European Commission

STANDARD OPERATING PROCEDURE

Handling, Maintenance and Quality Control of the GH3 cell line, version 1.0

EURL ECVAM validation study of a battery of mechanistic methods relevant for the detection of chemicals that can disrupt the thyroid hormone system

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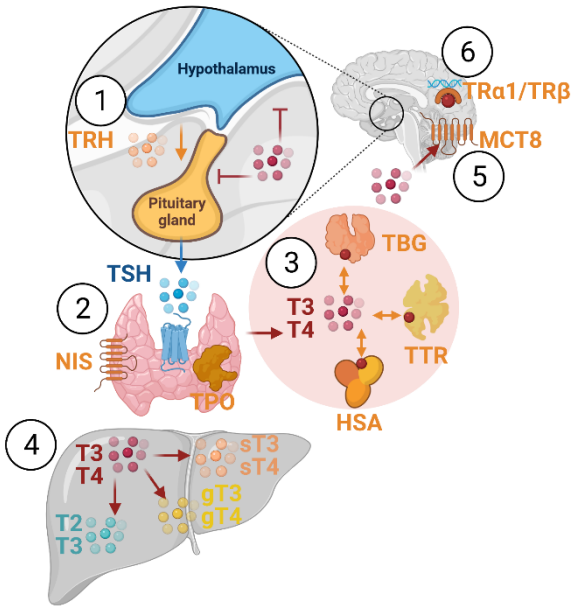

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Joint Research Centre

This Standard Operating Procedure (SOP) has been prepared within the context of a collaboration agreement with the Joint Research Centre (JRC) Directorate for Health, Consumers and Reference Materials (Chemicals Safety and Alternative Methods Unit F3 / EURL ECVAM), for the validation of mechanistic methods to identify potential modulators of thyroid hormone signalling. It aims to provide evidence-based scientific support to the European policymaking process. The contents of this publication do not necessarily reflect the position or opinion of the European Commission. Neither the European Commission nor any person acting on behalf of the Commission is responsible for the use that might be made of this publication. For information on the methodology and quality underlying the data used in this publication for which the source is neither Eurostat nor other Commission services, users should contact the referenced source. The designations employed and the presentation of material on the maps do not imply the expression of any opinion whatsoever on the part of the European Union concerning the legal status of any country, territory, city or area or of its authorities, or concerning the delimitation of its frontiers or boundaries.

This SOP is part of a series of 3 SOPs used to perform the "T-screen assay measuring cell proliferation of GH3 cells using alamar blue/ resazurin":

1. SOP "T-screen assay measuring cell proliferation of GH3 cells using alamar blue/resazurin" v1.0 (used in Part1 of the validation study)
2. **SOP "Handling, Maintenance and Quality Control of the GH3 cell line " v1.0** (used in Part 1 of the validation study)
3. SOP "Determination of cell proliferation in T screen assay" v1.0 (used in Part 1 of the validation study)

The method was developed by Arno Gutleb, LIST (Luxembourg) and subsequently implemented by the EU-NETVAL test facility NIOM (Poland) within the validation study.

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Standard Operating Procedure

Handling, Maintenance and Quality Control of the GH3 cell line

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Contents

1. Scope of the SOP

A rat pituitary tumour cell line (GH3) is the test system for T-screen assay. This Standard Operating Procedure describes how to handle and maintain the GH3 cells and the quality control to be performed. The GH3 cell line should fulfil acceptance criteria for absence of microbiological contamination and *Mycoplasma sp.* and the doubling time (Td).

1.1 Safety

All activities related to the culture of GH3 cells (except for counting cells in Bürker chamber) should be performed under sterile conditions under a laminar chamber.

