Standard Operating Procedure **SOP032**

Title: Revival of Cryopreserved Mammalian Cells

Location: CBE laboratories.

1. PURPOSE

This SOP describes how to revive cryopreserved mammalian cells from long-term cryostorage (See **SOP031**).

2. SCOPE

This SOP applies to all users within the CBE laboratories who maintain cells in cryostorage units.

The SOP describes the procedures for:

- Retrieving frozen cells from cryostorage **(Section 5.1)**
- Rapid thawing of frozen cells **(Section 5.2)**
- Cryoprotectant dilution **(Section 5.3)**
- Cell seeding (Section 5.4)

Read this SOP in conjunction with **SOP013** (Safe Use and Maintenance of Liquid Nitrogen Stores); **SOP031** (Cryopreservation and Storage of Mammalian Cells); **SOP036** (Maintenance of a Quality Laboratory Environment) and **SAFMM6405** Risk Assessment Liquid Nitrogen in CBE Laboratories).

3. RESPONSIBILITES

3.1 Authorised CBE Laboratory Users

- **Shall** understand the health and physical hazards of cryogenic liquids (read **SAFMM6405 Risk Assessment Liquid Nitrogen** 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 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** regularly check Pro-curo to ensure logging of material matches that of the database.

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).

4.2 Supplies

a. Aspirating pump with connected aspiration line.
b. Plastic Serological Pipettes (5 ml – 25 ml).
c. Aspirating pipettes (2 ml).
d. Polypropylene centrifuge tubes (15 ml and/or 50 ml).
e. Culture vessel(s) (e.g. T-flasks or multi-well plates)

4.3 Media and Chemicals

a. Growth medium (e.g. DMEM + 10 % Foetal Bovine Serum)
b. 70% IMS

5. PROCEDURE

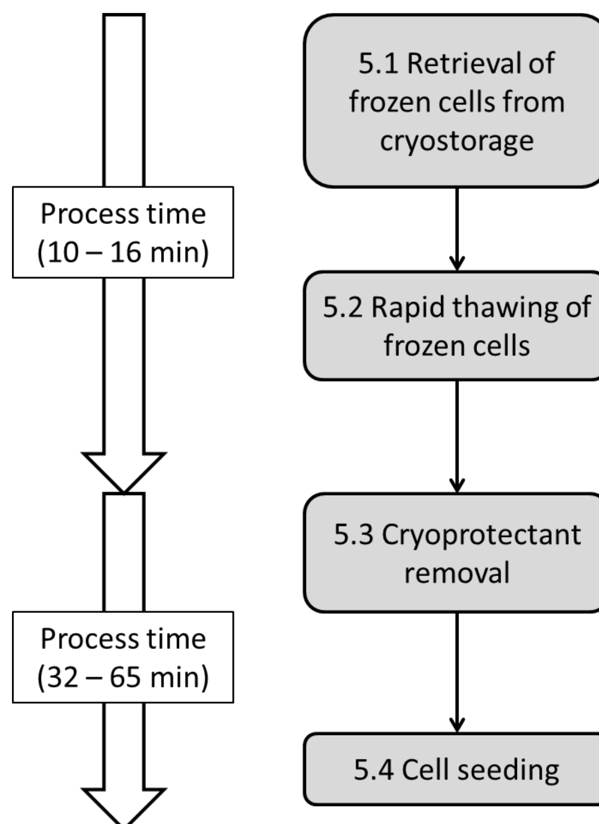

Figure 1: SOP overview. Process map defining process stages with suggested timing for process timing at each process stage.

5.1 Retrieval of frozen cells from cryostorage

This procedure describes the steps needed to identify and retrieve vials containing frozen cells from the CBE cryostorage unit(s).

