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CELL STORAGE-01-Freezing and Thawing Protocol for Adherent Cell Lines

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ABSTRACT

In our institute, cell lines are stored after freezing procedure that provides for a constant temperature lowering by 1 degree/minute. This procedure is useful to minimizes ice crystals formation during freezing process, this allow to reduce the cells damage and, in turn increasing cells viability after thawing. After storage at -80°C or in liquid nitrogen (in relation to subsequent applications or the expected freezing time) cells are thawed rapidly in a water bath at 37°C and the cryopreserving-medium is immediately remove by gentle centrifugation in order to reduce as soon as possible, the DMSO toxic effect.

Currently, in our institute, the freezing/thawing procedure is validated for the following cell lines:

- **MCF7**, Human Caucasian breast adenocarcinoma, epithelial-like morphology, adherent growth mode.
- **HUVEC**, Human Umbilical Vein Endothelial Cells, endothelial morphology, adherent growth mode.
- **A549**, Human Caucasian lung carcinoma, epithelial-like morphology, adherent growth mode.
- **Synovial fibroblasts**, Human synoviocytes, fibroblast-like cells morphology, adherent growth mode.

After freezing/thawing procedure, cells quality must be assessed by cell morphology evaluation (using Optical Microscopy technique) and time required to reach confluence in the T25 cm^2 culture flask, which must not exceed the times indicated in the **Table 1**.
After quality test cells must be resuspended according to specific protocols (for details see **Table 2**).

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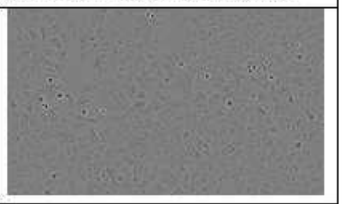
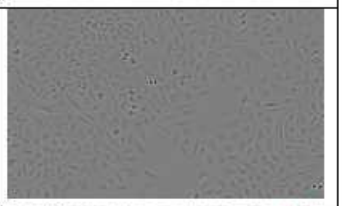
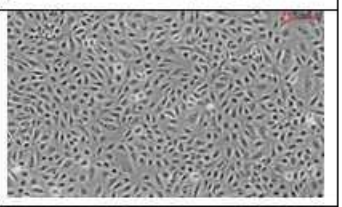
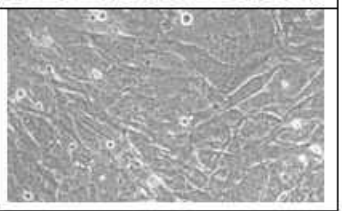
Cell lines	Time to Reach Confluence	Reference for morphology evaluation
MCF7	48 hours	
NSCLC A549	24 hours	
HUVEC	72 hours	
Synovial fibroblast	96 hours	

Table 1

Cell line	Culture medium	Applications post thawing	Our publication using this applications
NSCLC A549	RPMI 1640+10%FBS+100 U/ml penicillin/streptomycin	Cell cultures	Coelho et al., 2019
HUVEC	EndoGRO™basal medium+ 2% FBS+10 mM L-glutamine+0.75 U/ml Heparin sulfate+ 5 ng/ml rh-VEGF + 5 ng/ml rh-EGF + 5 ng/ml rh-FGF basic+ 15 ng/ml rh-IGF-1 + 50 µg/ml ascorbic acid+ 100 U/ml penicillin/streptomycin	Cell cultures	Marino et al., 2017
MCF7	DMEM without phenol red+10% FBS+100 U/ml penicillin/streptomycin+2 mM L-glutamine	Cell cultures	Spagnuolo et al., 2014; Cosentino et al., 2007
Synovial Fibroblast	RPMI 1640 without phenol red+10% FBS+100 U/ml penicillin/streptomycin	Cell cultures	Capellino et al., 2014

Table 2

List of published work by our institute that using this procedure

- Coelho M, Imperatori A, Chiaravalli AM, Franzi F, Castiglioni M, Rasini E, Luini A, Legnaro M, Marino F, Ribeiro L, Cosentino M. **Beta1- and Beta2-Adrenoceptors Expression Patterns in Human Non-small Cell Lung Cancer: Relationship with Cancer Histology.** J Neuroimmune Pharmacol. 2019 Dec;14(4):697-708. doi: 10.1007/s11481-019-09879-6. Epub 2019 Oct 16. PMID: 31620969.

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- Marino F, Schembri L, Rasini E, Pinoli M, Scanzano A, Luini A, Congiu T, Cosentino M. **Characterization of human leukocyte-HUVEC adhesion: Effect of cell preparation methods**. J Immunol Methods. 2017 Apr;443:55-63. doi: 10.1016/j.jim.2017.01.013. Epub 2017 Feb 3. PMID: 28167274.