1.2 Test System

- GH3-cell line – a rat pituitary tumour cell line (ATCC® CCL-82.1™)

1.3 Apparatus and Materials

- Clean glass vials for the preparation of stock solutions (e.g. gas chromatography vials or Sarstedt #86.1509)
- 96-well microplates (e.g., Nunc # 167008)
- Freezer below -16 °C
- Freezer below -70 °C
- Freezer below -135 °C or a liquid nitrogen tank
- Refrigerator 2-10 °C
- CO_2 humidified incubator at 37°C +/- 2 °C, 5% CO_2 +/- 0.5%
- 37 °C water bath
- Pipette Aid
- Pipettors (e.g. p1000-100, p100-10, p10-0.5)
- Centrifuge
- Vacuum aspirator
- Sterile Laminar Flow Hood
- Culture Flasks (T75, T25; e.g. Nunc, Falcon)
- Serological pipettes (1-2 mL, 5 mL, 10 mL, and 25 mL)
- Sterile, filter pipette tips (e.g. 10 µL, 200 µL, and 1000 µL)
- Sterile, pipette tips without filter (e.g. 10 µL, 200 µL, and 1000 µL)
- Conical tubes (5 mL, 15 mL, and 50 mL)
- Syringe filters (0.22 µm)

1.4 Reagents and Chemicals

- Foetal calf/bovine serum (e.g. Gibco #10270106)
- Dulbecco's modified Eagle's medium/Ham's Nutrient Mixture F-12 (DMEM/F-12; 1:1 mixture with phenol red and HEPES (15 mM) (e.g. Sigma-Aldrich#D6421)
- DMEM/F-12 w/o L-Glutamine, with 15 mM HEPES and w/o phenol red (e.g. Sigma-Aldrich # D6434)
- HEPES 1M solution (e.g. Sigma-Aldrich #H0887)
- Penicillin-Streptomycin Solution (e.g. Sigma-Aldrich #P0781)

- Bovine insulin (e.g. Sigma-Aldrich #I0516)
- Ethanolamine (e.g. Sigma-Aldrich #E0135)
- Sodium selenite (e.g. Sigma-Aldrich #S5261)
- Human apotransferrin (e.g. Sigma-Aldrich #T2036)
- Bovine serum albumin (e.g. Sigma-Aldrich #A9418)
- PBS without Ca^{2+}/Mg^{2+} (e.g. Biological Industries #02-023-1A)
- Accutase (e.g. Sigma-Aldrich #A6964)
- Deionized water (dH_2O)
- *Mycoplasma sp.* detection kit (e.g. MycoBlue Mycoplasma Detector; VAZYME# D101-01)

2. Handling and Maintenance of Test System

2.1. Stock solutions of reagents

2.1.1. Ethanolamine [50 mM]

Prepare a 50 mM stock solution of ethanolamine in dH_2O by diluting a pure ethanolamine (16.6 M) 332x, i.e. add 25 µL of ethanolamine (16.6 M) to 8.275 mL of dH_2O, and filter (0.22 µm pore size). The stock should be prepared freshly before to use or stored at room temperature in closed glass containers.

2.1.2. Sodium selenite [50 µg/mL]

Prepare a stock solution of sodium selenite (50 µg/mL) in dH_2O, filter (0.22 µm pore size), and store in aliquots below -16 °C.

2.1.3. Human apotransferrin [2 mg/mL]

Prepare the 2 mg/mL stock solution of human apotransferrin in dH2O, filter (0.22 µm pore size), and store in aliquots below -16 °C.

2.1.4. Bovine serum albumin [100 mg/mL]

Prepare a 100 mg/mL stock solution of bovine serum albumin in dH_2O, filter (0.22 µm pore size), and store in aliquots at 2-8 °C.

2.2. Media

2.2.1. Cell culture medium (cDMEM/F12; completed cDMEM/F12)

Dulbecco's modified Eagle's medium/Ham's Nutrient Mixture F-12 (DMEM/F-12; 1:1 mixture; with phenol red and 15 mM HEPES) supplemented with 10% Foetal calf serum, 2.5 mM L-Glutamine and additional amount of HEPES to obtain 25 mM.