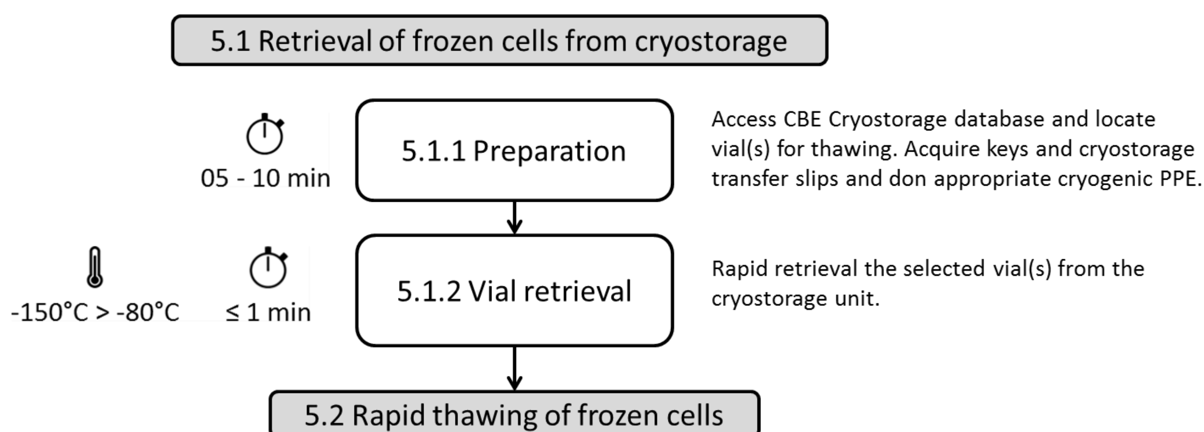

Figure 2: Retrieval of frozen cells from cryostorage overview. A process map describing the retrieval of frozen cells from cryostorage stage of SOP032. Operating ranges for temperature and time are shown with broad descriptions of each task.

5.1.1 Preparation

a. Access the Cryostorage database on the CBE -QUAL workspace and identify the location of your frozen cryovials. These can also be identified on Procuro.
b. Retrieve the cryostorage keys and a cryostorage transfer slip from the Responsible Person/Laboratory Manager.
c. Unlock and the relevant cryostorage unit(s) (kept in H29 or H30).
d. Deploy the spill-tray next to the cryostorage unit(s).
e. Don cryogenic PPE (Face visor or safety goggles, a heavily-insulated gauntlet glove instead of a nitrile glove, covered footwear and laboratory coats outlined in **SOP013 Safe Use and Maintenance of Liquid Nitrogen Stores**.

5.1.2 Vial Retrieval

Note: It is recommended that a maximum of 12 vials are thawed and processed at any one time when following this SOP. This maximum limits the potential for cell damage during time-sensitive steps (e.g. cryoprotectant washing) and is pragmatic when considering centrifuge capacity within the CBE.

a. Open the unlocked cryostorage unit and carefully place the cryostorage lid face up on the metal spill tray.
b. Retrieve the target cryostorage rack and hold the rack 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 3**). 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.

CAUTION: Perform steps **5.1.2b – 5.1.2h** quickly (within **60 seconds**). If a large number of vials are to be retrieved, split the task into multiple batches and allow the cryostorage rack time to thermally equilibrate (5 minutes) before retrieving another batch of frozen vials.

c. Place the cryostorage rack onto the spill tray then replace the cryostorage unit lid.
d. Remove the metal support rod from the rack to access the cryostorage boxes.
e. Quickly retrieve the required cryostorage box, collect the target cryovials and place them into a pre-chilled -80°C container (e.g. a dry-ice box or chilled metal water bath beads) and start the tasks outlined in **Section 5.2.1a** – **Section 5.2.1b** before completing **Section 5.1.2f** – **Section 5.1.3b**).

Note: If the expected vial(s) are not found within the cryostorage locations identified in **Section 5.1.1a** then:

i. Quickly check that the correct cryostorage box has been obtained and that the box is in the correct orientation.
ii. Quickly replace the cryostorage box into the cryostorage rack. iii. Replace the metal support rod into the cryostorage rack. iv. Replace the cryostorage rack into the cryostorage unit.
v. Replace the cryostorage unit lid.
vi. Note which cryostorage locations are inconsistent with the electronic database.
vii. Notify the Responsible Person/Laboratory Manager that a conflict between the electronic database and the cryostorage units exists.
viii. Identify an alternative location containing the desired preserved cell material using the electronic database and begin the process of vial retrieval again (See **Section 5.1.1**).

f. Replace the cryostorage box (if multiple boxes are needed from the same cryostorage unit, repeat steps 5.1.2.e and f).
g. Replace the metal support rod into the cryostorage rack and remove the cryostorage unit lid.
h. Place the cryostorage rack into the cryostorage unit and replace the cryostorage unit lid.
i. Once all vials are retrieved, relock the cryostorage unit(s).

