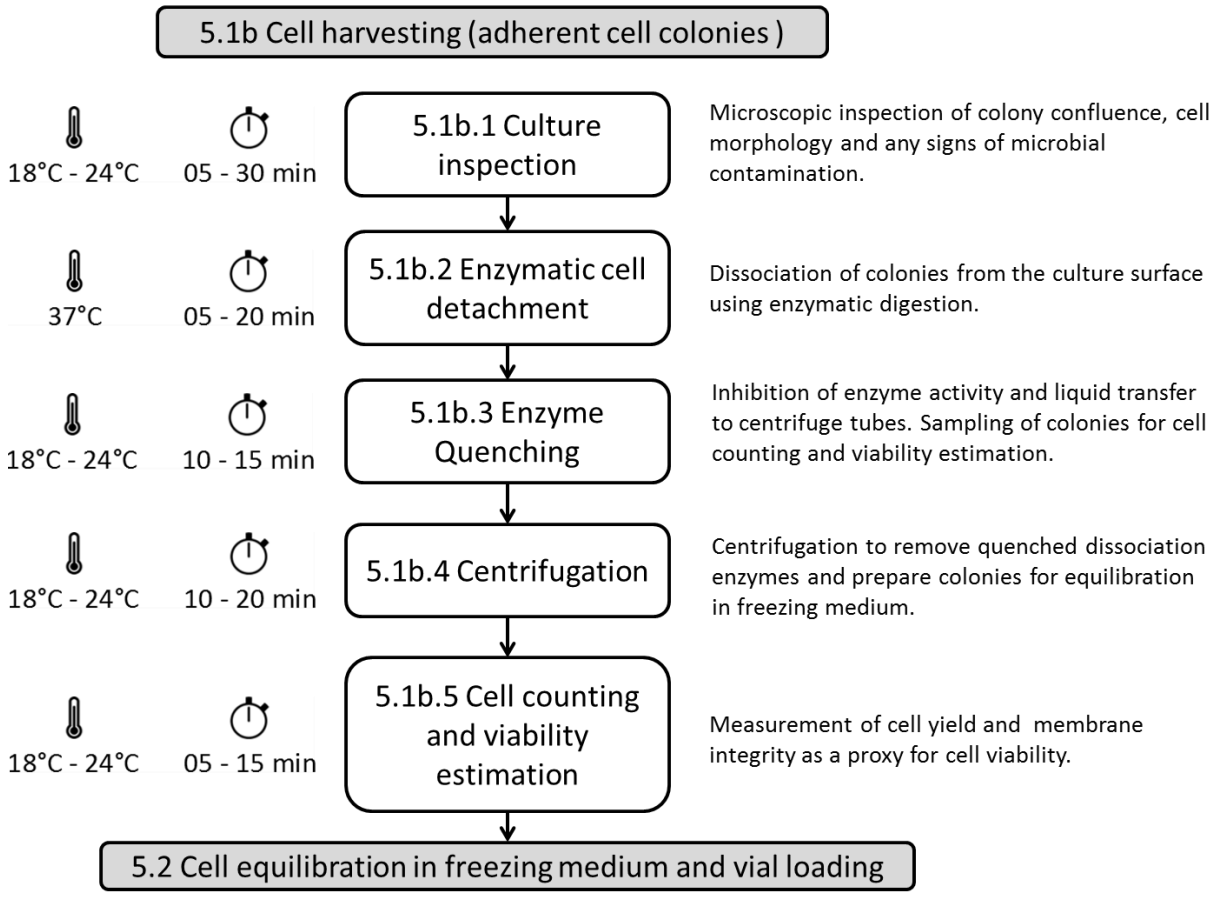


Figure 3: Cell harvesting (adherent cell colonies) overview. A Process map describing the cell harvesting (adherent cell colonies) stage of SOP031. Operating ranges for temperature and time are shown with broad descriptions of each task.

5.1b.1 Culture inspection

a. Remove culture vessels (e.g. T-flasks or multiwell plates) from the incubator.
b. Observe the culture using light microscope:

If:

I. Colonies are separately located and ideally sized for passaging (cell-type specific).
II. Cells within the colony core display the appropriate cell-specific morphology.
III. There is no sign of microbial contamination.

Then proceed to enzymatic cell detachment (**Section 5.1b.2**)

Otherwise:

IV. Incubate until colonies are correctly sized with the correct morphology and no sign of contamination before proceeding to enzymatic cell detachment (**Section 5.1b.2**).
V. Discard the culture if a large proportion of colonies display abnormal cell morphology (See **SOP003**).
VI. Implement infection control procedures and discard the culture if microbial contamination is detected (See **SOP003 Section 7.1.3.5**).
VII. Selectively remove abnormal colonies or colony-regions ("culture editing") using a light microscope fitted with a colony marker, followed by selective aspiration of marked colonies using a vacuum line fitted with a small pipette tip aperture (e.g. 300 µl pipette tip). Once abnormal colonies have been removed, incubate the plate for at least 1 hour in fresh medium before moving on to enzymatic cell detachment (**Section 5.1b.2**).