To prepare *cDMEM/F12* the following volume of supplements should be added:

	the final concentration	Volume [mL]
DMEM/F-12 w/o L-Glutamine, with 15 mM HEPES (e.g. Sigma-Aldrich #D6421)*		500
Heat inactivated FCS/FBS*	10%	57.6
200 mM L-Glutamine	2.5 mM	7.2
1 M HEPES	up to 25 mM	5.8
Penicillin-Streptomycin solution (100x)	1%	5.8
Total		**576.4**

*if different DMEM/F-12 is used the final concentration of HEPES and L-Glutamine should be adjusted to 2.5 mM and 25 mM, respectively.

** if heat-inactivated FCS/FBS was purchased, it should be defrost at 2-10°C e.g. during the night and stored in aliquots at below -16°C.
If non-inactivated FCS/FBS was purchased, it should be heat-inactivated in water bath at 56-57°C for 30-35 min and filtered (0.2 µm) before aliquoting.

2.2.2. Cell cryopreservation medium

Complete cell culture medium described in Section 2.1.1. supplemented with 5% (v/v) DMSO. The volume of the freeze medium prepared depends on the amount of vials. To prepare 1 mL of the freeze medium mix: 50 µL of DMSO with 950 µL of cDMEM/F12.

2.2.3. PCM medium

PCM medium is a Dulbecco's modified Eagle's medium/Ham's Nutrient Mixture F-12 (DMEM/F-12; 1:1 mixture) without phenol red supplemented with 10 µg/mL bovine insulin, 10 µM ethanolamine, 10 ng/mL sodium selenite, 10 µg/mL human apotransferrin, 500 µg/mL bovine serum albumin. To prepare *PCM medium* the following volume of supplements should be added:

	Final concentration	**Volume [mL]**
DMEM/F-12 w/o L-Glutamine, with 15 mM HEPES (e.g. Sigma-Aldrich # D6434)*		500
200 mM L-Glutamine	2.5 mM	6.5
1 M HEPES	up to 25 mM	5.2
Penicillin-Streptomycin solution (100x)	1%	5.2
10 mg/mL bovine insulin	10 µg/mL	0.523
50 mM ethanolamine (Section 2.1.1)	10 µM	0.105
50 µg/mL sodium selenite (Section 2.1.2)	10 ng/mL	0.105
2 mg/mL human apotransferrin (Section 2.1.3)	10 µg/mL	2.6
100 mg/mL bovine serum albumin (Section 2.1.4)	500 µg/mL	2.6
Total		**523.0**

*if different DMEM/F-12 is used the final concentration of HEPES and L-Glutamine should be adjusted to 2.5 mM and 25 mM, respectively.

2.3. Maintenance and handling of GH3 cells

2.3.1. Restoring GH3 cells

a. Restoring a purchased vial of GH3 cells

1. Thaw the GH3 cells (a purchased vial) according to the supplier's instructions, using the culture medium described in Section 2.1.1.
2. Seed at the cell density provided on the certificate of analysis.
3. Culture GH3 cells at 37 ± 2 °C and 5 ± 0.5% (v/v) CO_2 in a humid atmosphere in cDMEM/F12 medium

b. Restoring GH3 cells frozen in the laboratory

1. Prepare a tube with 9 mL of cDMEM/F12 medium
2. Thaw the vial in a 37°C water bath as rapidly as possible using gentle hand agitation. Immerse the vial in 70% alcohol to sterilize outside.
3. Unscrew the cap of the vial and transfer the contents of the vial to the pre-warmed medium in the tube.
4. Centrifuge
5. Discard the supernatant
6. Resuspend the pellet in the fresh warm cDMEM/F12 medium
7. Transfer the appropriate number of cells into a T25 flask

   Recommended number of cells per a T25 flask to subculture in 3-4 days: $6x10^5$ cells/ a T25 flask ($5\text{-}7\ x10^5$ cells/ a T25 flask)

Culture GH3 cells at 37 ± 2 °C and 5 ± 0.5% (v/v) CO_2 in a humid atmosphere in cDMEM/F12 culture medium.

Prepare both a Master Cell Bank (e.g. 10-20 vials) and Working Cell Bank (e.g. 20-30 vials).

2.3.2. Cryopreservation

General protocol of ATCC provides the following instructions:
Always harvest cells in exponential growth.