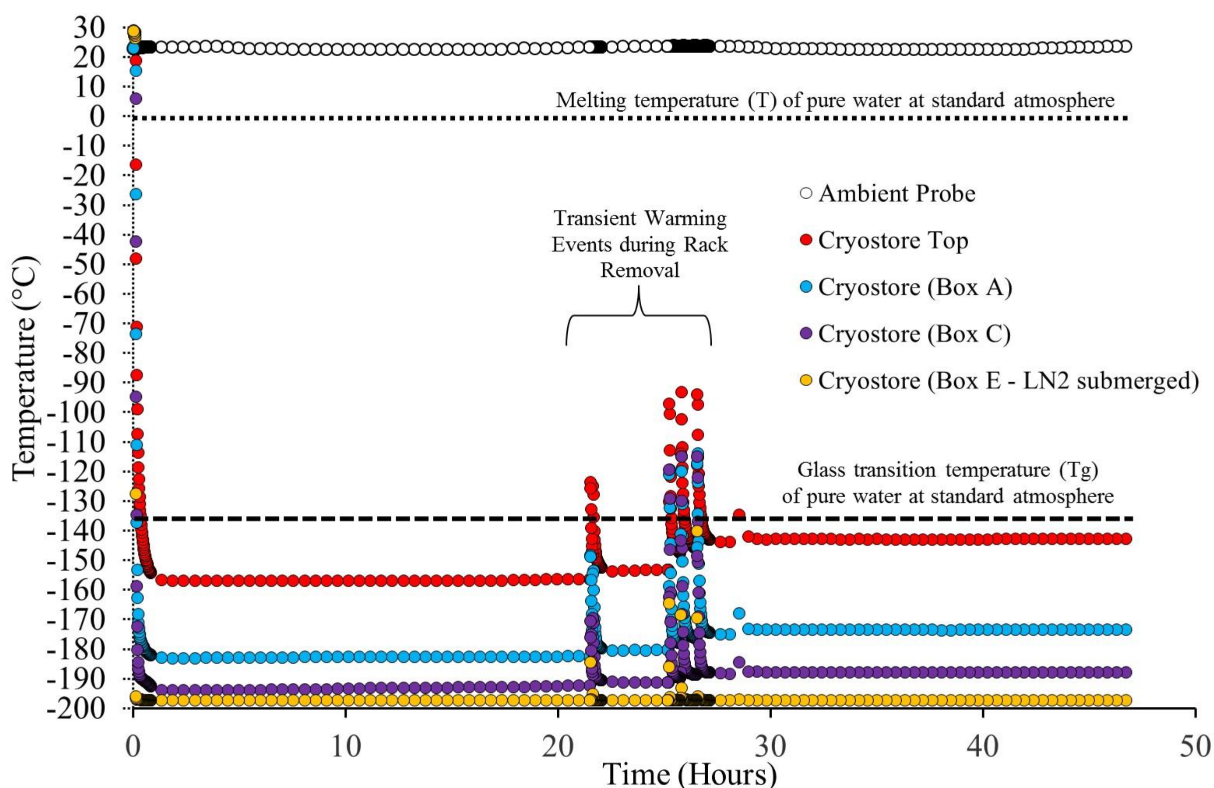

Figure 3: Thermal stability and transient warming event responsiveness of the current CBE cryostorage environment.
Temperatures were cyclically recorded from thermocouples housed in a modified cryostorage lid once per minute. Transient warming events were mimicked by fully removing the cryostorage lid and associated thermocouples for 1 minute before rehousing the modified lid within the cryostorage unit.

5.2 Rapid thawing of frozen vials

This procedure describes the steps needed to rapidly thaw cells retrieved from cryostorage.

