Standard Operating Procedure @ CNR- IREA

PROTOCOL	**HaCaT cell culture conditions and handling**
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1. Purpose

This procedure describes the materials and the protocols used for maintenance and storage of HaCaT cell line.

2. Background

HaCaT cells are *in vitro* spontaneously transformed keratinocyte from the adult human skin of a 62-year-old male. Frozen cryovial ($2x10^6$ cells/ml) was purchased from CLS (Lot. 300493-4820, p32; Eppelheim, Germany) and arrived at IREA on 12 October 2022. Upon arrival, the cells were amplified, then some stocks were prepared according to the manufacturer's instructions and stored in liquid

nitrogen (master bank of cells at passage 3-4). A working bank of HaCaT cells was established from a master bank vial in order to control the number of cell passages for NextGEM experiments.

The HaCaT cells grow as adherent cells in monolayer (figure 1). Their size, measured with Luna II cell counter, varies between 14-19 micrometer. The doubling time depends on the number of cells at seeding and is about 24 hours.

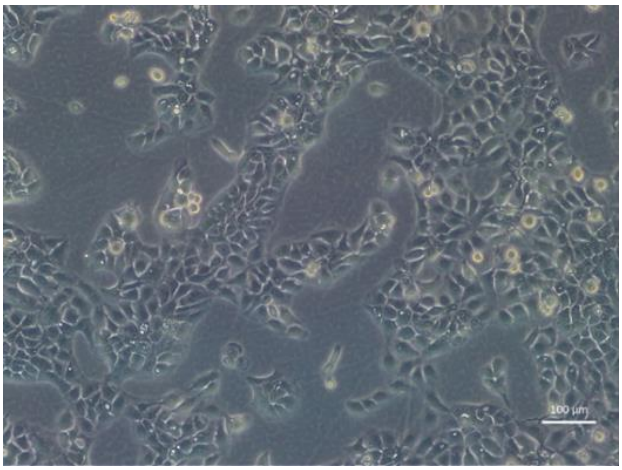

Figure 1: HaCaT cell line at CNR-IREA lab. Inverted microscope images, scale bar: 100 µm.

3. Procedure

The reagents and materials used are sterile and all the procedures are performed under a laminar flow cabinet.

3.1. Equipments

- Cell culture incubator (Thermo Scientific Forma, Model 311)
- Laminar flow cabinet (GELAIRE, BH24TG)
- Water bath (Grant Instruments, J SUB)
- Inverted microscope (Leica, DM IL)
- Refrigerated centrifuge (Thermo Electron, PK 131 R)
- Automated cell counter (Logos Biosystems, Luna II)
- Liquid nitrogen container (MVE XC 47/11-6)

3.2. Materials

- 35 mm cell culture dish (Corning, cod. 430165)
- 100 mm cell culture dish (Corning, cod. 430167)

3.3. Reagents

- DMEM (Dulbecco's Modified Eagle Medium), supplemented with 4.5 g/L glucose, sodium pyruvate and sodium bicarbonate (Microgem, cod. AL007) is stored at +4°C
- Fetal Bovine Serum (Dominique Dutscher, cod. RM10432) is stored at -20°C
- 200 mM L-glutamine (Dominique Dutscher, cod. X0550) is stored at +4°C
- 100X Penicillin-Streptomycin solution (Dominique Dutscher, cod. L0018) is stored at -20°C
- 1X PBS (Dulbecco's Phosphate Buffered Saline) w/o Calcium w/o Magnesium (Dominique Dutscher, cod. L0615 is stored at +4°C
- Trypsin – EDTA 1X in solution w/o Calcium w/o Magnesium w/ Phenol Red (Microgem, cod. L0930) is stored at -20°C
- Cryoprotective media CM-1 (CLS, cod. 800050)
- Trypan blue stain, 0.4% (Logos Biosystems, cod. TB0BJC2301) is stored at RT

3.3.1. Complete medium preparation

The HaCaT culture medium is composed by DMEM supplemented with 10% heat inactivated FBS, 2 mM L-glutamine, 1X Penicillin-Streptomycin solution.

For 100 ml complete medium: add 10 ml FBS, 1 ml L-glutamine and 1 ml Penicillin-Streptomycin to 88 ml DMEM. The culture medium can be stored at 4°C for 1-2 weeks.

3.4. Subculturing procedure

Before splitting:

- Warm trypsin and complete medium to 37°C
- Label tubes and dishes with cell name, passage and date

Splitting:

The following volumes are referred to 100 mm cell culture dish

a. Remove the culture medium and wash the cells with 4 ml PBS

b. Add 2 ml trypsin and incubate for 10 minutes at 37°C

c. Check the detachment of cells and resuspend them in 5 ml complete medium

d. Transfer cells into centrifuge tube and spin at 300 g for 5 minutes

e. Discard the medium and resuspend the cell pellet in 4 ml fresh complete medium

f. Collect an aliquot to count the cells before dispensing the required amount into new dish containing 10 ml of fresh medium

Note:

- For maintenance: split HaCaT cells twice a week and seed $1x10^6$ cells in 100 mm cell culture dish
- For the experiments: use HaCaT cells for a maximum of 16 passages

3.5. Freezing procedure

1. When cells are confluent, perform steps a-d described under “splitting”
2. Resuspend the cells in cryoprotective medium CM-1 at a concentration of $2x10^6$/ml
3. Aliquot 1 ml of cell suspension in sterile cryovials
4. Place the cells for 1 hour at -20°C, then overnight at -80°C. Finally transfer them into liquid nitrogen for long term storage

Note:

- Perform mycoplasma test (fluorescence DAPI test) before freezing cells
- Following the manufacturer's instructions, the day before freezing, change the culture medium to stimulate proliferation
- Use only the cryoprotective media CM-1 purchased from CLS to freeze the HaCaT cells

3.6. Thawing procedure

1. Take the cryovial out of liquid nitrogen and quickly thaw by hand
2. Transfer the cells into centrifuge tube containing 5 ml pre-warmed culture medium and spin at 300 g for 5 minutes
3. Discard the medium and resuspend the cell pellet in 5 ml complete medium
4. Transfer the cells to the culture dish and incubate at 37°C and 5% CO_2