

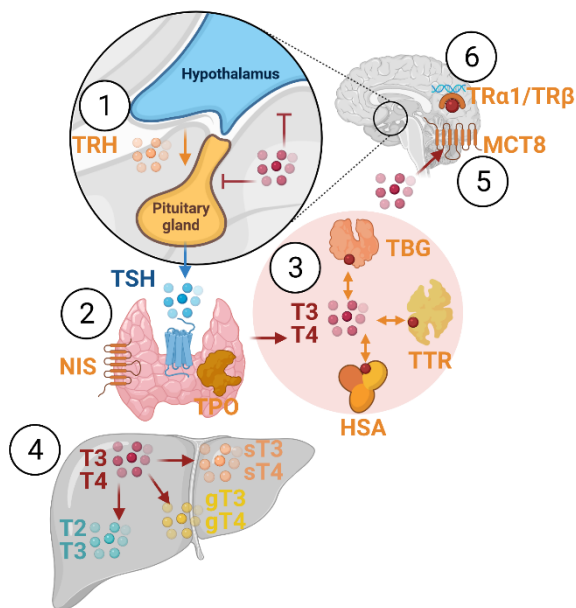
European  
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# STANDARD OPERATING PROCEDURE

## Determination of cell proliferation in T screen assay, version 2.0

*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.



2023

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" v2.0 (used in Part 2 of the validation study)
2. SOP "Handling, Maintenance and Quality Control of the GH3 cell line" v2.0 (used in Part 2 of the validation study)
3. **SOP "Determination of cell proliferation in T screen assay" v2.0** (used in Part 2 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. For the purpose of running the method on a robotic platform during part 2 of the validation study, EURL ECVAM prepared a 4<sup>th</sup> SOP.

4. SOP "T-Screen assay using a QUANTITATIVE HIGH THROUGHPUT screening approach" v1.0 (used in Part 2 of the validation study)

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JRC133180

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## Standard Operating Procedure

### Determination of cell proliferation in T-screen assay

**Author:** EU-NETVAL laboratory NIOM, Joanna Roszak

**Version:** 02

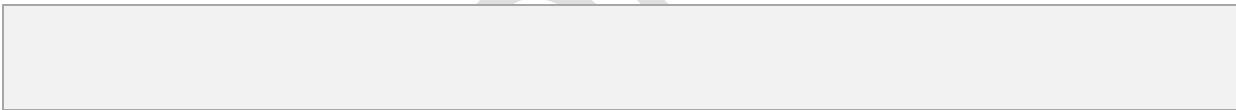
**Date:** 27.12.2021

#### Reference(s)

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#### Required Standard Operating Procedures

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



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33 **1. Scope of the SOP**

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

37 **1.1 Description of the method**

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

43 **1.2 Safety**

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

50 **1.3 Apparatus and Materials**

- 51 • Refrigerator 2-10 °C
- 52 • CO<sub>2</sub> humidified incubator at 37°C +/- 2 °C, 5% CO<sub>2</sub> +/- 0.5%
- 53 • Pipettors (e.g. p1000-100, p100-10, p10-0.5)
- 54 • Microplate Reader; for measuring Absorbance.
- 55 • Fume Hood
- 56 • Sterile, pipette tips with or without filter (e.g. 10 µL, 200 µL, and 1000 µL)

57 **1.4 Reagents and Chemicals**

- 58 • AlamarBlue® (e.g. Bio-Rad #BUF012B)

59 **2. AlamarBlue Assay**

60 **2.1. Procedure**

61 **2.1-1**(only if applicable; perform once ) After 96-h exposure, observe under the microscope wells in  
62 the additional plate for testing interference of AlamarBlue reagent with test items (samples  
63 incubated in PCM medium without cells) and record the following:

64 - Confirmed absence or observed microbiological contamination. In the case microbiological  
65 contamination is observed in any of the wells, the whole plate is discarded.

66 - Confirmed absence or observed precipitation of test items. In the case precipitates are  
67 observed, their presence at the specified concentration(s) should be noted. The proliferation  
68 test should be performed but these wells should be excluded from the calculations.

69 **2.1-2** After 96-h exposure, observe the cells under the microscope and record the following:

70 - Confirmed absence or observed microbiological contamination. In the case microbiological  
71 contamination is observed in any of the wells, the whole plate is discarded.