5.1b.2 Enzymatic cell detachment

a. Aseptically transfer the culture vessel(s) into a Class II BSC.
b. Aspirate the growth medium from the vessel(s).

Note: **Do not** let vessel(s) dry out; perform **step c** within 60 seconds of aspiration.

c. Wash each vessel once with 1 culture volume of 1 x PBS (See **Table 1** for culture vesselspecific definitions of culture volume) of PBS, then aspirate the PBS.

Note: **Do not** let vessel(s) dry out; perform **step d** within 60 seconds of aspiration.

d. Add 0.1 – 0.2 culture volumes of detachment solution to each culture vessel (e.g. 2 mg/ml Dispase in DMEM/F12) then incubate @ 37°C for 10 – 15 minutes.
e. Tap the vessel(s) to dislocate the enzyme-treated colonies and confirm the presence of suspended colonies using light microscopy.

5.1b.3 Enzyme quenching

a. Aseptically transfer the culture vessel(s) into a Class II BSC.
b. Gently dilute the colony suspension(s) with 1 culture volume of growth medium.
c. Transfer the colony suspension(s) in each well into a 15 ml centrifuge tube and rinse each well with1 additional culture volume of growth medium.

Note: **Do not** mechanically scrape colonies or aggressively pipette during transfer. Doing so will disaggregate colonies resulting in lower recovery of healthy colonies post-preservation.

d. Pool the rinse volume from each vessel into the corresponding 15 ml centrifuge tube used in **step c**.
e. Collect at least one 200 µl sample of the pooled colony suspension(s) for cell counting with gentle pipetting (**do not** disaggregate the colonies).

Note: Repeated sampling ($n \geq 2$) is recommended for colony suspensions to minimise the effect of sampling variability on the resulting cell counts.

5.1b.4 Centrifugation

a. Centrifuge the colony suspension(s) at 200 x g for 5 minutes.
b. While the colonies are being centrifuged, begin cell counting and viability estimation (**Section 5.1b.5**)
c. Aseptically transfer the centrifuge tubes(s) into a class II BSC.
d. Aspirate the supernatant from each centrifuge tube.
e. Tap the centrifuge tube(s) to dislodge the cell pellet(s).

5.1b.5 Cell counting and viability estimation

Measure cell density using one of the following protocols:

i. Manual counting with trypan blue exclusion haemocytometry (**SOP034**).

II. Cedex automated trypan blue exclusion (**SOP041**).
III. Countess automated trypan blue exclusion (**SOP102**).
IV. Nucleocounter automated cell counting (**SOP121**).
V. Or another suitable method (e.g. custom flow cytometry).

Note: Colonies may require enzymatic dissociation prior to counting to minimise artefacts caused by cell aggregation.

5.1c Cell harvesting (suspension cells)

This procedure describes a basic manual cell harvesting protocol for use with suspended mammalian cells.

