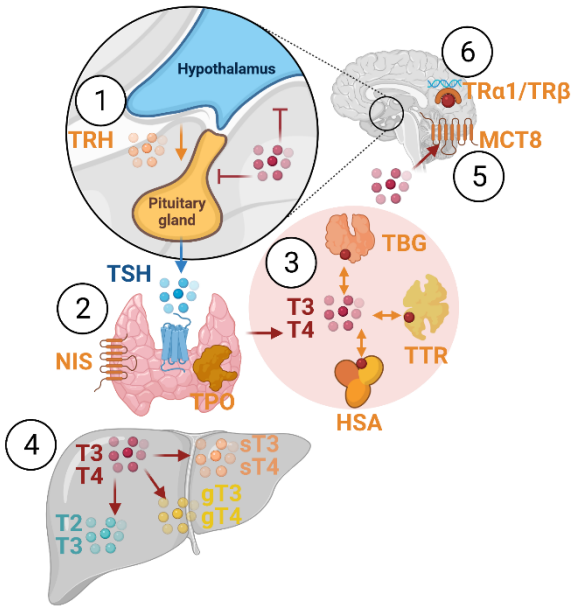
European Commission

# STANDARD OPERATING PROCEDURE

## *for thyroperoxidase activity assay with Amplex UltraRed (AUR-TPO), 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*

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)" v2.0** (used in Part2 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.

**Contact information**
**Method developer**:
US EPA
Steven O. Simmons, Ph.D.
Rapid Assay Development Branch
Biomolecular and Computational Toxicology Division
Center for Computational Toxicology and Exposure
U.S. EPA Office of Research and Development
109 TW Alexander Dr.
MD B243-03
Research Triangle Park, NC 27711
+01 (919) 541-1475

**EU-NETVAL facility**
RISE Research Institutes of Sweden AB
Sara Bogren, Facility Manager GLP
Box 857, 501 15, Borås, Sweden
Arvid Wallgrens Backe 20 | SE-413 46 Göteborg
M: +46 70 915 18 08
sara.bogren@ri.se
https://www.ri.se/en

**EU Science Hub**
https://joint-research-centre.ec.europa.eu

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# Thyroperoxidase Activity Assay with Amplex UltraRed (AUR-TPO)

Content:

# 1 Introduction

## 1.1 Aim and use of this SOP

This standard operating procedure is used to measure thyroperoxidase (TPO) activity after exposure to a test item, in extracts from cells expressing recombinant human TPO. This is done using the reagent Amplex™ UltraRed, a commercially available peroxidase substrate (AUR-TPO assay). Two control assays are also included in this SOP, these are performed to identify possible sources of nonspecific assay signal loss (i.e. to identify false positives): one cell-free luciferase inhibition assay to identify nonspecific enzyme inhibition (QuantiLum® Inhibition Assay, QLI) and one cytotoxicity assay using a human cell line to estimate the cellular tolerance limit (Cell Titer Glo, cytotoxicity assay, CTG).

## 1.2 References

US Environmental Protection Agency, OPERATING PROCEDURE: NHEERL-H/ISTD/SBB/JMH/2012-06-r0, SOP A352-031 THYROPEROXIDASE ACTIVITY ASSAY WITH AMPLEX ULTRARED

Life Technologies/Invitrogen Technical Bulletin for Catalog #A36006 Amplex UltraRed, http://products.invitrogen.com/ivgn/product/A36006.

Promega CellTiter-Glo® Luminescent Cell Viability Assay Technical Bulletin for catalog # G7570, G7571, G7572 and G7573: https://se.promega.com/-/media/files/resources/protocols/technical-bulletins/0/celltiter-glo-luminescent-cell-viability-assay-protocol.pdf?la=en

Promega Luciferase Assay System, Technical bulletin for Products E1483, E1500, E1501, E1531, E4030, E4530 and E4550: https://se.promega.com/-/media/files/resources/protocols/technical-bulletins/0/luciferase-assay-system-protocol.pdf?la=en

Dong H et al A rapid assay of human thyroid peroxidase activity. Toxicology in Vitro, 62, 104662, 2020, https://doi.org/10.1016/j.tiv.2019.104662

Jomaa B et al. Simple and Rapid In Vitro Assay for Detecting Human Thyroid Peroxidase Disruption, Altex 32(3), 2015. http://dx.doi.org/10.14573/altex.1412201

