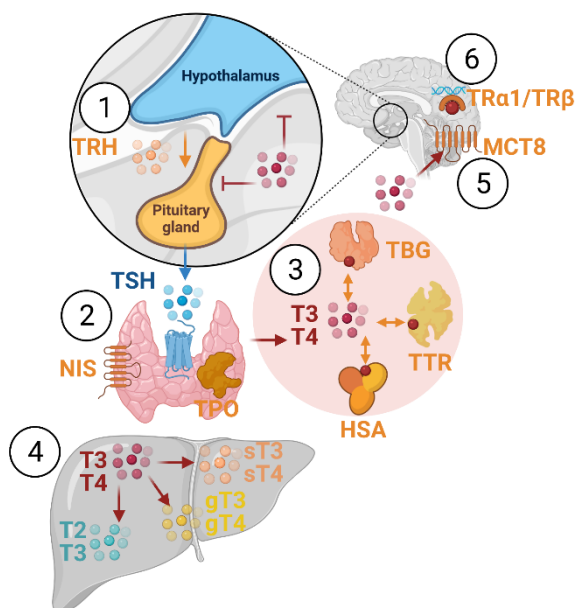


STANDARD OPERATING PROCEDURE

Determination of cell proliferation in T screen assay, 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

Roszak J.



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.

Contact information

Method developer:

Arno Gutleb
PhD, ERT
Luxembourg Institute of Science and Technology (LIST)
Environmental Research and Innovation (ERIN) Department
41, rue du Brill
L-4422 Belvaux
Grand-duchy of Luxembourg
Tel: +352 275 888 481
e-mail: arno.gutleb@list.lu

EU-NETVAL facility

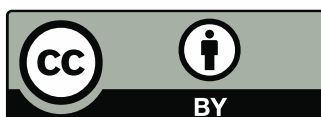
Joanna Roszak
National Center for Alternative Methods to Toxicity Assessment (CMA)
Department of Translational Research
Nofer Institute of Occupational Medicine
91-348 Lodz, 8 Sw. Teresy St, POLAND
e-mail: Joanna.Rozsak@imp.lodz.pl
<https://www.imp.lodz.pl/krajowe-centrum-metod-alternatywnych-do-oceny-toksycznosci>

EU Science Hub

<https://joint-research-centre.ec.europa.eu>

JRC133183

Ispra: European Commission, 2023
© European Union, 2023



The reuse policy of the European Commission documents is implemented by the Commission Decision 2011/833/EU of 12 December 2011 on the reuse of Commission documents (OJ L 330, 14.12.2011, p. 39). Unless otherwise noted, the reuse of this document is authorised under the Creative Commons Attribution 4.0 International (CC BY 4.0) licence (<https://creativecommons.org/licenses/by/4.0/>). This means that reuse is allowed provided appropriate credit is given and any changes are indicated.

For any use or reproduction of photos or other material that is not owned by the European Union, permission must be sought directly from the copyright holders. The European Union does not own the copyright in relation to the following elements:

- Cover page illustration, © BioRender.com

How to cite this report: Roszak J., Standard Operating Procedure for Determination of cell proliferation in T screen assay, version 1.0, applied in Part 1 of the EURL ECVAM thyroid validation study, European Commission, Ispra, 2023, JRC133183.

Standard Operating Procedure

Determination of cell proliferation in T-screen assay

Author: EU-NETVAL laboratory NIOM, Joanna Roszak

Version: 01

Date: 03.03.2021

Reference(s)

-

Required Standard Operating Procedures

- SOP "T-screen assay using GH3 cell line"

21 **Contents**

22	1. Scope of the SOP	3
23	1.1 Description of the method.....	3
24	1.2 Safety	3
25	1.3 Apparatus and Materials.....	3
26	1.4 Reagents and Chemicals	3
27	2. AlamarBlue Assay.....	4
28	2.1. Procedure.....	4
29	2.2 Data analysis	5
30	2.2.1. Calculation provided in the Bio-Rad manual (only applicable for Bio-Rad kit).....	5
31	2.2.2. Calculation universal for different brands of AlamarBlue	6

1. Scope of the SOP

The rat pituitary tumour cell line (GH3) is the test system for T-screen assay. The Standard Operating Procedure describes how to determine the proliferation of the GH3 cells with the Bio-rad AlamarBlue® reagent.

