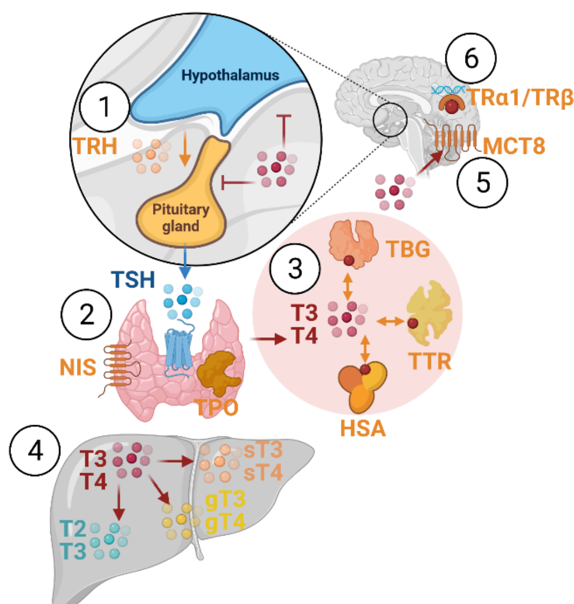
European Commission

STANDARD OPERATING PROCEDURE

for culturing FTC-238 and FTC-238/hrTPO cells, used in the thyroperoxidase (TPO) inhibition assay based on oxidation of Amplex UltraRed (AUR-TPO), version 3.0

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

Fant K.



2023

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 4 SOPs used to perform the “Thyroperoxidase Inhibition Assay based on Amplex UltraRed (AUR-TPO)”:

1. SOP “Thyroperoxidase Activity Assay with Amplex UltraRed (AUR-TPO)” v1.0 (used in Part1 of the validation study)
2. SOP “Solubility determination by visual inspection” v2.0 (used in Part 1 and Part 2 of the validation study)
3. **SOP “Culture of FTC-238 and FTC-238/hrTPO cells” v3.0** (used in Part 1 and Part 2 of the validation study)
4. SOP “Thyroperoxidase (TPO) extract preparation” v1.0 (used in Part 1 and Part 2 of the validation study)

SOP “Thyroperoxidase Activity Assay with Amplex UltraRed (AUR-TPO)” has been updated after Part 1, the other SOPs were considered complete to be used also in Part 2.

The method was developed by US EPA and subsequently implemented by the EU-NETVAL test facility RISE (Sweden) within the validation study.

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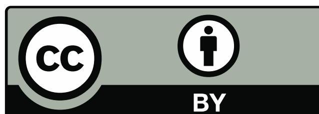

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Culture of FTC-238 and FTC-238/hrTPO cells

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1. Introduction

1.1 Aim and use of this SOP

The aim of this standard operating procedure is to cover all aspects of cultivating FTC-238 and FTC-238/hrTPO cells; medium preparation, initiation, subcultivation and cryopreservation.

1.2 References

Product Information Sheet for ECACC General Cell Collection: FTC-238 (ECACC No. 94060902). ECACC web page, accessed on Sept 5th, 2018: www.phe-culturecollections.org.uk/products/celllines/generalcell/detail.jsp?refId=94060902&collection=ecacc_gc

Generation of FTC-238/hrTPO clone: Schmutzler C et. al. The Ultraviolet Filter Benzophenone 2 Interferes with the Thyroid Hormon Axis, 148(6):2835–2844, 2007.

For more information on enzymatic dissociation and subculturing of cell lines consult Chapter 13 in Culture of Animal Cells, a manual of Basic Technique by R. Ian Freshney, 5th edition, published by Alan R. Liss, N.Y., 2005.

For more information on cryopreservation consult Chapter 20 in Culture of Animal Cells, a manual of Basic Technique by R. Ian Freshney, 5th edition, published by Alan R. Liss, N.Y., 2005.

GCCP: Coecke, S., Balls, M., Bowe, G., Davis, J., Cstraunthaler, G., Hartung, T., Hay, R., Merten, O., Price, A., Schectman, L., Stacey, G. And Stokes, W. Guidance on Good Cell Culture Practice, A Report of the Second ECVAM Task Force on Good Cell Culture Practice, ATLA, 33, pp. 261-287, 2005

1.3 Cells

FTC-238 cells (ECACC No. 94060902, European Collection of Cell Cultures) were originally established from a lung metastasis of a follicular thyroid carcinoma from a 42-year-old male. The cells are polymorphic showing flat polygonal to spindle-like morphologies. Complex chromosomal changes were detected as well as a p53 mutation.

