Standard Operating Procedure: Sciensano

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

This procedure describes the storage, culturing and maintenance of the HaCaT cell culture used in the laboratory of experimental toxicology.

2. Definitions et abbreviations

DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethylsulfoxide
FBS	Foetal Bovine Serum

3. Procedure

3.1. Security

Protective Clothing: Eye-shield, laboratory coat and cryogenic gloves

All manipulations involving the culturing of the cell cultures should be performed in a biosafety cabinet.

3.2. Chemicals

Amphotericin B *(Thermo Fisher Scientific)*
DMEM (Dulbecco's Modified Eagle Medium) *(Thermo Fisher Scientific)*
DMEM (Dulbecco's Modified Eagle Medium) (Microgem)
DMSO (Dimethylsulfoxide) *(Sigma-Aldrich)*
FBS (Foetal Bovine Serum) (Thermo Fisher Scientific)
Gentamycin (Thermo Fisher Scientific)
Glutamax (Thermo Fisher Scientific)
Non Essential Amino Acids (Thermo Fisher Scientific)
PBS pH 7.2 (Thermo Fisher Scientific)
Sodium Pyruvate (Thermo Fisher Scientific)
TrypLE™ (Thermo Fisher Scientific)
Trypsin-EDTA (Thermo Fisher Scientific)

3.3. Solutions

3.3.1. Complete Medium adherent cells (Micronucleus, comet assay, etc.

Add to a 500 ml DMEM bottle:

- 6 ml Gentamycin, Glutamax, Sodium Pyruvate & Non Essential Amino Acids
- 60 ml FBS (2 aliquots)
- 600 µl Amphoterecin B (1 aliquot)
- Label & store at 4°C for maximum 1 month.

3.3.2. Complete Medium adherent cells (TempO-Seq)

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

3.3.3. Complete Medium adherent cells (Epigenetics)

The HaCaT culture medium is composed by DMEM (Thermo Fisher Scientific) supplemented with 10% heat inactivated FBS, 2 mM L-glutamine, 1X Penicillin-Streptomycin solution and 1mM sodium pyruvate.

3.3.4. 2x freezing DMEM medium

- Add 5 ml DMSO to 45 ml of complete DMEM medium
- Keep on ice
- Prepare freshly and don't store

3.4. Protocol for Passaging Cell Lines

3.4.1. HaCaT Cell Lines

The HaCaT cell density should be maintained between 1.000.000 and 10.000.000 cells in a T75 culture flask. by counting and diluting the cells in a new culture bottle (passaging at the beginning and the end of the week).

Heathy growing cell lines usually are passaged at a cell line specific sub-cultivation ratio before becoming fully confluent, usually at the beginning and the end of the week.

- Aspirate the spent cell culture media from the 75 cm^2 culture flask with a Pasteur pipette
- Wash the cells briefly with minimum 3 ml PBS (enough to cover the cell layer) with a disposable serological pipet
- Aspirate the PBS with a new Pasteur Pipette
- Add 5ml TrypLE. Gently rock the culture flask to obtain complete coverage of the cell layer. Alternatively, trypsin-EDTA can be used, but in general, we prefer to use TrypLE over Trypsin, because we have observed slightly less cell clumping when using TrypLE.
- Incubate the culture flask for 10-15 minutes (37°C, 5% CO2).
- Check for detachment of the cells (visual or under microscope). If necessary, incubate longer (max another 5 minutes) and check every 1-2 minutes for dissociation. Tapping the flask to help cell detachment is also possible although this might cause cell clumping for some cell lines.
- Add 10 ml of pre-warmed growth medium to the detached cells and transfer the cells to a 30 ml Sterelin reservoir. Make sure that all cells are harvested (only if needed pipet over the cell layer surface several times). Use the same disposable serological pipet for this step.
- Centrifuge 5 minutes at 200 x g
- Aspirate the supernatant
- Re-suspend the cell pellet in 1 ml fresh, pre-warmth culture medium with a 1ml micropipette (pipette the suspension 25-30 times up and down). To reduce cell clumping, also use a 100

µL micropipette to pipette the cell suspension up and down repeatedly (20-30 times).

- Label one or more new culture flasks (passaging date, passage number, cell line, splitting rate) and dilute cell suspension to the appropriate volume for sub-culturing (for a total volume of 20 mL) and return the cells to the incubator.

Remark:

- When cells are used to perform a test, the same protocol is followed but cells are counted, diluted to the correct cell number and transferred to the desired well-plate or cell culture dish format.

3.4.2. Passage Number

- All cells stored in the liquid nitrogen are considered to be in passage 1.
- When cells are thawed from the cryogenic storage reservoir they go to passage 2
- Passage number increases when cells are passaged (transferred to a new 75 cm^2 culture flask)
- Cells are used up to maximum passage 18

3.4.3. Population Doubling Time

The population doubling time (PDT) for a cell line is the time taken for the cells in a culture medium to double its cell population. The PDT is specific for each cell line and can be determined experimentally. Cell density should be measured at the beginning and at the end of the incubation time of cells in the log-phase/exponential phase. PDT can be calculated using following formula.

PDT=T ln2/ln(Xe/Xb)

T = incubation time in any units.

Xb = cell number at the beginning of the incubation time.

Xe = cell number at the end of the incubation time.

A very common misuse of calculation of population doubling time (PDT) is frequently seen in many papers: number of cells at seeding time versus number of cells at harvest time. This approach does not account for the lag phase, ie. the time from seeding until the cells begin to proliferate, which span from hours to several days depending on the specific cell line or cell type. Hence, calculation will be reflected by a less steep curve resulting in falsely increased PDT.

The definition of PDT is the average time it takes a cell population to double in the log-phase/exponential phase, ie. during linear growth.