OECD (2018), Guidance Document on Good In Vitro Method Practices (GIVIMP), OECD Series on Testing and Assessment, No. 286, OECD Publishing, Paris. https://doi.org/10.1787/9789264304796-en

Paul K B et al. Development of a Thyroperoxidase Inhibition Assay for High-Throughput Screening. Chemical Research in Toxicology, 27(3), 387–399, 2014. http://doi.org/10.1021/tx400310w

Paul Friedman, K et al. Tiered High-Throughput Screening Approach to Identify Thyroperoxidase Inhibitors Within the ToxCast Phase I and II Chemical Libraries. Toxicological Sciences, 151(1), 160–180, 2016. http://doi.org/10.1093/toxsci/kfw034

Schmutzler C. et al. Endocrine disruptors and the thyroid gland – a combined in vitro and in vivo analysis of potential new biomarkers. Environ. Health Persp. 115, Suppl 1, 77-83, 2007. (a)

Schmutzler C et al. The Ultraviolet Filter Benzophenone 2 Interferes with the Thyroid Hormone Axis. Endocrinology 148(6), 2835–2844, 2007. (b)

Taurog, A. Hormone synthesis. In: Werner and Ingbar's The Thyroid: A Fundamental and Clinical Text (Braverman LE, Utiger, RD eds). Philadelphia: Lippencoott, Williams and Wilkins, 47-81, 2005.

## 1.3 Background

TPO plays a central role during synthesis of the thyroid hormones thyroxine (T4) and triiodothyronine (T3). In living animals, the heme protein thyroperoxidase catalyses all the essential steps in the synthesis process (Taurog et al, 2005). The process begins with the oxidation of iodide, the iodination of tyrosyl residues of thyroglobulin (Tg) and the coupling of two iodotyrosyls to give Tg-coupled T4 and T3. Then these hormones, after hydrolysis, are released by the thyroid gland in response to the appropriate stimulus by the pituitary hormone, thyroid stimulating hormone (TSH). Hydrogen peroxide ($H_2O_2$), generated by the thyrooxidase enzymes ThOX1 and ThOX2, is an essential cosubstrate in this reaction sequence because it serves as a source for oxidative equivalents (Schmutzler et al, 2007 (a)).

The Thyroperoxidase Activity Assay with Amplex™ UltraRed (AUR-TPO) test method was originally developed by researchers at the US Environmental Protection Agency (EPA). Amplex™ UltraRed (AUR) (Life Technologies, cat. no. A36006) is sold for the sensitive detection of $H_2O_2$ released from biological samples, including cells, in the presence of excess horseradish peroxidase; AUR is a fluorogenic substrate that is converted from AUR to Amplex™ UltroxRed by horseradish peroxidase in the presence of $H_2O_2$. EPA repurposed the AUR substrate to detect peroxidase activity (i.e., TPO activity) in the presence of excess $H_2O_2$ for the AUR-TPO assay (Paul et al, 2014).
The method described in this SOP is based on the protocol described in the US Environmental Protection Agency SOP A352-031 Thyroperoxidase Activity Assay With Amplex UltraRed, scientific publications of the method (Paul et al, 2014; Paul-Friedman et al, 2016), and publications of TPO isolation from human cell lines (Jomaa et al, 2015; Schmutzler et al, 2007 (b); Dong et al, 2020). The method in this SOP is transformed to 1) use an in vitro source for the TPO instead of thyroid glands, 2) be functional in a GLP environment, and 3) use commonly accessible equipment and software, 4) have a more simple data analysis which does not require sophisticated statistical knowledge.

## 1.4 Principle of the method

This test is based on the measurement of TPO activity (peroxidase activity) in the presence of excess $H_2O_2$ using the fluorogenic substrate Amplex™ UltraRed (AUR). TPO is prepared as a whole cell extract from cultures of a human cell line producing recombinant TPO. The TPO is incubated at 37 °C for 30 minutes with the test item, AUR reagent, and $H_2O_2$, after which the TPO inhibition is quantified with a fluorescence measurement. The dose-response for a test item is determined, and parameters regarding the response are quantified.

To identify false positives, two luminescence-based control assays are performed:

- To identify nonspecific enzyme inhibition, QuantiLum® recombinant luciferase is incubated with the test item under conditions mimicking the main experiment with

Amplex™ UltraRed. Luciferase detection reagent is then added to quantify the nonspecific enzyme inhibition with a luminescence measurement.