FTC-238/hrTPO cells are normal FTC-238 cells, stably transfected with the eukaryotic vector pcDNA3 with cDNA encoding human TPO inserted by Schmutzler et al (see reference above). The FTC-238/hrTPO cells are Genetically Modified Organisms Class 1 (GMO1) and the work must follow national requirements for GMO work.

Cells shall be free from mycoplasma or other contaminations. Mycoplasma testing shall be performed by DNA extraction followed by real-time PCR method .

2. Waste handling

According to the laboratory's internal procedures. Old cell cultivation vessels and redundant cell suspension must be disposed of as biologically hazardous waste and according to national requirements for GMO work.

3. Equipment and consumables

- Incubator, 37 °C, humidified, 5 % CO_2
- Centrifuge, preferably with swing-out rotor, capable of a relative centrifugal force of ~130×g
- -150°C freezer or equivalent cryostorage for mammalian cells
- -80°C freezer
- Biological Safety Cabinet class II
- Water bath, 37 °C
- Inverted phase contrast microscope
- Controlled-rate freeze chamber (CoolCell LX, Mr Frosty or equivalent)
- Centrifuge tube 50ml
- Cryovials 1.8-2 ml + coloured caps (optional)
- Tissue culture flasks
- Cryomarker pen
- Bürker counting chamber or other equipment for accurate determination of cell numbers
- Autoclave (if solutions and reagents are not sterile)
- Pipettes capable of dispensing 10µl -1 ml
- Pipette tips
- Pipetting aid
- Serological pipettes
- 0.22 µm sterile filter + syringes (if solutions and reagents are not bought sterile)
- Sterile Eppendorf tubes (for storage of reagents)
- Sterile polypropylene centrifuge tubes (for storage of reagents)
- pH sticks, capable of measuring pH in the interval 6.0 to 9.0 (since culturing is performed without phenol red)

All equipment should be regularly cleaned, maintained and calibrated, according to the cell culture facility's internal procedures. Both equipment and consumables should be fit for purpose and their use shall be documented according to the cell culture facility's internal procedures.

4. Medium, Chemicals and reagents

For cell culture-related work, ready-to-use sterile solutions shall be used as far as possible, otherwise solutions must be sterilized using a syringe filter (pore size ≤ 0.22µm) or autoclaved. Depending on the stability of the solutions they can be mixed in advance and stored in a fridge/freezer, or should be prepared just before use, see details for each solution/medium. All preparations should be performed using aseptic techniques. Handling, storage, aliquotation, labeling and documentation of media and other reagents should follow the cell culture facilities internal procedures.

Reagents needed:

- Iscove's modified Dulbecco's medium (1×) buffered with $NaHCO_3$ (Gibco Life Technologies, cat. no. 21056-023, or equivalent)
- Fetal Bovine Serum (Gibco Life Technologies, cat. no. 10270-098, or equivalent)
- Geneticin selective antibiotics (G-418 sulfate) (Gibco Life Technologies cat.no. 10131-035, or equivalent)

- TrypLE-EDTA (Gibco Life Technologies cat. no. A12177 and Gibco Life Technologies, cat. no. 15040033, or Gibco Life Technologies cat. no. 12563, or equivalent)
- DPBS without Ca^{2+}, Mg^{2+} (Cytiva Hyclone, cat. no. SH30028.02, or equivalent)
- Trypan blue stain (Gibco Life Technologies, cat. no. 15250061, or equivalent)
- Cell culture grade Dimethyl sulfoxide, DMSO (cat. no. Sigma Aldrich D2650, or equivalent)
- *Optional:* Penicillin-streptomycin (Cytiva Hyclone, cat. no. SV30010 or equivalent)

4.1 Complete culture medium 1× FTC:

Iscove's modified Dulbecco's medium (1×) buffered with $NaHCO_3$ supplemented with

- 10 % Fetal Bovine Serum
- 250 µg/ml G-418 (from stock solution 50 mg/ml), shall be included only for the recombinant cells (FTC-238/hrTPO)
- Optional: 100 IU/ml penicillin and 100 µg/ml streptomycin (from stock solution 100×)
  - Where possible, the use of antibiotics should be avoided. It should not become routine in the cell culture laboratory, and can never be relied on as a substitute for effective aseptic techniques.