1. Prepare a freeze medium consisting of complete culture medium and 5% DMSO (Section 2.1.2). Do not add undiluted DMSO to a cell suspension as dissolution of DMSO in aqueous solutions gives off heat (exothermic).
2. Label the appropriate number of vials with the name of the cell line and the date and the passage number.
3. Collect cells by gentle centrifugation (10 min at 125 x g) and resuspend them in the freeze medium at a concentration of 1×10^6 to 5×10^6 viable cells/mL.
4. Add 1 mL of the cell suspension to each of the vials and seal.
5. Allow cells to equilibrate in the freeze medium at room temperature for a minimum of 15 minutes but no longer than 60. This time is usually taken up in dispensing aliquots of the cell suspension into the vials. After 60 minutes, cell viability may decline due to the DMSO.
6. Place the vials into a controlled-rate freeze chamber, such as ATCC ACS-6000, CoolCell LX, and place the chamber in a -70°C (or colder) mechanical freezer for at least 24 hours. Alternately, use a programmable freezer unit set to cool the cryovials at -1°C per minute until a temperature below -70°C is achieved.
7. Quickly transfer the vials to a liquid nitrogen or a -130°C (or colder) freezer. Frozen material will warm up at a rate of 10°C per minute and cells will deteriorate rapidly if warmed above -50°C.
8. Record the location and details of the freeze.

After at least 24 hours at below -130°C, remove one cryovial, restore the cells in culture, and determine their viability and sterility as described in Section 2.3.3.

2.3.3. Quality control to be performed

Before cryopreservation and after restoring the first cryovial of the cell bank; check your cell culture for absence of microbiological contamination such as fungi, bacteria, yeast, which can be verified by

microscopic observation after 1-2 weeks culturing of the cells without antibiotics assuring proper safety conditions or out-sourcing.

Before cryopreservation and during normal use, check your cell culture for *Mycoplasma sp.* contamination using a PCR-based mycoplasma detection procedure or out-sourcing.

The culture that is contaminated (microscopic observation or a positive result in a mycoplasma detection kit) should be disposed in accordance with the appropriate system procedure for the disposal of hazardous wastes. A new frozen vial with the GH3 cells should be used for testing or for multiplication and cryopreservation.

2.3.4. Detachment of GH3 cells

GH3 cells should be detached by using Accutase.

1. View the GH3 culture using an inverted microscope to assess the degree of confluency and confirm the absence of bacterial and fungal contaminants
2. Remove spent medium and wash the cell monolayer with PBS without Ca^{2+}/Mg^{2+} (5 mL/a T25 flask or 10 mL/a T75 flask)
3. Pipette Accutase (RT) into the washed cell monolayer using 1-2 mL/T25 or 3-6 mL/T75. Gently rotate the flask to cover the monolayer with Accutase.
4. Return the flask to the incubator and leave for 3-5 minutes.
5. Examine the cells using an inverted microscope to ensure that all the cells are detached and floating. DO NOT tap or shake the flask to release any remaining attached cells since it increases cell mortality.
6. Add fresh serum-containing medium (cDMEM/F12) to the cells to inactivate Accutase (5 mL/a T25 flask or 10 mL/a T75 flask)
7. Transfer the content to a new labeled vial and centrifuge the cells 1500 rpm; 3-5 min.
8. Resuspend the pellet into a known volume of fresh medium cDMEM/F12 and count the cells using Trypan blue to determine density and viability as described in Section 2.3.6

2.3.5. Passaging of GH3 cells

Passage GH3 cells in T25 or T75 tissue culture flasks every three-four days by using Accutase. Every two-three days culture medium should be exchanged with the fresh one.