- To estimate the cellular tolerance limit, a cytotoxicity test is performed where the cells are incubated with the test item and the viability after 24h is determined using the commercial CellTiter-Glo® Luminescent Cell Viability Assay.

A more detailed overview of the test strategy is provided in section 6.

### 1.5 Test system

The TPO needed for the experiments is prepared as a whole cell extract from cultures of FTC-238 cells (human follicular thyroid carcinoma) transfected with human recombinant TPO (FTC-238/hrTPO), according to SOP "Thyroperoxidase (TPO) extract preparation". The generation of the cell line is described in Schmutzler et. al. 2007 (b).

During implementation of the assay, the activity of extracts from FTC-238/hrTPO cells in the AUR-TPO assay shall be compared with the activity of extracts from the wildtype FTC-238 cells. If prepared correctly and with correct implementation of the assay, extracts from the FTC-238/hrTPO cause a linear concentration-dependent increase in AUR fluorescence signal while extracts from the wildtype cells have very little effect on fluorescence. This control experiment shows clearly that the human TPO transgene in the transformed cells is active and the only source of peroxidase activity in extracts from these cells.

### 1.6 Required additional SOPs

- SOP"Culture of FTC 238 and FTC 238/hrTPO cells"
- SOP "Thyroperoxidase (TPO) extract preparation"
- SOP "Solubility determination by visual inspection"

## 2 Waste handling and cleaning

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

# 3 Equipment and consumables

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 qualified for use, and their use shall be documented according to the cell culture facility's internal procedures.It is of special importance to verify that no consumables give rise to background noise in the assay, as might happen with many types of plastics in sensitive assays for endocrine disruption.

## 3.1 Preparation of test, reference and control item stock solutions and DMSO dilution series

### 3.1.1 Equipment for preparation of test, reference and control item stock solutions and DMSO dilution series

- Single and multi-channel pipettes (e.g. 0.2-2 µl, 1-10 µl, 10-100µl, 20-200 µl, 100-1000 µl and 500-5000 µl)
- Precision balance with capacity of minimum 50 g and readability of 0.1 mg for weighing of test, reference and control items
- Fume hood or other equipment for safe handling of undissolved test, reference and control items
- Vortex shaker
- Water bath, 37 °C
- Ultrasonic bath

### 3.1.2 Consumables for preparation of test, reference and control item stock solutions and DMSO dilution series

- Clean glass vials, for weighing of test and reference items, e.g. Infochroma ag G075S-14/030-H (2.5 ml) or G075S-27/047-H (15 ml), or similar
- 96-well compound storage plates, e.g. Corning® 96 Well Storage Microplates, Corning Costar cat # 3365, or similar, for long-term storage of DMSO stock solutions and efficient preparation of dilution series, optionally equipped with sealing mat (e.g. Corning Costar cat # 3080).
- Clean glass tubes or vials, e.g. Duran group, Fiolax 12 mm × 75 mm 261100803 (for preparation of dilutions of stock and work solutions, if compound storage plates are not used)
- Pipette tips
- Glass pipettes, for pipetting DMSO.

## 3.2 AUR-TPO assay

### 3.2.1 Equipment AUR-TPO assay

- Equipment for safe handling of chemicals, e.g. fume hood or Biological Safety Cabinet class II with VOC-filter.
- Single and multi-channel pipettes (e.g. 0.2-2 µl, 1-10 µl, 10-100µl, 20-200 µl, 100-1000 µl and 500-5000 µl)
- Pipetting aid

- Precision balance with capacity of 50 g minimum and readability of 0.1 mg for weighing of potassium phosphate.
- Plate reader with fluorescence measurement function + software , equipped with appropriate filters and mirrors for detection of the Amplex UltraRed reagent
- pH meter (calibrated), for preparation of potassium phosphate buffer
- -80°C freezer, for storage of TPO extracts

### 3.2.2 Consumables AUR-TPO assay

- Black solid 96-well plates, e.g. Corning Costar cat # 3356 or similar
- *Optional:* 96-well compound storage plates, e.g. Corning Costar cat # 3365 or similar, for efficient preparation of working solutions (if not prepared in an assay plate)
- Serological pipettes 2-50 ml
- Pipette tips
- Disposable reagent reservoirs
- Polypropylene centrifuge tubes, 15 and 50 ml