Store at 2-8 °C for up to two weeks.

4.2 Complete freezing medium 1× FTC:

Base medium 1× FTC supplemented with

- 20% Fetal Bovine Serum
- 100 IU/ml penicillin and 100 µg/ml streptomycin (from stock solution 100×)
- 250 µg/ml G-418 (from stock solution 50 mg/mL), shall be included only for the recombinant cells (FTC-238/hrTPO)
- 7 % DMSO
  NOTE: Care should be used when handling any DMSO solution as it will rapidly penetrate intact skin and may carry toxic contaminants along with it.

Prepare freshly for each cryopreservation.

4.3 TrypLE-EDTA (Versene) 1×

TrypLE is an animal origin-free recombinant enzyme for cell dissociation. If not using a ready-to-use TrypLE-EDTA solution, TrypLE Select 10× is diluted in 0.48 mM EDTA (Versene) to 1× before use. Alternatively, e.g. a 0.25 % (w/v) Trypsin-0.53 mM EDTA can be used. Aliquots are labelled and stored according to the laboratory's internal procedures.

5. Cell culture

Cell culture work shall be performed under aseptic conditions. When a new cell line is brought into the laboratory from an external provider, internal cell banks (master and working cell banks) should be established in line with GCCP and internal procedures.

Table 1 below suggest culture volumes for different sizes of vessels, applicable to the procedures described below.

Table 1. Culture volume for different culture vessels (TPP brand).

Name	Area (cm^2)	Culture volume	TrypLE-EDTA	DPBS (ml)

		(ml)	(ml)*	
T25	25	7	1	5-10
T75	75	13	2.5	10-20
T150	150	25	5	20-30
T300	300	50	10	40-60

*The TrypLE-EDTA volumes are based on the solutions described in section 4.3 and may be adjusted if using other concentrations

5.1 Initiation

Principle

Cells are quickly thawed in a 37°C water bath, resuspended in complete medium, centrifuged, and finally resuspended in complete medium and incubated at 37°C and 5% CO_2 for further culture.

Medium, Chemicals and reagents

- Complete culture medium 1× FTC
- 70 % ethanol (for disinfection)

Method

1. Select the size of the culture vessel and prepare an appropriate volume of complete culture medium (see table 1). For cells cryopreserved according to section 5.3 below, the recommended seeding density is 2-4×10^4 cells/cm^2. For new cells from an external provider, see the specific batch information for the culture recommended dilution ratio. Check the medium for contamination by macroscopic and microscopic inspection.
2. Pre-warm at least 9.0 ml complete culture medium to 37°C.
3. Retrieve the vial from the freezer. It is important that the cells are thawed rapidly. *Optionally*, keep the vial in a pre-cooled (-80°C) controlled-rate freeze chamber or cooling rack until inside the lab, in order to avoid thawing.
4. Thaw the vial by gentle agitation in a 37°C water bath. To reduce the possibility of contamination, keep the O-ring and cap out of the water. Thawing should be rapid (approximately 2 minutes).
5. Remove the vial from the water bath as soon as the contents are thawed, check and record the text on the label and then decontaminate the vial by dipping in or spraying with 70% ethanol. All of the operations from this point on should be carried out under strict aseptic conditions.
6. Carefully transfer the vial content to a new centrifuge tube (e.g. 50 ml). Add 9.0 ml of pre-warmed complete culture medium dropwise during approximately 1 min. Spin down the cells at approximately 130×g for 5-7 minutes.
7. Discard the supernatant and resuspend the cell pellet with complete culture medium (preferably pre-warmed and buffered with CO_2). Transfer the cells to the culture vessel. *Optionally*: remove a small amount of cells for counting.
8. Check the cells with the microscope at, e.g., 10× magnification.

9. Label the culture vessel with the name of the cell line ("FTC-238" or "FTC-238/hrTPO"), a unique vessel ID, operator, date of initiation and passage number.
10. Incubate the culture at 37°C and 5% CO_2 in a humidified incubator.
11. Check the cells with the microscope at after ~24 hours (record and save images if desired) and change the medium (check that the pH is as expected). Alternatively: split/expand the culture.

5.2 Subcultivation

Principle

A sub confluent monolayer of cells (70-80 % confluent) is detached from the culture surface and subcultured in one or several new culture vessels (expansion/split). It is important to keep track of the cell passage number. Work should only be done on one cell line at a time.