1. Detach the GH3 cells as described in Section 2.3.4
2. After determination of density and viability of GH3 cell suspension (Section 2.3.6) transfer an appropriate number of the viable cells into a new flask. Recommended number of cells to subculture in 3-4 days is:
   - 6×10^5 cells/T25 flask ($5\text{-}7 \times 10^5$ cells/T25 flask) or,
   - 1.8×10^6 cells/T75 flask ($1.5\text{-}2.1 \times 10^6$ cells/T75 flask)

   Alternatively, if the cells cannot be counted, then a sub-cultivation ratio of 1:2 to 1:4 is recommended
3. Culture GH3 cells at 37 ± 2 °C and 5 ± 0.5% (v/v) CO_2 in a humid atmosphere in culture medium (Section 2.1.1)

2.3.6. Determination of cell suspension density and cell viability

Density and vability of cell suspension is determined in Trypan blue exclusion test using Bürker chamber. To this end:

- Take 100 µL representative sample of cell suspension into Eppendorf
- Add 100 µL Trypan Blue solution
- Count cell number at least in 3 squares
- Calculate cell density [cells/mL] using a formula below:

Cell density [cells/mL] = X/Y * Z *10000

where,
X= the sum of all cells (#) counted in the large squares (min. 3 max 9)
Y = the number of the counted large squares
Z = dilution in Trypan Blue solution (usually 2x)
10000 = conversion factor to 1 mL

(#) IMPORTANT! All cells means all viable cells or all/total cell number (viable+nonviable), depending on the aim of counting. For further culture (passage) or for the test calculate density of viable cells. For determination of the doubling time total number of cells is counted (density of viable+nonviable cells).

- Calculate cell viability [cells/mL] using a formula below:

Viability [%] = X*100/(X+Y)

where,
X = number of viable cells (cells with clear cytoplasme) counted in the large squares (min. 3 max 9)
Y = number of nonviable cells (cells with blue cytoplasme) counted in the same large squares as X has been counted

2.4. Determination of the doubling time (Td) of GH3 cells

Every time when the GH3 cells are restored the doubling time (Td) of GH3 culture should be determined both in cDMEM/F-12 and PCM medium. To this end, an appropriate number of T25 flasks with the known number of cells will be set (5.5-6.5x10^5 cells/T25) in cDMEM/F12 medium and in PCM medium (the number of flasks depends on the number of time points; at least 6 time points should be planned). The exact number of cells applied to the culture flasks will be given in the source materials. The cells should be cultured for 10 days without changing medium and the total no. of cells should be counted everyday (with exception of weekend). In every time point (e.g. every 24 h) cells from one cell culture in cDMEM/F12 and one cell culture in PCM should be detached using Accutase and total no. of cells (live and dead) should be counted.

Detachment of GH3 cells should be performed as described in Section 2.3.4 with one exception: spent medium and PBS after washing should not be discarded but they should be collected in the same vial with the cells.

Determine density of cell suspension (total number of cells/viable+nonviable)using Trypan Blue as described in Section 2.3.6

Doubling time (Td) should be calculated based on total number of cells in time point (live+dead) according the following formula (ATCC, 2014):

$$\mathbf{Td} = (t2 - t1) \times \frac{\log(2)}{\log(q2/q1)}$$

where:
t2-t1 – incubation time in any units e.g. hours (the beginning of the incubation time - the end of the incubation time)
q1- the cell number at the beginning of the incubation time
q2- the cell number at the end of the incubation time

It is recommended to start the measurement on Friday and calculate the Td based on results obtained in 72- and 96-h culture of the cells when the GH3 cell growth is exponential.

2.5. Acceptance Criteria

The GH3 cells can be used for the T-screen assay when:

1. Absence of mycoplasma is confirmed
2. Absence of microbiological contamination is confirmed
3. The doubling time of the GH3 cells in cDMEM/F12 medium is 42 ± 5h
4. Cell number in PCM in 72h±1h and 96h±1h of culture should be at least 40% lower that cell number in cDMEM/F12, when determined simultaneously.

3. Reference(s)

1. Characteristics of GH3 provided by The Global Bioresource Center (ATCC) https://www.lgcstandards-atcc.org/products/all/CCL-82.1.aspx?geo_country=pl#
2. ENV/JM/MONO(2018)19: Guidance Document on Good In Vitro Method Practices (GIVIMP, 2018)
3. ATCC (2014), Animal Cell Culture Guide, ATCC. https://www.atcc.org/~/media/PDFs/Culture%20Guides/AnimCellCulture_Guide.ashx.

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