1.1 Description of the method

GH3 cell growth is increased in the presence of TH agonists and decreased in the presence of TH plus TH antagonists. Cell growth can be measured with different types of cell proliferation assays using a standard plate reader. In this SOP a colorimetric assay is used, where resazurin is reduced from a blue oxidized form into its violet reduced form of resorufin. The change of colour can be detected as a change in absorbance using a microplate reader.

1.2 Safety

Alamar Blue reagent should be added into wells under sterile conditions under a laminar chamber. Because the proliferation assay is performed using cells both unexposed and exposed to chemicals, personal protection suitable to a chemical tested should be applied. If T3 (an endocrine disrupting chemical, that can be extremely hazardous) is used, precautions such as using gloves, protective goggles and masks under a laminar flow hood should always be taken during operating the cultures treated with T3.

1.3 Apparatus and Materials

- Refrigerator 2-10 °C
- CO₂ humidified incubator at 37°C +/- 2 °C, 5% CO₂ +/- 0.5%
- Pipettors (e.g. p1000-100, p100-10, p10-0.5)
- Microplate Reader; for measuring Absorbance.
- Fume Hood
- Sterile, pipette tips with or without filter (e.g. 10 µL, 200 µL, and 1000 µL)

1.4 Reagents and Chemicals

- AlamarBlue® (e.g. Bio-Rad #BUF012B)

2. AlamarBlue Assay

2.1. Procedure

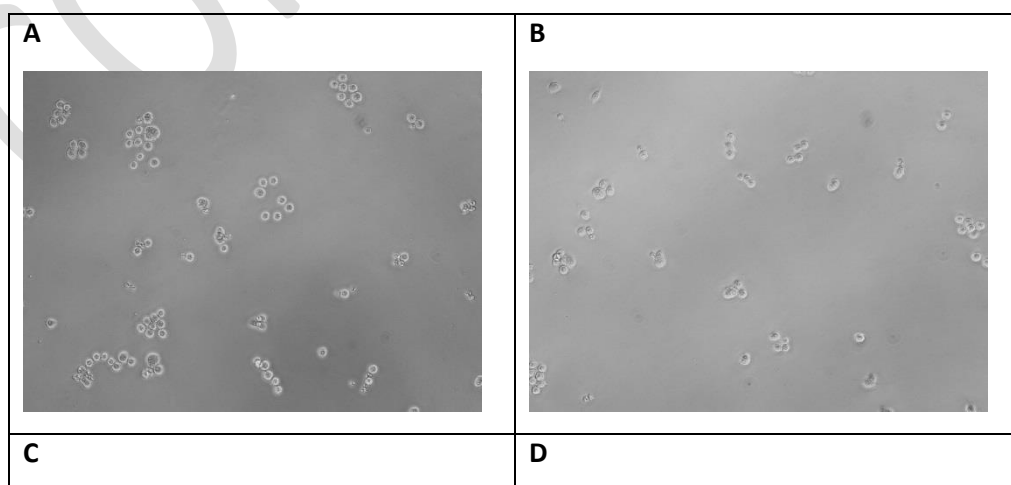
1.1-1 (only if applicable; perform once) After 96-h exposure, observe under the microscope wells in the additional plate for testing interference of AlamarBlue reagent with test items (samples incubated in PCM medium without cells) and record the following:

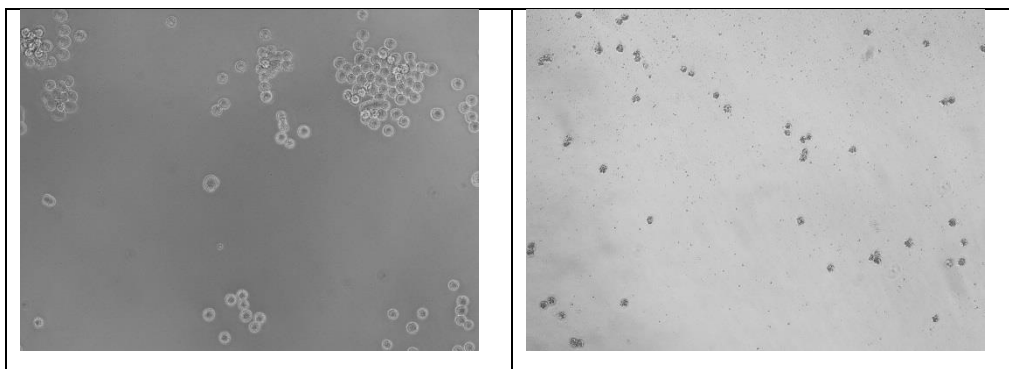
- Confirmed absence or observed microbiological contamination. In the case microbiological contamination is observed in any of the wells, the whole plate is discarded.
- Confirmed absence or observed precipitation of test items. In the case precipitates are observed, their presence at the specified concentration(s) should be noted. The proliferation test should be performed but these wells should be excluded from the calculations.

1.1-2 After 96-h exposure, observe the cells under the microscope and record the following:

- Confirmed absence or observed microbiological contamination. In the case microbiological contamination is observed in any of the wells, the whole plate is discarded.
- Confirmed usual or abnormal growth characteristics of control cells (Solvent Control). In case the solvent control shows abnormal growth, e.g. proliferating effect (Figure 1C) or cytotoxic effect (Figure 1D), the whole plate is discarded.
- Cytotoxicity observed in test item-exposed cells. In the case cytotoxicity is observed, the cytotoxic effect at the specified concentration(s) should be noted. The proliferation test should be performed without excluding any wells from calculations unless the cytotoxic effect is observed for all test concentration.
- Precipitation observed in test-item-exposed cells. In the case precipitates are observed, their presence at the specified concentration(s) should be noted. The proliferation test should be performed but these wells should be excluded from the calculations.

Figure 1. Exemplary images of cells observed under an inverted microscope equipped with a camera. A) Untreated GH3 cells; B) Solvent (0.2% DMSO) treated GH3 cells; C) GH3 cells treated with 20 nM T3 (proliferating effect); D) GH3 cells treated with 20 μ M Amiodarone (cytotoxic effect).





- 1.1-3** Discard PCM medium from columns 1 and 12 (Blank and Blank w/o AB) and add 200 µL of fresh PCM medium (to avoid an outsized effect of evaporation on the method performance)
- 1.1-4** Add 10 µL of Alamar blue reagent to every well (B1-G11) and incubate the plate for 3.5 h ± 0.5 h
- 1.1-5** Shake the microplate for 1 minute in the plate shaker using 800 rpm when used as a separate instrument or intensive shaking when used as a component of a microplate reader
- 1.1-6** Read the absorbance spectrophotometrically (OD570 and OD600 nm)
- 1.1-7** Perform data analysis as described below (Section 2.2). Analyse also the additional plate for testing interference of AlamarBlue reagent with test items (samples incubated in PCM medium without cells). Perform data analysis of results obtained for exposed cells only if results on the additional plate (incubation without cells) was accepted, i.e. they do not indicate any interference between AlamarBue and test item.

2.2 Data analysis

Results can be calculated in two ways – the first way is applicable only for Bio-Rad kit (Section 2.2.1), whereas the second one is universal for different brands of AlamarBlue (Section 2.2.2). Although %DR is not equivalent to %AR, the results can be comparable when they are converted to the relative proliferative effect (RPE) or the relative inhibitory effect (RIE) according to formulas given in SOP “T-screen assay using GH3 cell line” Section 3.4.1 or Section 3.4.2, respectively.