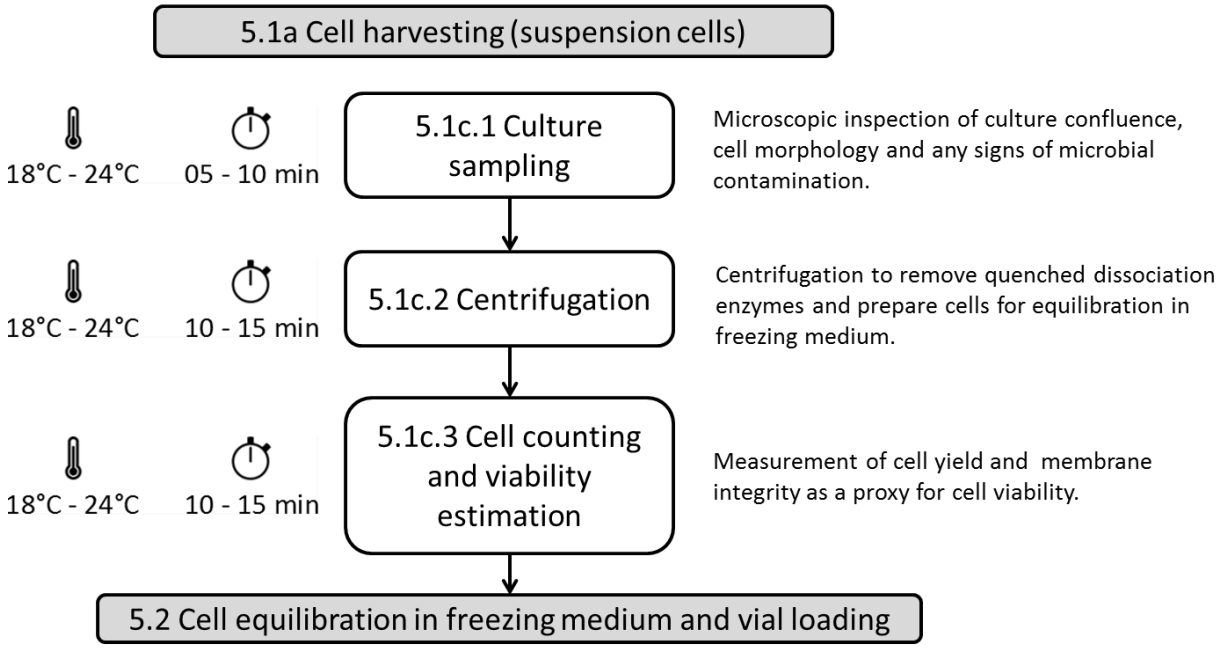


Figure 4: Cell harvesting (suspension cells) overview. A Process map describing the cell harvesting (suspension cells) stage of SOP031. Operating ranges for temperature and time are shown with broad descriptions of each task.

5.1c.1 Culture sampling

a. Aseptically transfer the culture vessel(s) into a Class II BSC.
b. Transfer the remaining cell suspension(s) into appropriately sized (e.g. 50 ml) polypropylene centrifuge tubes.
c. Aseptically collect at least one 200 µl sample of each cell suspension for cell counting (**Section 5.1c.3**).

Note: Repeated sampling ($n \geq 2$) is recommended for large volume (e.g. > 5 ml) suspensions to minimise the effect of sampling variability on the resulting cell counts.

5.1c.2 Centrifugation

a. Centrifuge the cell suspension(s) at 200 x g for 5 minutes.
b. While the cells are being centrifuged, begin cell counting and viability estimation (**Section 5.1c.3**)
c. Aseptically transfer the centrifuge tubes(s) into a class II BSC.
d. Aspirate the supernatant from each centrifuge tube.
e. Tap the centrifuge tube(s) to dislodge the cell pellet(s).

5.1c.3 Cell counting

Measure cell density using one of the following protocols:

I. Manual counting with trypan blue exclusion haemocytometry (**SOP034**).
II. Cedex automated trypan blue exclusion (**SOP041**).
III. Countess automated trypan blue exclusion (**SOP102**).
IV. Nucleocounter automated cell counting (**SOP121**).
V. Or another suitable method (e.g. custom flow cytometry).

Note: Some mammalian cells will form aggregates during suspension culture. Aggregates can be removed using a 100 µm nylon-mesh cell strainer at the point of harvest, or the cell suspension can be enzymatically treated to dissociation aggregates before cell counting and subsequent seeding.

5.2 Cell equilibration in freezing medium

This procedure describes the suspension of harvested cells into an appropriate vehicle solution, which is supplemented with cryoprotective agents to form a freezing medium. This procedure also describes the loading of equilibrated cells into cryovials in preparation for subsequent slow-freezing (**Section 5.3**).

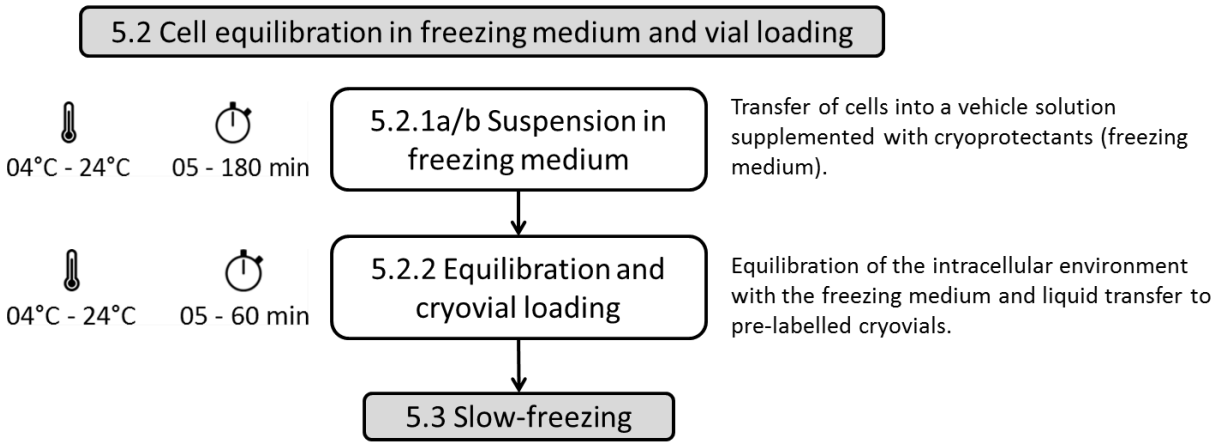


Figure 5: Cell equilibration in freezing medium and vial loading overview. A Process map describing the cell equilibration in freezing medium and vial loading stage of SOP031. Operating ranges for temperature and time are shown with broad descriptions of each task.