## 3.3 QLI assay

### 3.3.1 Equipment QLI assay

- Equipment for safe handling of chemicals, e.g. fume hood or Biological Safety Cabinet class II with VOC-filter.Plate reader with luminescence measurement function and one dispenser + software Precision balance with capacity of 50 g minimum and readability of 0.1 mg for weighing of BSA
- Single and multi-channel pipettes (e.g. 0.2-2 µl, 1-10 µl, 10-100µl, 20-200 µl, 100-1000 µl and 500-5000 µl)
- Pipetting aid

### 3.3.2 Consumables QLI assay

- White solid 96-well plates, e.g. Corning Costar cat # 3912 or similar
- *Optional:* 96-well compound storage plates, e.g. Corning Costar cat # 3365 or similar, for efficient preparation of working solutions (if not prepared in an assay plate)
- Serological pipettes 2-50 ml
- Pipette tips
- Disposable reagent reservoirs
- Polypropylene centrifuge tubes, 15 and 50 ml

### 3.4 CTG assay

#### 3.4.1 Equipment CTG assay

- Equipment for safe handling of chemicals, e.g. fume hood or Biological Safety Cabinet class II with VOC-filter.Plate reader with luminescence measurement function + softwareSingle and multi-channel pipettes (e.g. 0.2-2 µl, 1-10 µl, 10-100µl, 20-200 µl, 100-1000 µl and 500-5000 µl)
- Pipetting aid
- Vortex shaker
- Centrifuge for pelleting of cells, capable of centrifuging at ~200×g
- Incubator, 37 °C, humidified, 5 % $CO_2$ -150°C freezer or equivalent cryostorage for mammalian cellsBiological Safety Cabinet class II
- Shaker, for microtiter plates
- Water bath, 37 °C
- Inverted phase contrast microscope
- Cell counter or hemocytometer

#### 3.4.2 Consumables CTG assay

- White 96-well plates with transparent bottom, e.g. Corning Costar cat # 3610 or similar
- *Optional:* 96-well compound storage plates, e.g. Corning Costar cat # 3365 or similar, for efficient preparation of working solutions (if not prepared in an assay plate)
- Serological pipettes 2-50 ml
- Sterile pipette tips
- Sterile disposable reagent reservoirs
- Sterile polypropylene centrifuge tubes, 15 and 50 ml
- Tissue culture flasks, of appropriate size (T75-T300)
- pH sticks, capable of measuring pH in the interval 6.0 to 9.0 (for culturing without phenol red)

## 4 Medium, chemicals and reagents

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, labelling and documentation of media and other reagents should follow the cell culture facilities internal procedures. 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.

### 4.1 AUR-TPO assay

#### 4.1.1 Chemicals AUR-TPO assay

- Amplex UltraRed reagent (Thermo Fisher cat # A36006)

- $KH_2PO_4$, CAS 7778-77-0, Potassium phosphate monobasic, 99% (Sigma (Merck) cat # P5379, or equivalent)
- $K_2HPO_4$, CAS 7758-11-4, Potassium phosphate dibasic, 98% (Sigma (Merck) cat # P3786, or equivalent)
- Anhydrous DMSO (Sigma (Merck) cat # 276855 or equivalent)
- Hydrogen peroxide solution, 30% (w/w) in $H_2O$, CAS 7722-84-1, (Sigma (Merck) cat # H1009 or equivalent)
- Deionized water
- Sodium deoxycholate 0.1%, according to section 4.1.2 in SOP "Thyroperoxidase (TPO) extract preparation", for dilution of TPO extracts and evaluation of background.

### 4.1.2 Reference and Control Items AUR-TPO assay

Reference Item:

- 2-Mercapto-1-methylimidazole (MMI), CAS 60-56-0 (Sigma (Merck) Cat # 301507, or equivalent)

Control Item(s):

- Positive control (PC): 6-Propyl-2-thiouracil (PTU), CAS 51-52-5 (Sigma (Merck) Cat # P3755)
- Negative control (NC): 2-Hydroxy-4-methoxy-benzophenone (BP3), CAS 131-57-7 (Sigma (Merck) Cat # H36206, or equivalent)

Vehicle Control (VC): Maximal thyroperoxidase activity with solvent at same level as test items

Blank 1 (BC1): hydrogen peroxide free wells, otherwise the same content as vehicle control wells – gives the background signal in the assay

Blank 2 (BC2): TPO-free wells – also a measure of the background signal, used to determine the activity of the TPO extract

### 4.1.3 Reagent preparation AUR-TPO assay

4.1.3.1 Potassium phosphate buffer, 0.2M, pH 7.4

First prepare:

- Solution A: 27.2 g $KH_2PO_4$ per litre (0.2M)
- Solution B: 34.8 g $K_2HPO_4$ per litre (0.2M)

Then, for 100mL:

1. Make 0.2M buffer by combining 19 mL solution A + 81 mL solution B
2. Adjust pH to 7.4 by adding solution A or B accordingly

Store at room temperature for up to one month.