2.2.1. Calculation provided in the Bio-Rad manual (only applicable for Bio-Rad kit)

Calculate the results according the formula given below:

- (1) the average **OD (AVG), SD and %CV** for each sample (triplicates), also for Blank/column no. 1 (results for Blank w/o AB/column no. 12 are not used for this calculations). The average values will be used for the next steps
- (2) **% AlamarBlue reduction (%AR)** for each sample (OD(AVG)) measured according the following formula:

$$\%AR = \frac{(O2 \times A1) - (O1 \times A2)}{(R1 \times N2) - (R2 \times N1)} \times 100$$

Where:

O1 = molar extinction coefficient (E) of oxidized AlamarBlue (Blue) at 570 nm

O2= E of oxidized AlamarBlue at 600 nm

R1 = E of reduced AlamarBlue (Red) at 570 nm R2= E of reduced AlamarBlue at 600 nm

A1 = AVG absorbance of test wells at 570 nm

A2 = AVG absorbance of test wells at 600 nm

N1 = AVG absorbance of Blank (medium plus AlamarBlue but no cells) at 570nm

N2 = AVG absorbance of Blank (medium plus AlamarBlue but no cells) at 600nm

Molar extinction coefficients for AlamarBlue at different wavelengths

Wavelength	Reduced (R)	Oxidized (O)
570 nm	155677	80586
600 nm	14652	117216

2.2.2. Calculation universal for different brands of AlamarBlue

Calculate the results according the formula given below:

(1) the average **OD (AVG)**, **SD** and **%CV** for each sample (triplicates), also for *Blank* (column no. 1) and *Blank w/o AB* (column no. 12). The average values will be used for the next steps.

(2) **AOLW** (absorbance of oxidized form at the lower wavelength) and **AOHW** (absorbance of oxidized form at the higher wavelength) by subtracting AVG for background (*Blank w/o AB*; only PCM medium without cells and without AlamarBlue reagent) at 570 nm and 600 nm from the AVG values for each sample measured at the same wavelength. Calculate AOLW and AOHW also for Blank (medium without cells and with AlamarBlue reagent).

(3) **Correction factor Ro** based on data for the dye used in medium (*Blank*; without cells with AlamarBlue reagent) adjusted for background (*Blank w/o AB*; only PCM medium without cells and without AlamarBlue reagent); according the following formula:

$$Ro = AOLW[Blank]/AOHW[Blank]$$

(4) **% Dye reduction (%DR)** at 570 nm; according the following formula:

$$\%DR = (AOLW - AOHW \times Ro) \times 100$$

GETTING IN TOUCH WITH THE EU

In person

All over the European Union there are hundreds of Europe Direct centres. You can find the address of the centre nearest you online (european-union.europa.eu/contact-eu/meet-us_en).

On the phone or in writing

Europe Direct is a service that answers your questions about the European Union. You can contact this service:

- by freephone: 00 800 6 7 8 9 10 11 (certain operators may charge for these calls),
- at the following standard number: +32 22999696,
- via the following form: european-union.europa.eu/contact-eu/write-us_en.

FINDING INFORMATION ABOUT THE EU

Online

Information about the European Union in all the official languages of the EU is available on the Europa website (european-union.europa.eu).

EU publications

You can view or order EU publications at op.europa.eu/en/publications. Multiple copies of free publications can be obtained by contacting Europe Direct or your local documentation centre (european-union.europa.eu/contact-eu/meet-us_en).

EU law and related documents

For access to legal information from the EU, including all EU law since 1951 in all the official language versions, go to EUR-Lex (eur-lex.europa.eu).

Open data from the EU

The portal data.europa.eu provides access to open datasets from the EU institutions, bodies and agencies. These can be downloaded and reused for free, for both commercial and non-commercial purposes. The portal also provides access to a wealth of datasets from European countries.



The European Commission's science and knowledge service

Joint Research Centre

JRC Mission

As the science and knowledge service of the European Commission, the Joint Research Centre's mission is to support EU policies with independent evidence throughout the whole policy cycle.



EU Science Hub
joint-research-centre.ec.europa.eu



@EU_ScienceHub



EU Science Hub - Joint Research Centre



EU Science, Research and Innovation



EU Science Hub



EU Science