CAUTION: Prolonged exposure (> 30 minutes) to hypothermic temperatures (< 35°C) will result in a cold shock response in mammalian cells. Adherent cells also experience stress when maintained without a surface to attach to and proliferate upon. Care must be taken to minimise the overall processing time (ideally < 4 hours) from the point of harvest (**Section 5.1**) to the point of freezing (**Section 5.3**). Otherwise, the yield of functional cells post-thaw may be diminished. This is particularly important when preserving large banks of material (e.g. Master or Working Cell Banks).

5.2.1a Suspension in freezing medium (direct suspension technique)

a. Directly suspend the cell pellet(s) in freezing medium (either refrigerated 4°C - 8°C or ambient 18 - 24°C) with gentle pipetting to the target preservation cell density (**Table 2**). Proceed to **Section 5.2.2**.

Note: Many freezing media exist, including homebrew (10 wt% dimethylsulfoxide in Foetal Bovine Serum) or commercial formulations (e.g. CryoStor® CS10, Biolife Solutions, USA). Care should be taken to select a medium which is appropriate for your research needs.

> **Note:** In many cases, $1e^6$ cells/ml is suitable for preserving master and working cell banks of material for future research efforts.

Table 2: Suggested freezing cell densities and freezing volumes. Volumes shown are per 1.8 ml cryovial. For multiple vials or other containers (e.g. cryobags), scale volumes accordingly.

Equilibration Technique	Target freezing cell density ($1e^6$ cells)	1		5		10	
	Freezing volume (ml)	0.5	1	0.5	1	0.5	1
Direct suspension	Add sufficient 1 x freezing medium to obtain target freezing cell density						
Indirect suspension	2 x Freezing medium (ml)	0.25	0.5	0.25	0.5	0.25	0.5
	Cell suspension @ 2 x Target cell density		0.5		0.5		0.5
	Cell suspension @ 4 x Target cell density	0.25		0.25		0.25	

5.2.1b Suspension in freezing medium (indirect suspension technique – homebrew only)

a. Suspend the cell pellet(s) in vehicle solution (e.g. Refrigerated 4°C - 8°C or ambient 18°C - 24°C Foetal Bovine Serum) with gentle pipetting above (e.g. 2 x or 4 x) the target preservation cell density (**Table 2**).
b. Dilute the cell suspension with 2 x freezing medium and mix with gentle pipetting to obtain the target cell density in 1 x freezing medium. Proceed to **Section 5.2.2**.

5.2.2 Equilibration and cryovial loading

a. Maintain cells at equilibration temperature (either refrigerated 4°C - 8°C or ambient 18 - 24°C) for between 10 and 60 minutes to ensure that cryoprotectants in the extracellular freezing medium are equilibrated with the intracellular environment.

Note: Cryoprotectant equilibration follows Arrhenius kinetics. Therefore, colder temperatures will necessitate longer equilibration times. Furthermore, optimal equilibration times will depend on the intrinsic permeability of the cells to cryoprotectants and water.

Note: Cellular viability and functionality will be progressively compromised as equilibration time increases beyond 60 minutes. Some cell types (e.g. human Embryonic Stem Cells) are particularly sensitive to being dissociated and placed into freezing medium. Consult an experienced user or reputable source (e.g. UK Stem Cell Bank) for advice on how to adapt this SOP to successfully preserve these sensitive cell types.

a. Load pre-labelled 1.8 ml cryovials (or other suitable freezing container) with 1 x freezing volume of cryoprotectant equilibrated cell suspension (e.g. 0.1 ml – 1 ml).

Note: For 1.8 ml cryovials, a maximal volume of 1 ml is recommended to (i) accommodate the expansion of solid water upon freezing while (ii) retaining sufficient headspace in the vial to enable aseptic collection of the contents upon thawing.

5.3a Slow-freezing (passive cooling)

This procedure describes slow-freezing of freezing medium-suspended cells using a passive cooling device and an ultralow temperature (-80°C) freezer.