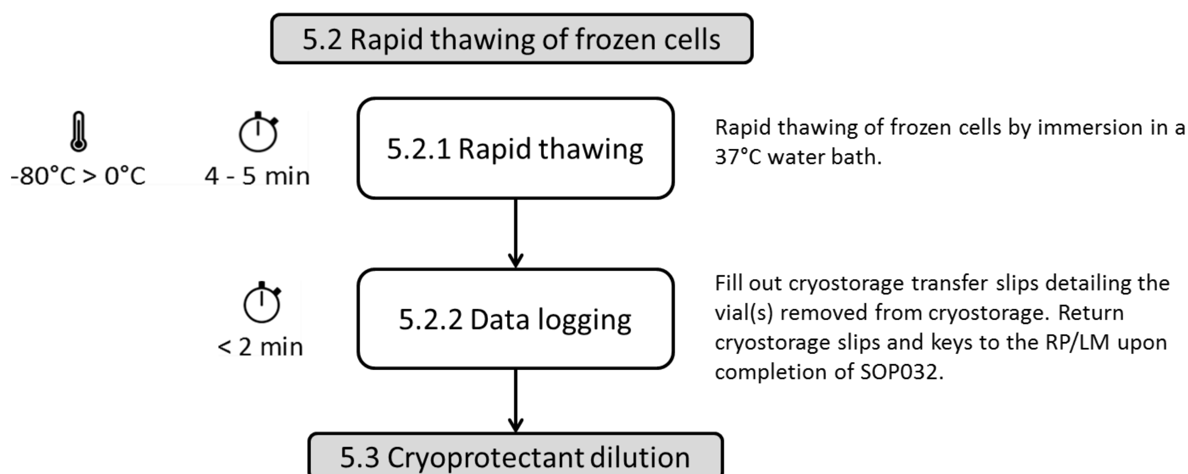

Figure 4: Rapid thawing of frozen cells overview. A process map describing the rapid thawing of frozen cells stage of SOP032. Operating ranges for temperature and time are shown with broad descriptions of each task.

5.2.1 Rapid thawing

a. Transfer the chilled secondary container containing the frozen vials from the cryostore(s) (H29 or H30) to a cell culture lab (e.g. H25) containing a water bath set to 37°C.

   Note: this step may be omitted if the cryostorage unit is accessed adjacent to a 37°C water bath.

b. Quickly place the vials into the 37°C water bath using a sample holder or foam sample float or other suitable vial holder.

CAUTION: Cryovials should not be submerged in water beyond the vial-lid interface. This is because standard cryovials are not hermetically sealed and can be contaminated by the ingress of non-sterile water.

c. Incubate the vials in the water bath to thaw them until a slither of ice remains (approximately 3.5 - 4 minutes for 1 ml vial volume).

5.2.2 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 after completion of this SOP.

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.
It is the individuals responsibility to update any removal of vials on Pro-CURO.

5.3 Cryoprotectant removal

This procedure describes the steps needed to aseptically remove cryoprotectants found in the freezing medium by dilution in growth medium followed by centrifugation.