- Spagnuolo P, Rasini E, Luini A, Legnaro M, Luzzani M, Casareto E, Carreri M, Paracchini S, Marino F, Cosentino M. **Isoflavone content and estrogenic activity of different batches of red clover (Trifolium pratense L.) extracts: an in vitro study in MCF-7 cells.** Fitoterapia. 2014 Apr;94:62-9. doi: 10.1016/j.fitote.2014.01.027. Epub 2014 Feb 5. PMID: 24508860.

- Capellino S, Cosentino M, Luini A, Bombelli R, Lowin T, Cutolo M, Marino F, Straub RH. **Increased expression of dopamine receptors in synovial fibroblasts from patients with rheumatoid arthritis: inhibitory effects of dopamine on interleukin-8 and interleukin-6.** Arthritis Rheumatol. 2014 Oct;66(10):2685-93. doi: 10.1002/art.38746. PMID: 24965369.

- Cosentino M, Marino F, Ferrari M, Rasini E, Bombelli R, Luini A, Legnaro M, Delle Canne MG, Luzzani M, Crema F, Paracchini S, Lecchini S. **Estrogenic activity of 7-hydroxymatairesinol potassium acetate (HMR/lignan) from Norway spruce (Picea abies) knots and of its active metabolite enterolactone in MCF-7 cells.** Pharmacol Res. 2007 Aug;56(2):140-7. doi: 10.1016/j.phrs.2007.05.001. Epub 2007 May 22. PMID: 17572100.

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MATERIALS TEXT

Reagents

- **FBS** catalog number: ECS0180L-500 ml, Euroclone
- **RPMI 1640** catalog number: ECM0495L-500 ml, Euroclone
- **RPMI 1640 without phenol red** catalog number: ECM0505L-500 ml, Euroclone
- **DMEM** catalog number: ECM0160L-500 ml, Euroclone
- **Penicillin/streptomycin** catalog number: ECB3001D-500 ml, Euroclone
- **L-Glutamine** catalog number: ECB3000D-500 ml, Euroclone
- **EndoGRO™-VEGF complete media kit** catalog number: SCME002, Millipore
- **Trypsin/EDTA** catalog number ECB3052D, Euroclone
- **PBS** catalog number ECB4053L, Euroclone
- **DMSO** catalog number 276855, Sigma-Aldrich

Sterile plastic disposable:

- **Cryogenic vials** catalog number: CC430659, Corning
- **T25 cm^2 culture flasks for adherent cells** catalog number: CC430639, Corning
- Generic laboratory plastic disposable

Instrumentation required:

- Laminar flow hood
- Centrifuge
- -80°C freezer

BEFORE STARTING

Work under laminar flow hood when you are processing samples (from the beginning to the end of the following procedure). Make sure you are using **sterile plastic disposable and medium**.

CELL FREEZING PROCEDURE FOR ADHERENT CELLS (MCF7, HUVEC, A549, SYNOVIAL FIBROBLAST)

1 At confluence remove culture medium from the flasks, wash twice the cell monolayer with **5 mL** of sterile PBS.

2 Add **1 mL** (for T25 cm^2 culture flasks) of trypsin/EDTA for 5-10 minutes, observe the cells under a microscope to make sure that cells are detached.

3 Add **4 mL** of complete medium to inactivate trypsin, recover the cell suspension in 15 mL conical tube and centrifuge for **00:05:00** at **200 x g** **Room temperature** .

4 Remove supernatants and resuspend the cell pellet in **1 mL** of cryopreserving medium (90% FBS + 10% DMSO).

5 Immediately aliquot **1 mL** of cell suspension into a cryogenic vial.

Place the cryogenic vial(s) in cotton wool, and then place it in a polystyrene box.

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7 Put the polystyrene box in -80°C freezer.

-80°C Freezer
Eppendorf **B U9230-0001**

8 After a minimum of **24:00:00** transfer the cryogenic vials into a box for storage in -80°C freezer.

CELL THAWING PROCEDURE

9 Remove cryogenic vial from -80°C and keep it at **37 °C** in water bath, this procedure should take approximately 1-2 minutes or until ampoule is slightly thawed.

10 Clean the cryogenic vial outside with 70% EtOH, open the cryogenic vial and add **1 mL** of prewarmed complete medium for specific cells lines **(Table 2)**.

11 Transfer the cell suspension into a 15 mL conical tube containing **10 mL** of prewarmed complete medium for specific cells lines.

12 Centrifuge for **00:05:00** at **200 x g** at **Room temperature**

13 Resuspended the cells into appropriate cell culture medium (**Table 2**) and start quality control procedure (**Table 1**).

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