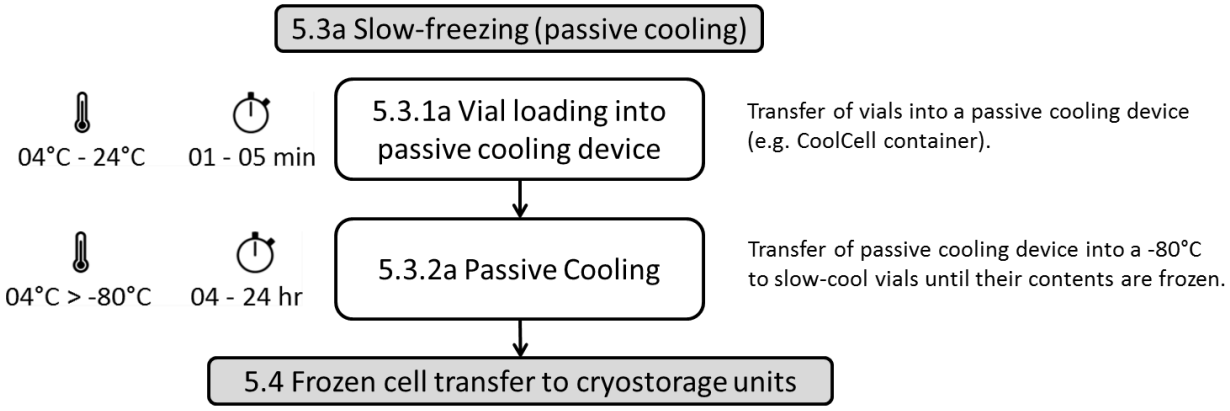


Figure 6: Slow-freezing (passive cooling) overview. A Process map describing the slow freezing (passive cooling) stage of SOP031. Operating ranges for temperature and time are shown with broad descriptions of each task.

5.3a.1 Load vials into passive cooling device

a. Confirm each loaded cryovial is properly closed.

Note: Vials should be hand tightened with an extra twist to ensure closure without damaging O-rings or the vial threads.

b. Transfer the closed vials to a passive cooling device (e.g. CoolCell®) pre-cooled to 4°C - 8°C if a chilled equilibration was performed.

5.3a.2 Passive cooling

a. Transfer the passive cooling device to a -80°C freezer shelf and close the freezer door.
b. Leave the vials to passively cool for 4 - 16 hours to ensure their contents freeze and equilibrate with the freezer temperature. After freezing and thermal equilibration, proceed to **Section 5.4**.

Note: Do not allow the freezer shelf to be opened during the first 4 hours of cooling, or the rate of cooling will deviate from the anticipated -1°C/min.

Note: While cells can be preserved in a -80°C freezer over the short-term (days – weeks), it is not sustainable for long-term preservation (years) as the storage temperature must be below the glass transition temperature of the freezing medium (e.g. -124°C to -130°C).

5.3b Slow-freezing (active cooling)

This procedure describes slow-freezing of freezing medium-suspended cells using a controlled-rate freezing device (e.g. EF600 Stirling cryocooler).

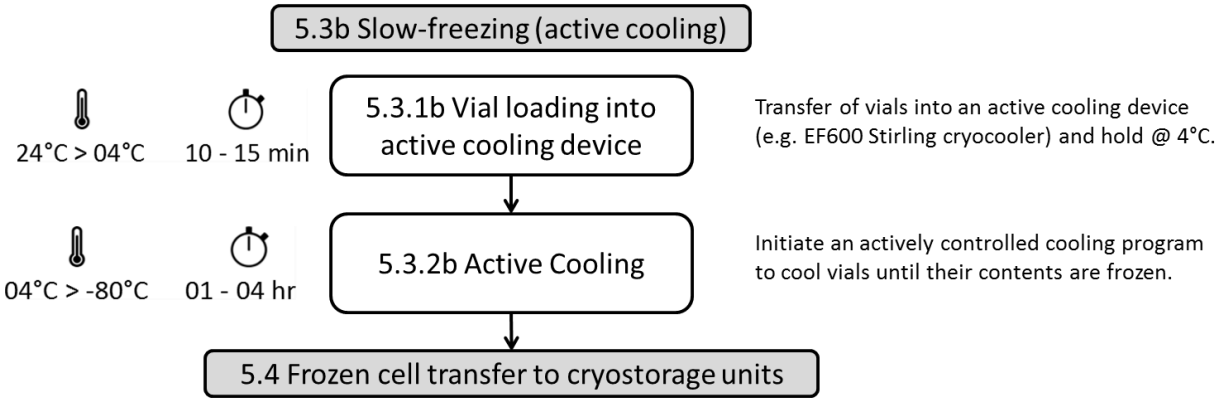


Figure 7: Slow-freezing (active cooling) overview. A Process map describing the slow freezing (active cooling) stage of SOP031. Operating ranges for temperature and time are shown with broad descriptions of each task.

5.3b.1 Load vials into active cooling device

a. Confirm each loaded cryovial is properly closed.

Note: Vials should be hand tightened with an extra twist to ensure closure without damaging O-rings or the vial threads.

b. Transfer the closed vials to an active cooling device (e.g. EF600 Controlled Rate Freezer – **SOP123**) pre-cooled to 4°C.
c. If cryoprotectant equilibration was performed at ambient temperatures (18°C - 24°C), then hold the vials in the active cooling device for 5 minutes to allow the vials time to thermally equilibrate with the chilled environment.

5.3b.2 Active cooling

a. Run a suitable cooling programme to freeze the vial contents at a controlled rate. After freezing and thermal equilibration, proceed to **Section 5.4**.

Note: The following cooling profile should be effective with most mammalian cell types:

i. Start @ 4°C
ii. Ramp @ -1°C/min to -80°C
iii. Hold @ -80°C for 5 minutes.

