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Freezing of cells

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Major changes as compared to to the previous version:

- Minor adaptations were added in the quality control check under 7.1.1.
- Addition of alpha-MEM medium to the list of Materials (4.1) as ‘Growth medium’ option
- Administrator and content check have been changed.

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1 Subject and field of application

Freezing of cells for all bio-assays.

2 Introduction

Mammalian cells can be stored in liquid nitrogen for many years. To prepare the cells for nitrogen storage, healthy cells are trypsinated and then taken up in growth medium containing extra serum together with DMSO as a cryo protective agent. The cells are transferred to cryo vials, which are stored at -80°C for one day to allow for a rapid - but not too rapid - decrease in temperature. The next day, the cryo vials are transferred to the liquid nitrogen to allow for long term storage.

This protocol describes the procedure used for freezing mammalian cells. It is based on freezing the cells derived from 25 cell culture flasks, resulting in 75 cryo vials. If 25 flasks are too many flasks to handle in one time, the cells are frozen in batches of 5 flasks at a time. When freezing smaller or larger batches of cryo vials, simply adapt the required amounts of vials, solutions etc.

3 Health and Safety

Be careful with (unknown) chemicals! Don’t inhale, avoid skin contact, wear gloves and glasses and work in the fume hood. If needed, plasticized paper is available as underlayment.
Also check the guidelines stated in p-bds-022; Safety and health guidelines for CALUX® technology.
Furthermore, wear isolated gloves and wear glasses when working with liquid nitrogen. Also be sure to work in a well-ventilated room to avoid suffocation as nitrogen can replace the oxygen.

4 Materials, Chemicals and Apparatus

More information on the products can be found in protocol p-bds-050.

4.1 Materials

4.1.1 75 cm^2 and/or 175 cm^2 culture flasks, for tissue culture
4.1.2 Pipettes (10 ml), sterile
4.1.3 Pipettes (25 ml), sterile
4.1.4 Pipet (range to 20 µl)
4.1.5 CALUX® cells (BioDetection Systems)
4.1.6 Plastic tubes (50 ml) with cap, sterile
4.1.7 Bürker-Türk counting chamber counting chamber; see counting cells (p-bds-074)
4.1.8 Cryo vials
4.1.9 Storage container for cryo vials
4.1.10 Tweezers
4.1.11 Mr. Frosty freezing containers
4.1.12 Garbage-barrel for biologically material
4.1.13 Ice

4.2 Chemicals

4.2.1 Freezing medium - p-bds-168 (Preparation of freezing medium)
4.2.2 Growth medium U2OS - p-bds-76 (Prep. of DMEM/F12 containing 7.5% FCS)
H4IIE - p-bds-63 (Prep. of αMEM containing 10% FCS)
4.2.3 Liquid nitrogen
4.2.4 PBS pH 7.2
4.2.5 Trypsin solution - p-bds-64; Preparation of trypsin solution)

4.3 Apparatus

4.3.1 Autoclave
4.3.2 Centrifuge
4.3.3 Safety-cabinet
4.3.4 CO2-incubator
4.3.5 Inverted microscope (culture microscope) with 4X, 10X and 20X objective
4.3.6 Pipette controller
4.3.7 Freezer -80 °C
4.3.8 Liquid nitrogen cell storage facility
4.3.9 Mr. Frosty freezing containers

5 Time schedule

- Preparation of cell culture flasks should start up to 1.5 week in advance.
- Preparation of the cryo vials can be done in one day. The next day, the cryo vials should be transferred to the liquid nitrogen.
- After at least one day in liquid nitrogen, one cryo vial should be tested for sterility, speed of grown and cell line performance.

6 Procedure

6.1 Freezing of cells

(For freezing of cells without centrifuge see **Appendix A**)

6.1.1 Prepare 75 cm^2 or 175 cm^2 culture flasks with cells (start 1.5 week in advance).

6.1.2 Check whether the flasks are approximately 90% confluent before starting the freezing procedure.

6.1.3 Label closed cryo vials with: cell line number (php), batch number (php), type of batch (alfa-, intermediate- or work-batch), passage number, freezing date, BDS (see figure 1 for example). For each 75 cm^2 flask, mark 3-4 cryo vials; for each 175 cm^2 flask, mark 7 cryo vials.

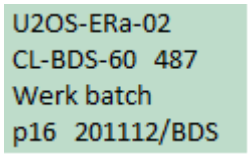

Figure 1: Label for batch of seeding vials

6.1.4 Trypsinate all cells according to the procedure described in the corresponding cell culture protocol.

6.1.5 Suspend the cells in growth medium (10 ml for each 75 cm^2 flasks; 15 ml for each 175 cm^2 flasks) (4.2.2).
Note: H4IIE cells are resuspended in αMEM growth medium whereas U2OS cells are resuspended in DMEM/F12 growth medium!

6.1.6 Pool and count the cells (see p-bds-74, counting of cells).

6.1.7 Determine the final volume of freezing medium required to prepare a final cell suspension of 1.5 x 10^6 cells/ml freezing medium.