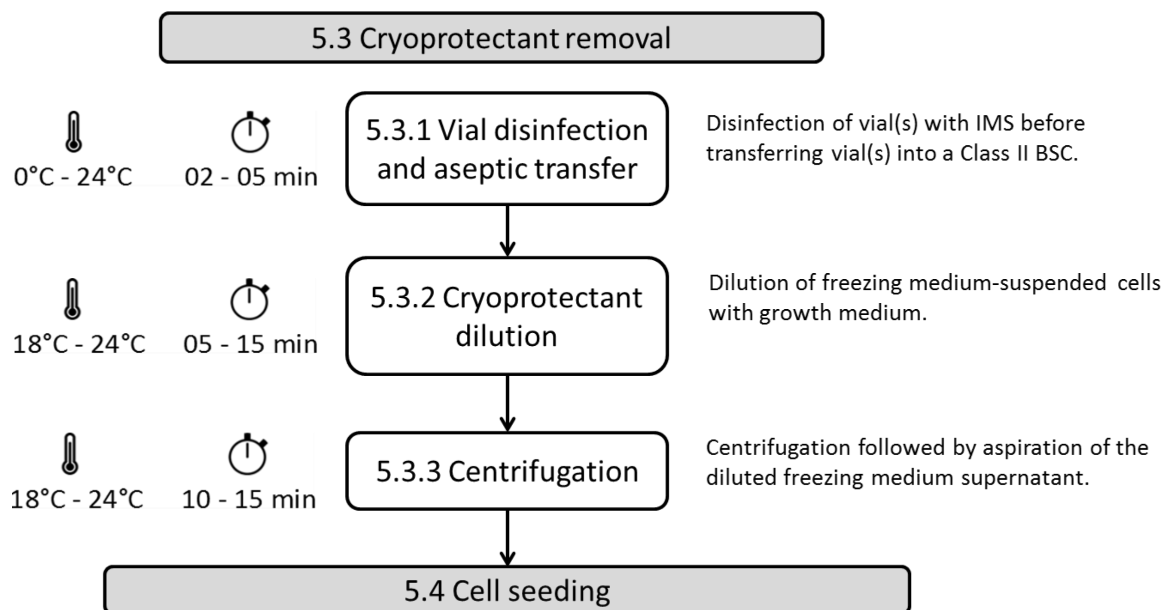

Figure 5: Cryoprotectant removal overview. A process map describing the cryoprotectant removal stage of SOP032. Operating ranges for temperature and time are shown with broad descriptions of each task.

5.3.1 Vial disinfection and aseptic transfer

a. Retrieve the vial(s) and wipe each with IMS saturated white paper towel.
b. Aseptically transfer the vial(s) into a Class II BSC.

5.3.2 Cryoprotectant dilution.

a. For each vial, carefully remove and discard the cryovial lid
b. Sequentially dilute the vial contents 1:1 using pre-warmed (37°C) growth medium and gentle pipetting (twice is sufficient), then smoothly transfer the diluted cell suspension into a 15 ml polypropylene tube containing additional growth medium (1:5 dilution).

Example: If 1 ml of cell suspension was preserved, dilute the thawed cells with 1 ml warm growth medium and then transfer the diluted suspension into a further 8 ml of warm growth medium.

Note: Some cryopreservation protocols described in the literature or provided by freezing medium manufacturers require the drop-wise addition of thawed cells into growth medium to

"gently washout" the cryoprotectant. The author considers this unnecessary in his experience, with such an over-elaborate technique only slowing down the processing of cells post-thaw.

5.3.3 Centrifugation.

a. Centrifuge the diluted cell suspension at 200 x g for 5 minutes.
b. Aseptically transfer the centrifuge tube into a Class II BSC.
c. Carefully aspirate the supernatant (avoiding the cell pellet).
d. Tap the polypropylene tube(s) to dislodge the cell pellet.

5.4. Cell seeding

This procedure describes the steps needed to suspend cells in growth medium and seed them for further culture.

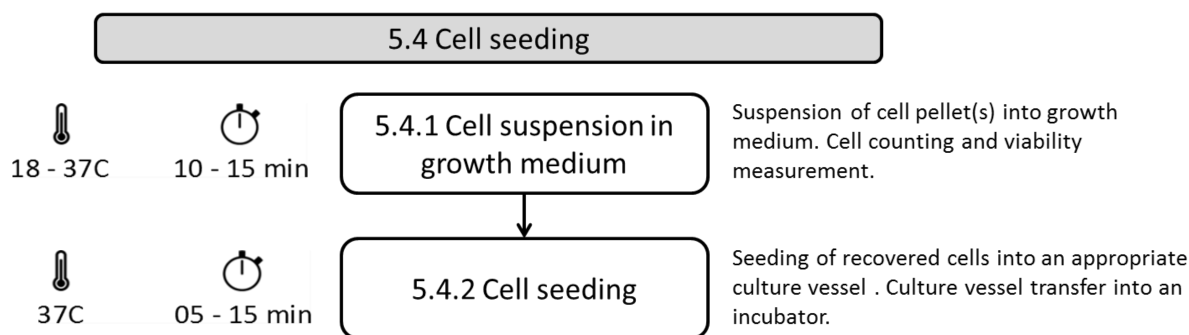

Figure 6: Cell seeding overview. A process map describing the cell seeding stage of SOP032. Operating ranges for temperature and time are shown with broad descriptions of each task.