Note: More nuanced cooling profiles which allow controlled ice nucleation, or which facilitate the rapid removal of the latent heat of fusion released as ice forms, may provide better cell recovery. It is difficult to know in advance what the best cooling profile will be, as many cryobiological factors (e.g. cell-type, cryoprotectant type, and cooling profile) interact to determine the outcome of a cryopreservation process.

5.4 Frozen cell transfer to cryostorage units

This procedure describes the transfer of vials (containing frozen cells) to cryostorage units containing liquid nitrogen (vapour-phase liquid nitrogen storage). This procedure also describes the necessary data logging required to maintain an accurate record of preserved cells held long-term within CBE cryostorage units.

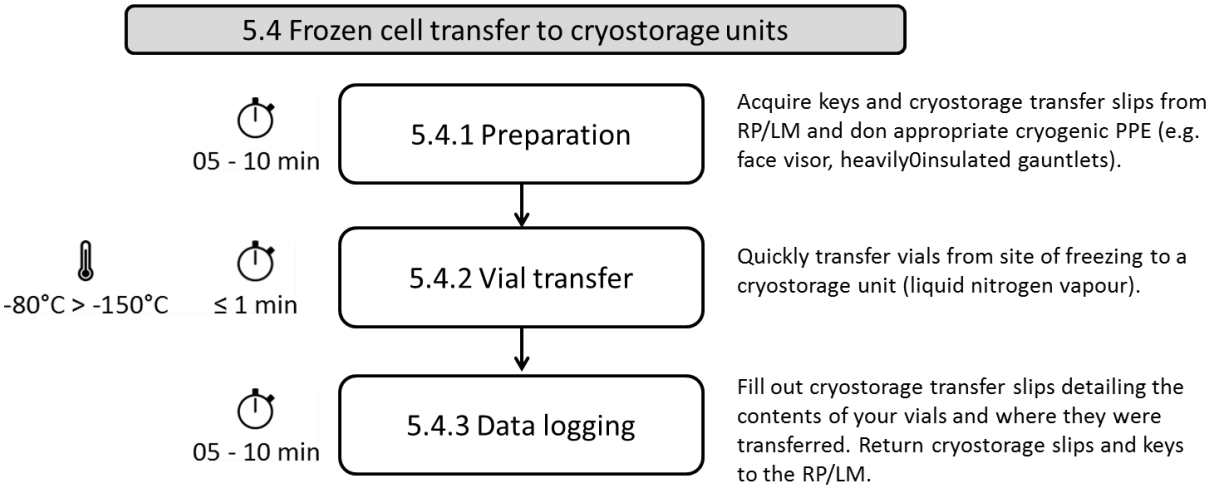


Figure 8: Frozen cell transfer to cryostorage units overview. A Process map describing the frozen cell transfer to cryostorage units stage of SOP031. Operating ranges for temperature and time are shown with broad descriptions of each task.

5.4.1 Preparation

a. Access the Cryostorage database on the CBE-QUAL Workspace (Cell Banking Records) and identify a suitable location to deposit your frozen cryovials. The cells location are also found on Procuro. Note space has been allocated per group.
b. Retrieve the cryostorage keys and a cryostorage transfer slip from the folder in the labs internal corridor.
c. Unlock and the relevant cryostorage unit(s) (kept in H31, H30 & H34).
d. Deploy the spill-tray next to the cryostorage unit(s).
e. Don cryogenic PPE (Face visor or safety goggles, covered footwear, laboratory coats outlined in **SOP013 Safe Use and Maintenance of Liquid Nitrogen Stores**.

5.4.2 Vial transfer

a. Quickly collect the frozen vial(s) and transfer then to a pre-chilled (-80°C) secondary container (e.g. a dry-ice box or chilled metal water bath beads).
b. Transfer the secondary container from the site of freezing to the cryostore(s) (H30/H31/H34).
c. Open the unlocked cryostore unit (repeat **steps 5.4.2 c – 5.4.2 i** if vials are to be deposited in multiple cryostore units).
d. Retrieve the target cryostorage rack and hold at the neck of the cryostorage unit to allow residual liquid nitrogen to drain back into the cryostore (requires approximately 30 seconds).

CAUTION: Once exposed to ambient air, the rack and its contents will rapidly begin to warm (transient warming event or TWEs, see **Figure 2**). Repeated and/or prolonged exposure of frozen cells to TWEs above the glass transition temperature of the freezing medium will result in decreased cell viability upon thawing.