Taken together, proper calculation of PDT should be done by counting cells on say daily basis for e.g. 7 days using 6-wells, T25 or similar. Next, draw a growth curve from the data (number of cells versus time) and calculate the PDT from the linear part of the curve using this equation: (t2 - t1)/3,32 x (log n2 - log n1) where t is time and n number of cells.

3.4.4. Number of Population Doublings

The number of population doublings (PD) of a cell culture refers to the total number of times the cells in the population have doubled since a given time point (eg seeding of cells).

PD = log(Xe/Xb) / log(2)

Xb = cell number at the beginning of the incubation time.

Xe = cell number at the end of the incubation time.

3.5. Cryopreservation cell lines

3.5.1. HaCaT Cell Lines

- Prepare 2x freezing DMEM medium and store at 2-8°C until use.
- Estimate the number of cells (culture flasks) needed to make the cryopreserved cell stock (usually 30-40 cryogenic storage vials with cell density $1*10^6$ cells/ml). Label the cryogenic storage vials (cell line, date).
- Only use pre-confluent healthy cells in the log phase of growth with a passage number as low as possible.
- Detach the cells following the protocol for passaging adherent cell lines
- Collect the cells in fresh DMEM culture medium
- Count the cells with a Bürker haemocytometer
- Calculate the required volume of freezing DMEM medium (final cell density 2-5 10^6 cells/ml)
- Dilute the cells with fresh culture medium to ½ volume of the required volume of freezing DMEM medium (on ice) (cell density 4-10 10^6 cells/ml)
- Slowly add 2x ice cold freezing DMEM medium and dilute the cells to the required freezing DMEM medium volume (on ice)
- Distribute 1 ml cell suspension in each labelled cryogenic storage vials.
- Put the cells overnight in the -80°C freezer
- Put the cryogenic storage vials into a labelled cryogenic storage box and transfer to the liquid nitrogen container
- Complete the cryogenic storage container file with the storage information from the cryopreserved cells (frozen cell stock).

The cell stocks are stored in cryogenic boxes (max 25 cryogenic storage vials) which are fixed in 2 racks (5 storage boxes/rack) that are kept in the liquid nitrogen cryogenic storage container. Each vial from the same cell stock is labelled with the same coloured stopper.

The temperature (-185°C) and liquid nitrogen level (min 40%) from the liquid nitrogen is monitored continuously to assure optimal storage conditions.

3.5.2. Refill Liquid Nitrogen

When the liquid nitrogen level drops to approximately 40% the container should be refilled, usually every 6-7 weeks only on Tuesdays by an external company at the mycology service.

- The day before filling the container should be transported to the mycology service (after informing the people from this service by email).
  Contact person = Yves Bastin (or Sam Roesems in the absence of Yves)
  Yves Bastin Yves.Bastin@sciensano.be
  Sam Roesems Sam.Roesems@sciensano.be
- The transportation always needs to be performed with 2 persons. The container is placed in the elevator with the inscription "Temporary no person transportation" and is send to the -1st floor. The 2 persons take the stairs. It's not allowed to use the elevator together with the container!
- The follow-up FORM (annual calendar) for the liquid nitrogen is completed after the container is filled together with the expected next refill date.
  \\sciensano.be\fs\1442_ExpToxi_Employee\L1 Calibration Material & Products\Cell Stock Liquid Nitrogen
  The most recent new, empty FORM liquid nitrogen tank can be found at the server:
  \\\sciensano.be\fs\1442_ExpToxi_Employee\L1 Calibration Material & Products\kwaliteit labo\FORM Labo\FORM apparaten

3.5.3. Follow-up Cell Stock

Every year an up-to-date excel file for follow-up of the cell stocks in the cryogenic storage container is created.

This file contains

- A visual overview of the content of the liquid nitrogen reservoir (exact place of every cryogenic storage vial in the container)
- The colour of the stopper from each cell stock is displayed
- General information (date of purchase & storage, passage number) from every cell line present in the container is displayed at the bottom of the file

New vials or new cell stocks are added to the file.

Every vial that is taken out of the container is marked (strikethrough) and labelled (date) on the file.

3.5.4. Handling Liquid Nitrogen

Wearing a face mask and cryogenic gloves is obligatory when adding or taking out cryogenic vials into the liquid nitrogen.

Before opening the container be sure where you will add or take out cryogenic vials (check the overview file)

Work as quickly as possible!

- Take out the cryogenic stopper from the container and keep at a safe place
- Take out the correct rack
- Release the desired cryogenic storage box and take the cryogenic vial(s) or add new

vials (*)

- Immediately fix the storage box again into the rack, but the rack back in place and close the container.

(*) When a new cell stock is added the cryogenic vials are added as quick as possible into the cryogenic box

3.6. Thawing Frozen Cells

- Take one cryogenic storage vial of the desired cell stock out of the liquid nitrogen and indicate this in the cryogenic storage container excel file
- Leave the vial at room temperature for maximum 1 minute.
- Thaw cells for maximum 5 minutes in a 37°C water bath (as quick as possible).
- Transfer the cells carefully to 20 ml freshly pre-warmed of the appropriate culture medium in a 75 cm^2 culture flask
- Optionally: After thawing the cells can be transferred to 10 ml culture medium in a sterile container and centrifuged (5 minutes, 200xg). Gently re-suspend the cell pellet with 1 ml culture medium and transfer to the culture flask.
- Incubate the cells at 37°C and CO2 5%
- Renew the culture medium preferably the day after cell culturing for adherent cell lines

Remark:

- The thawing procedure is stressful to frozen cells and should be performed rapidly.
- New purchased cells should immediately after arrival be stored into the liquid nitrogen container or thawed and cultured following the same protocol.