5.4.1 Cell suspension in growth medium

a. Suspend the cell pellet in an appropriate amount of pre-warmed (37°C) growth medium (e.g. 1 ml) with gentle pipetting (5 times should suffice) using a P1000 pipette.

   Note: If cell colonies were preserved, avoid over-pipetting which will result in disaggregation of colonies and ultimately decrease the recovered cell yield.

b. Obtain a 200 µl sample of the thawed and washed cell suspension for cell counting and viability assessment.
c. 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).

5.4.2 Cell seeding

a. Determine the number of viable cells (based on membrane integrity measurements – **Step 5.4.1c**).
b. Seed a user-defined number of cells into a suitable culture vessel or vessels (e.g. T-flask or multiwell plate) containing pre-warmed (37°C) growth medium.

Note: The type of growth medium, culture vessel and desirable seeding density will be celltype and culture-format specific. It is recommended that the user seed cells as per a standard passage for their culture system.

Note: In some cases, it may be sensible to slightly over-seed (e.g. 120 % normal density) to compensate for delayed-onset post-thaw cell death which can occur in sensitive cell-types (e.g. Human Embryonic Stem Cell lines).

c. Incubate the culture vessels in a permissive culture environment (e.g. 37°C in a humidified incubator containing a 5 % CO_2 in air atmosphere).

CAUTION: Avoid disturbing culture vessels containing recently thawed cells. Vibrations or physical movement may prevent or delay cell adhesion to the culture surface, decreasing the yield of recovered cells.

Note: It is recommended that cultures be inspected using light microscopy 24 hours after seeded to evaluate the relative recovery of the cells with correct morphology. For adherent cultures, it may be necessary to completely exchange the growth medium to remove debris and non-viable cells. Dead cells and debris in suspension cells generally deposit around the culture vessel and can be avoided during subsequent passaging. However, dead cells release free DNA into the culture environment which can promote cell aggregation in suspension cultures. The use of cell-strainers and anti-clumping agents may be necessary to minimise the carryover of aggregated cells during subsequent passages.

6. DOCUMENTATION

The following forms are outputs of this SOP:

6.1 FSOP031.1 for Cryostorage Transfer Slip Templates

Note: Examples of the labeling and storage system for cryopreservation are found in SOP031 - Cryopreservation and Storage of mammalian cells.

SOP Version History

Version Reviewed	Date Revised/ Reviewed	Revision Summary	New Version Number
001	26/04/2010	**Section 7** – rephrased procedure parts (ii) to (v) to include new procedure for locked cryo-storage system. **Section 8** – included sample diagram explaining how to read vial location references, as well as an example of the cell banking database and cryo-storage location map.	**002**
002	16/06/2010 Reviewed by C. Kavanagh	**References** – Added SOP029 ‘ Safe handling & disposal of Trypan blue’ & COSHH Risk Assessment for Trypan blue CBE32/review 1. **Section 4.2** – Added a section titled ‘Trypan blue’ & the statement Trypan blue is Carcinogenic. Please refer to SOP029 for details of how to handle & dispose of Trypan Blue safely	**003**
003	11/12/2012	i)Annual Review	**004**

	Reviewed by C. Kavanagh	ii)Transferred to lean template iii)Minor formatting amendments iv)**Section 5(.ii)** removed reference to use of key v)**Section 5 (iii)** Added statement referring to location of cell banking record folder. vi)**Section 5 (xiv)** Removed reference to use of key & added statement to return folder to shelf in H31. vii)**Section 6.1** Amended from 2 to three cell banks	
004	25/04/2014 A. Picken	Complete revision	**005**
005	26/11/2015 J.Bowdrey	Review – No amendments	**006**
006	02/06/18 K.Sikand	Review – No changes – kept version number	**006**
006	**09/06/2020 J.Bowdrey**	Review- Addition of use of Procuro	**007**