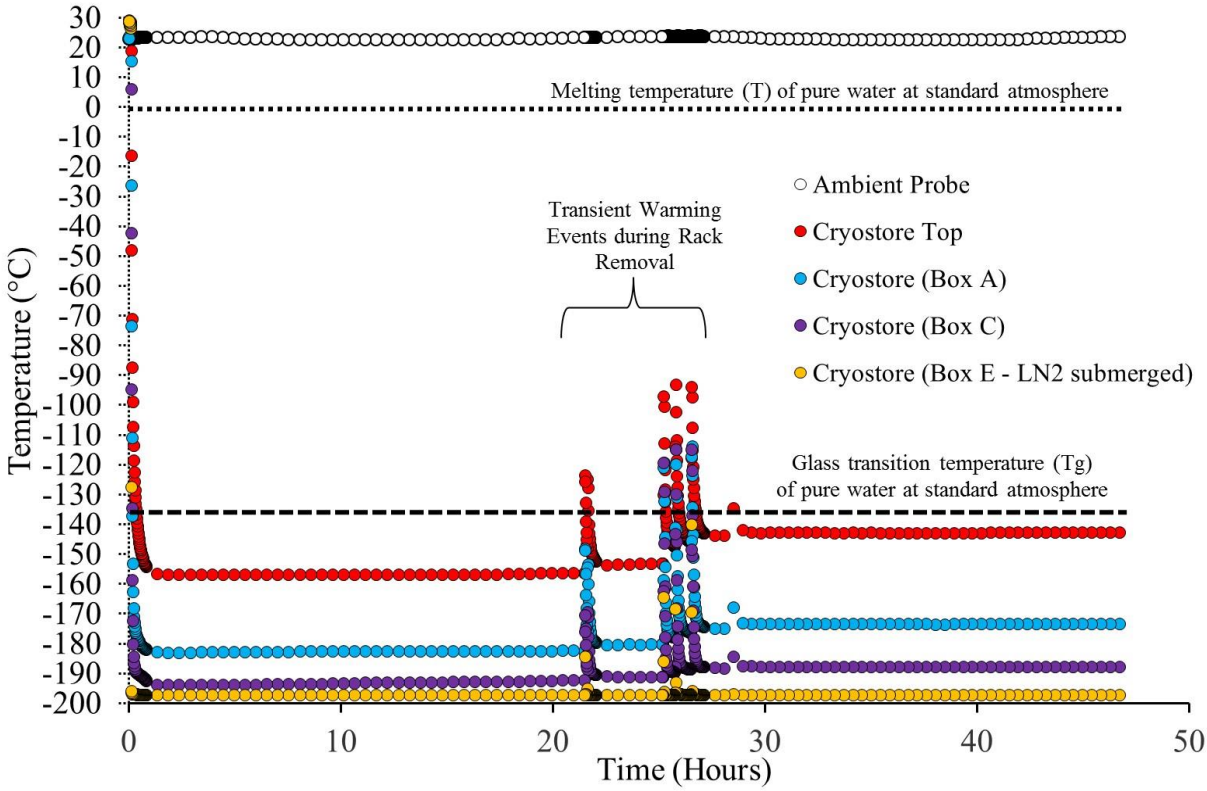


Figure 2: Thermal stability and transient warming event responsiveness of the current CBE cryostorage environment.

Temperatures were cyclically recorded from thermocouples housed in a modified cryostorage lid once per minute. Transient warming events were mimicked by fully removing the cryostorage lid and associated thermocouples for 1 minute before rehousing the modified lid within the cryostorage unit.

CAUTION: Perform **steps 5.4.2e – 5.4.2i** quickly (within **60 seconds**). If a large number of vials are to be transferred, split the task into multiple batches and allow the cryostorage rack time to thermally equilibrate (5 minutes) before transferring another batch of frozen vials.

e. Place the cryostorage rack onto the spill tray then replace the cryostorage unit lid.
f. Remove the metal support rod from the rack to access the cryostorage boxes.
g. Retrieve the required cryostorage box, deposit the cryovials into it then replace the cryostorage box (if multiple boxes are needed from the same cryostorage unit, repeat this step).
h. Replace the metal support rod into the cryostorage rack and remove the cryostorage unit lid.
i. Place the cryostorage rack into the cryostorage unit and replace the cryostorage unit lid.
j. Once all vial transfers are completed, relock the cryostorage unit(s).

Note: If the free cryostorage locations identified in **Section 5.4.1a** do not match the locations observed in the respective cryostorage boxes then:

i. Quickly deposit the cryovials into alternative observably free locations within the cryostorage box(es).
ii. Note which cryostorage locations are inconsistent with the electronic database and note the new location(s) in which your vials were deposited.
iii. Notify the Responsible Person/Laboratory Manager that a conflict between the electronic database and the cryostorage units exists.

Or

i. Quickly replace the cryostorage box(es) into their respective cryostorage rack.
ii. Replace the metal support rod into the cryostorage rack. iii. Replace the cryostorage rack into the cryostorage unit.
iv. Replace the cryostorage unit lid.
v. Place the frozen cryovials into a -80°C freezer to prevent damaging transient warming events.
vi. Notify the Responsible Person/Laboratory Manager that a conflict between the electronic database and the cryostorage unit exists. vii. Identify a new location to store the vials using the electronic database and begin the process of vial transfer again (See **Section 5.4.1**).