Medium, Chemicals and reagents

- Complete culture medium 1× FTC
- TrypLE-EDTA (Versene) 1×
- DPBS without Ca^{2+} and Mg^{2+}
- 70 % ethanol

Method

Below follows a protocol that describes the subculturing procedure. See table 1 for specific volumes of solutions. All media and solutions added to the cell culture should be at least room tempered (but not higher than 37°C), and preferably pre-warmed to 37°C.

1. Check the culture macroscopically and microscopically for microbial contamination.
2. Check the medium for contaminations.
3. Evaluate cell morphology with the microscope and record observations .
4. Remove and discard culture medium. Since culturing is performed without phenol red, measure the pH of the culture medium removed from the culture vessel. The pH is normally between 7.0-7.6.
5. Briefly rinse the cell layer with sterile DPBS to remove all traces of serum.
6. Add TrypLE-EDTA solution and incubate at 37°C until the cell layer is dispersed (usually within 5 to 15 minutes). Check that the cell layer is dispersed with the microscope.
   Note: To avoid clumping do not agitate the cells by hitting or shaking the flask while waiting for the cells to detach.
7. Add complete culture medium to the cell suspension to inactivate the TrypLE. Gently wash any remaining cells from the growth surface of the flask. Use the cell suspension and wash over the surface several times to make sure that most of the cells are collected.
8. Add appropriate aliquots of cell suspension to the new culture vessels. Make sure to distribute the cells evenly over the surface (carefully tilt the flask in all directions). Recommmended subcultivation ratios are:
   - FTC-238: 1:3 to 1:20
   - FTC-238/hrTPO: 1:3 to 1:6.

Optionally, remove a small portion, e.g. 100µl, of this suspension for counting.

9. Label the culture vessel with the name of the cell line ("FTC-238" or "FTC-238/hrTPO"), a unique vessel ID, name/initials of operator, date of initiation and passage number.
10. Place culture vessels in the incubator at 37°C, 5% CO_2, humidified.
11. Passage the cells when approximately 70-80% confluent.

5.3 Cryopreservation

Principle

The standard procedure for cryopreservation is to freeze cells slowly (-1°C per minute) until they reach a temperature below –70°C in medium that includes a cryoprotectant (DMSO). Vials are then transferred to a second freezer to maintain them at temperatures below –136°C.

Medium, Chemicals and reagents

- Complete freezing medium 1× FTC
  NOTE: Care should be used when handling any DMSO solution as it will rapidly penetrate intact skin and may carry toxic contaminants along with it. Do not add undiluted DMSO to a cell suspension as dissolution of DMSO in aqueous solutions gives off heat.
- Complete culture medium 1× FTC, for further culturing.
- TrypLE-EDTA (Versene) 1×
- DPBS without Ca^{2+}, Mg^{2+}
- Trypan blue solution (0.4%)
- 70 % ethanol for disinfection

Instructions for Mr Frosty

Assembly:

1. Remove high-density polyethylene vial holder and foam insert from polycarbonate unit. DO NOT discard foam insert.
2. Add 100% isopropanol (250 ml) to fill line. DO NOT overfill.
3. Carefully replace foam insert and vial holder

The Mr Frosty should be stored, preferably without isopropanol, at room temperature when not in use. When in use, the isopropanol shall be replaced after every fifth use.

Instructions for CoolCell LX

- Make sure the core (black ring) is at room temperature and seated in the bottom of the central cavity.
- Place sample vials containing 1 ml of cell suspension in each well. Each well should contain a filled vial. If freezing batch is fewer than 12 vials, fill each empty well with a BioCision CoolCell Filler Vial (2ml vial) or other vial that contains equivalent volume of freezing media.
  NOTE: For optimal results, CoolCell should be at the same temperature as the cell suspensions.
- Check that the tubes slide in and out freely.

- Fully seat the lid on CoolCell LX

After freezing of cells, the CoolCell LX is ready to freeze again as soon as the foam body and core (black ring) are at room temperature. To rapidly recycle CoolCell LX to room temperature, remove the center solid core ring. CoolCell LX body and lid will return to room temperature in 10 to 15 minutes. Check that all chambers are dry. Dry the core ring before re-inserting into the central chamber.