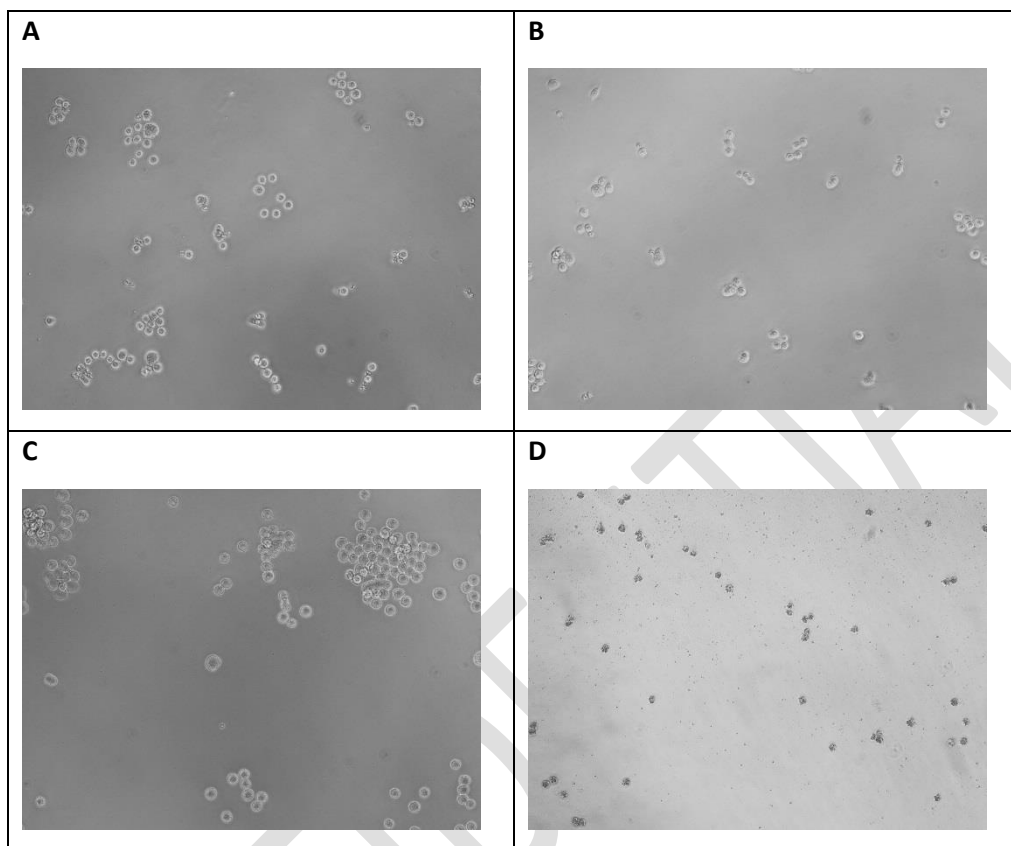
72 - Confirmed usual or abnormal growth characteristics of control cells (Solvent Control). In case  
73 the solvent control shows abnormal growth, e.g. proliferating effect (Figure 1C) or cytotoxic  
74 effect (Figure 1D), the whole plate is discarded.

75 - Cytotoxicity observed in test item-exposed cells. In the case cytotoxicity is observed, the  
76 cytotoxic effect at the specified concentration(s) should be noted. The proliferation test  
77 should be performed without excluding any wells from calculations unless the cytotoxic  
78 effect is observed for all test concentration.

79 - Precipitation observed in test-item-exposed cells. In the case precipitates are observed, their  
80 presence at the specified concentration(s) should be noted. The proliferation test should be  
81 performed but these wells should be excluded from the calculations.

82

83 **Figure 1.** Exemplary images of cells observed under an inverted microscope equipped with a camera.  
84 A) Untreated GH3 cells; B) Solvent (0.2% DMSO) treated GH3 cells; C) GH3 cells treated with 20 nM  
85 T3 (proliferating effect); D) GH3 cells treated with 20  $\mu$ M Amiodarone (cytotoxic effect).  
86



87

88

89 **2.1-3** Discard PCM medium from columns 1 and 12 (Blank and Blank w/o AB) and add 200  $\mu$ L of  
90 fresh PCM medium (to avoid an outsized effect of evaporation on the method performance)

91 **2.1-4** Add 10  $\mu$ L of Alamar blue reagent to every well (B1-G11) and incubate the plate for 3.5 h  $\pm$   
92 0.5 h

93 **2.1-5** Shake the microplate for 1 minute in the plate shaker using 800 rpm when used as a  
94 separate instrument or intensive shaking when used as a component of a microplate reader

95 **2.1-6** Read the absorbance spectrophotometrically (OD570 and OD600 nm)

96 **2.1-7** Perform data analysis as described below (Section 2.2). Analyse also the additional plate for  
97 testing interference of AlamarBlue reagent with test items (samples incubated in PCM  
98 medium without cells). Perform data analysis of results obtained for exposed cells only if  
99 results on the additional plate (incubation without cells) was accepted, i.e. they do not  
100 indicate any interference between AlamarBue and test item.

101

102 **2.2 Data analysis**

103 Calculate the results according the formula given below:

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

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

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

114 
$$Ro = AOLW[Blank]/AOHW[Blank]$$

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

116 
$$\%DR = (AOLW - AOHW \times Ro) \times 100$$

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117 **3. Change log**

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119 Standard Operating Procedure:

120 Determination of cell proliferation in T-screen assay

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ID	Version	Status	Date	List of changes in version 02 with respect to version 01:
1.	v. 01	Approved	03.03.2021	not applicable
2.	v. 02	Draft	09.12.2021	- Antagonist part of the method was removed throughout the SOP - %AR calculation was removed as the results were the same as for the %DR calculation
3.	v. 02	Final	27.12.2021	- Adding a section: Change log

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