6.1.8 Divide the cells-suspension equally over 50 ml Greiner tubes and centrifuge (250 g, 5 minutes).

6.1.9 During centrifugation, carefully open labelled cryo vials in the laminar flow cabinet (sterile) and set aside until further use. Each cryo-vial will contain 1 ml of cell suspension in freezing medium.

6.1.10 Following centrifugation, keep the vials and 50 ml Greiner tubes on ice at all times and work as quickly as possible to minimise the toxic effect of DMSO at room temperature!

6.1.11 In each 50 ml Greiner tube, discard medium and re-suspend the cells in freezing medium (4.2.1). The amount of freezing medium to use for resuspension equals the final total volume of freezing medium (see 6.1.7) divided by the number of 50 ml Greiner tubes used for centrifugation.

Note: H4IIE cells are resuspended in αMEM freezing medium whereas U2OS cells are resuspended in DMEM/F12 freezing medium!

6.1.12 Following resuspension of the centrifuged cells, pool the cells and gently homogenise.

6.1.13 Aliquot the re-suspended cells in the opened and cooled cryo vials. Each cryo-vial shall contain 1 ml of resuspended cells (1.5 x 10^6 cells/ml). Close the cryo-vials and place them in a storage box or Mr. Frosty freezing containers.

6.1.14 Cover the inside of the storage box of the cryo vials with tissues to facilitate a graduate freezing of the cells and store at -80 °C overnight. Alternatively, apply Mr. Frosty freezing containers to achieve a rate of cooling close to -1 °C/minute.

6.1.15 Transfer cryo vials to liquid nitrogen for storage.

***** When freezing a "work-batch" of cells a fictive passage number of 5 is used to obtain uniformity. The correct passage number should be noted in LIMS (p5 = p14 for example)!

7 Quality control

7.1 Check cells

Note: For this check of the cells (bacterial and/or mycoplasma contamination), use growth medium **without antibiotics**. These can inhibit the growth of bacteria and mycoplasma, whereas this test wants to confirm that there are indeed no contaminations of this kind in the newly prepared batch of cells.

7.1.1 Thaw one cryo vial after at least one day of storage in the liquid nitrogen, according to protocol p-bds-065. Culture the cells for at least one week according to the appropriate protocol (in appropriate growth medium without antibiotics) and dilute the cells to a cell density of 100.000 (U2OS) or 400.000 (H4IIE) cells/ml. Use this cell suspension for:

1. Seeding half a 96-microtiter plate with 100 µl cell suspension per well. After an o/n incubation, check the confluency of the cells and perform the CALUX assay as usual.
2. Seeding a 75 cm^2 culture flask with 1/4 of this cell suspension for testing bacterial contamination. Fill out the flask with extra growth medium to a total volume of 20 ml and culture the cells for 4-5 days.
3. Seeding a 75 cm^2 culture flask with 1/8 of this cell suspension for testing Mycoplasma contamination. Fill out the flask with extra growth medium to a total volume of 10 ml and culture the cells for 3-4 days until the confluence reaches 50-70% (p-bds-150).

Appendix A:
Freezing of cells without the use of a centrifuge

For freezing of cells without the use of a centrifuge, a different freezing medium has to be used:

H4IIE cell: αMEM growth medium (p-bds-063) containing 20 % DMSO (e.g., 8 ml DMSO with 32 ml growth medium).

U2OS cells: DMEM/F12 growth medium (p-bds-076) containing 20 % DMSO (e.g., 8 ml DMSO with 32 ml growth medium).

A-1 Prepare 5 culture flasks (75 cm^2) with cells (start one week in advance). Check whether the flasks are approximately 90 % confluent.

A-2 Label each cryo vial with cell line and batch number, cell line number (php), batch number (php), type of batch (alfa-, intermediate- or work-batch), passage number, freezing date, BDS (see figure 1 for example).

A-3 Open the cryo vials carefully and set aside in the laminar flow cabinet (sterile) until further use (see point A-6).

A-4 Trypsinate cells from five flasks according to the procedure described in the corresponding cell culture protocol and remove as much trypsin as possible.

A-5 Suspend the cells in the first flask with 5 ml growth medium. Use the same 5 ml of growth medium with cells to suspend the cells in the remaining 4 flasks and collect the cells in the fifth flask.

A-6 Transfer 0.5 ml of the cell suspension from the fifth culture flask into the cryo vials. Wait 10 minutes to allow the cells to sink to the bottom of the vial.

A-7 Slowly add (drop wise) 0.5 ml of freezing medium to each cryo vial.

A-8 Close the cryo-vials and place them in a storage box or Mr. Frosty freezing containers.

A-9 Cover the inside of the storage box of the cryo vials with tissues to facilitate a graduate freezing of the cells and store at -80 °C overnight. Alternatively, apply Mr. Frosty freezing containers to achieve a rate of cooling close to -1 °C/minute.

A-10 Transfer cryo vials to liquid nitrogen for storage.