Method

Volumes used in this protocol are for 150 cm^2 flasks; for other sizes of culture vessels change the volumes appropriately. Suitable volumes of DPBS, media and Trypsin-EDTA are summarized in table 1.

1. Check the culture macroscopically and microscopically for microbial contamination.
2. Check the medium for contaminations.
3. Evaluate cell morphology with the microscope and record observations.
4. Remove culture medium from a layer of near confluent cells (they should still be in the exponential growth phase) using a serological pipette. Use appropriate amounts of the supernatant for mycoplasma testing and sterile control (microbial), if applicable, and discard the rest.
5. Rinse cells with sterile DPBS to remove all traces of serum.
6. Add TrypLE-EDTA solution (preferably pre-warmed to 37°C) to flask and incubate the cells at 37°C until the cell layer is dispersed (usually within 5-15 minutes). Check that the cell layer is dispersed with the microscope.
   Note: To avoid clumping do not agitate the cells by hitting or shaking the flask while waiting for the cells to detach.
7. Add complete culture medium to the cell suspension to inactivate the TrypLE. Gently wash any remaining cells from the growth surface of the flask. Use the cell suspension and wash over the surface several times to make sure that most of the cells are collected.
8. Note the volume of the remaining cell suspension in milliliters. Remove a small portion, e.g. 100µl, of this suspension for counting.
9. Remove a fraction of the cell suspension for further cultivation. It is important to maintain the cells in culture until the viability of the recovered cells is confirmed (see Step 18). If cells are frozen from more than one culture flask, it is enough to keep cells from one of the flasks in culture.
10. Collect the cells by gentle centrifugation (e.g. 5-7 minutes at 125g).
11. During centrifugation count the cells, see SOP KM 11739.
12. Prepare the appropriate number of cryovials and one 50 ml centrifuge tube. Use appropriate racks for the vials. Label the cryovials (using a cryomarker pen!) according to the format "Cell line name YYMMDD:# pP XX", where YY = year, MM = month, DD = day, # = cryovial number (you probably have prepared more than one cryovial that day), P = passage number, and XX = your initials, e.g. "L929 100609:3 p6 EF". Colour code the cap if desired.
13. Discard the supernatant and resuspend the cell pellet in the freezing medium at a concentration of between 2 and 6×10^6 viable cells/ml.
14. Then add 1 ml of the cell suspension to each of the cryovials and seal the vials.
15. Allow cells to equilibrate in the freezing medium at room temperature for a minimum of 15 minutes but no longer than 40 minutes. This time is usually taken up in dispensing aliquots of the cell suspension into the vials. After 40 minutes, cell viability may decline due to the DMSO in the medium.

16. Place the vials into a room tempered controlled-rate freeze chamber (e.g. CoolCell or Mr Frosty, for the latter check the isopropanol level, see above). Chambers and cryovials should be dry to avoid tube sticking upon freezing. Place the chamber upright in a mechanical freezer at –80°C for 4-20h. This will assure cooling at a rate of ~1°C/minute. Ensure that there is at least one inch of free space clearance around the chamber.
17. Quickly transfer the vials to the long-term cryostorage (< -136 °C). Frozen material will warm up at a rate of 10°C per minute and cells will deteriorate rapidly if warmed above –50°C.
18. After >24 hours below –136°C, remove one vial, restore the cells in culture medium (see section 5.1 above), and verify that the quality/performance is consistent with previous batches of the cell line (for cryopreserved cells initiated from a new batch) or previous behaviour of the same batch (if, e.g., establishing a new working cell bank).

6. Record keeping and reporting

At a minimum, records should be kept for

- Media and reagents used
- Equipment and consumables used
- Passage history of cells, including observations regarding cell growth, e.g.
    - Confluency estimates.
    - If growth/appearance deviates from expected behaviour.
    - Signs of contamination of cells or media.
- Documentation of cell counting, when performed

7. Revision history of SOP

Edition*	Date	Author	Short information about the changes
1.0	2020-08-26	Emma Pedersen	Change of title to include also culture of the wildtype FTC-238 cell line.
2.0	2020-10-01, 2021-02-11	Emma Pedersen, Kristina Fant	Update to include culture details for the wildtype FTC-238 cell line. Also rewritten the SOP to make it more general. Rewrote parts of the instructions for cryopreservation to avoid ambiguity

* Edition of the SOP which is subject to revision. Once the changes are approved the SOP will obtain a new (higher) edition number.

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