5.4.3 Data logging

a. Fill out the cryostorage transfer slip(s) detailing the date, location, vial contents and owner of the newly stored cryovials.
b. Return the cryostorage keys and the filled out cryostorage transfer slip(s) to the Responsible Person/ Laboratory Manager.
c. Update Procure with the vial location and cell information.

Note: It is the RP/LM's responsibility to update and maintain the electronic cryostorage database, but you should assist this process by ensuring cryostorage transfer slips are handed to the RP/LM without delay. It is also recommended that you confirm that the electronic database has been updated with your stored vial information to ensure the electronic database accurately reflects the preserved contents of the CBE cryostorage units.

Note: It is the users responsibility to keep Procuro up to date with the correct information and cell location.

6. DOCUMENTATION

The following forms are outputs of this SOP:

FSOP031.1 Cryostorage Transfer Slip Template

The following are examples of the labeling & storage system for cryopreservation.

6.1. An example of fields required for the labeling of cryo-vials containing cryo-preserved cells
6.2. Biorack 750 map for storage of Cryo-vials in the Liquid Nitrogen Store

6.1 An example of fields required for the labelling of cryovials containing cryopreserved cells

Cell line, Passage number,   ***Date***   ***Owner/Group Initial HTA?***   ***Quarantine?***

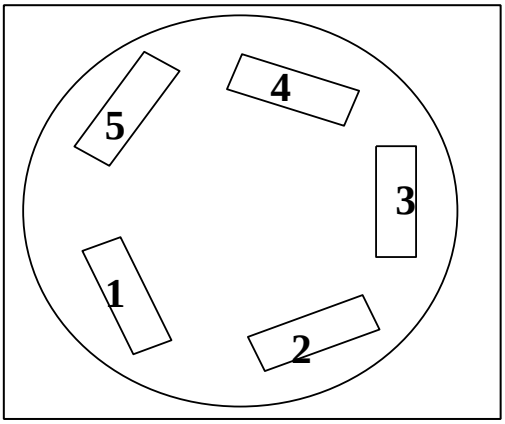
6.2 Biorack 750 map for storage of Cryovials in the Liquid Nitrogen Store

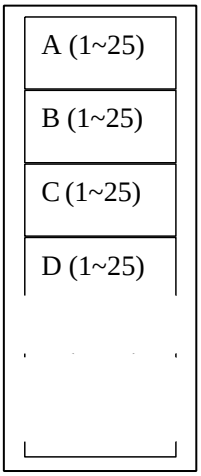
Bank - Available 6 Banks (B1 to B6) and 1 Experimental bank
Carrier Rack - Available 5 carrier racks per bank (R1, R2, R3, R4, & R5)
Cryo Box - 4 cryoboxes per carrier rack (A, B, C, D)
Position - 25 positions per box (01 – 25)

Location example **B1-R5-A-17**

Key: ***B1 = Bank 1*** ***R5 = Rack 5***
A = Cryobox A ***17 = Position 17***

Carrier





Cryobox Array

01	02	03	04	05
06	07	08	09	10
11	12	13	14	15
16	17	18	19	20
21	22	23	24	25

Standard Operating Procedure **SOP031**

Title: Cryopreservation And Storage of Mammalian Cells

Location: CBE laboratories.

SOP Version History

Version Reviewed	Date Revised/ Reviewed	Revision Summary	New Version Number
001	**26/04/2010 KB**	Annual Review – **Section 4** – included note to highlight that DMSO gels at low temperatures. **Section 7** – rephrased procedure parts 7.9 to 7.16 to include new procedure for labeling vials and using the cryostorage map and online database. **Section 8** – included sample vial label and diagram explaining how to allocate cell banking database numbers and vial locations, as well as an example of the cell banking database.	**002**
002	**16.06.10 Reviewed by C. Kavanagh**	**References** – Added reference SOP029 'Safe handling & disposal of Trypan blue'. **Section 4.1.7** – Added a statement to say that Trypan blue is Carcinogenic & to refer to SOP029 for details on how to safely handle & dispose of Trypan blue.	**003**
003	**11th December 2012 Reviewed by C. Kavanagh**	i) Annual Review ii)Transfer to new template iii)Minor formatting amendments iv)Section 5.14 – Removal of statement regarding use of key. vi)Section 6 – Amendments to cell bank number from 2 to 3.	**004**

Standard Operating Procedure SOP031

Title: Cryopreservation And Storage of Mammalian Cells

Location: CBE laboratories.

004	**15/09/2014 A. Picken**	Complete rewrite of SOP in response to Change Note SRN012.	**005**
005	**26/11/15** J. Bowdrey	Review- No amendments	**006**
006	**02/06/18** Reviewed by K.Sikand	No changes kept same version number.	**006**
006	**09/06/2020 J.Bowdrey**	Addition of Cryostickers, and also input of data on to Procuro	**007**