4.1.3.2 $H_2O_2$, 2.4 mM

1. Prepare a 100× dilution of the 9.75 M $H_2O_2$ (30% stock), e.g. 990 µL of di$H_2O$ + 10 µL of 9.754 M $H_2O_2$

2. Prepare 2.4 mM $H_2O_2$ from the 100× dilution, e.g. for 10 mL use 246 µL of the 97.5 mM $H_2O_2$ + 9.754 mL of di$H_2O$.

Prepare the $H_2O_2$ dilution fresh for each day.

Take care in handling concentrated 30% $H_2O_2$. Avoid splashing on exposed skin.

4.1.3.3 Amplex UltraRed reagent:

1. Prepare a 10 mM stock solution of Amplex® UltraRed reagent by adding 340 µL of fresh, high-quality DMSO to one vial of Amplex® UltraRed reagent. Vortex well to dissolve.
2. Dilute 150× to 67uM in 0.2M potassium phosphate buffer (e.g. 20µl for a total volume of 3 ml), this is below referred to as the AUR working solution. This solution will yield 25 µM final concentration in the assay.

Prepare the AUR right before use, and protect from excess exposure to light and air by tightly sealing caps and wrapping tubes in foil or storing in drawers.

Store remaining solution in the dark with desiccant at –20°C for future use. When stored properly, this solution is stable for at least 6 months. Pink colouring in Amplex® UltraRed reagent is an early indicator of compromised material.

## 4.2 QLI assay

### 4.2.1 Chemicals QLI assay

- Luciferase assay reagent (Promega cat # E1501, or equivalent)
- QuantiLum® Recombinant Luciferase (Promega, cat # E170)
- $KH_2PO_4$, CAS 7778-77-0, Potassium phosphate monobasic, 99% (Sigma (Merck) cat # P5379, or equivalent)
- $K_2HPO_4$, CAS 7758-11-4, Potassium phosphate dibasic, 98% (Sigma (Merck) cat # P3786, or equivalent)
- Anhydrous DMSO (Sigma (Merck) cat # 276855 or equivalent)
- Bovine serum albumin (BSA), e.g. GE Healthcare Life Sciences Hyclone Laboratories cat # SH30574.01 or equivalent
- Deionized water

### 4.2.2 Reference and Control Items QLI assay

Reference Item:

- Luciferase inhibitor II (LUCINH2), CAS 10205-56-8 (Calbiochem EMD Millipore Corporation Cat # 119114, or equivalent)

Control Item(s):

- Positive control (PC): Luciferase inhibitor I (LUCINH1), CAS 352341-26-5 (Calbiochem EMD Millipore Corporation Cat # 119113, or equivalent)
- Negative control (NC): 2-Hydroxy-4-methoxy-benzophenone (BP3), CAS 131-57-7 (Sigma (Merck) Cat # H36206, or equivalent)

Vehicle Control (VC): Maximal luciferase activity with solvent and BSA at same level as test items

Blank (BC): Luciferase-free wells, otherwise same content as vehicle control – gives the background signal in the assay

### 4.2.3 Reagent preparation QLI assay

4.2.3.1 Potassium phosphate buffer supplemented with BSA

The BSA may be dissolved directly in the 0.2M potassium phosphate buffer (prepared according to section 4.1.3.1 above) to the concentration needed for the QLI assay, however, it might be more time efficient to first establish a 10× concentrated stock solution that may be stored until use:

1. Weigh up the desired amount of BSA, e.g. 300-500 mg BSA in a 50 ml centrifuge tube.
2. Dissolve to 10 mg/ml in 0.2M potassium phosphate buffer prepared according to section4.1.3.1, and then sterile filter the solution.
3. Aliquot in suitable amounts. Store at 2-8°C for up to 12 months.
4. Dilute 10× before use

4.2.3.2 Luciferase Assay Reagent

To prepare the Luciferase Assay Reagent (Promega E1501), add Luciferase Assay Buffer (10ml for E1501) to the vial containing the lyophilized Luciferase Assay Substrate.

Avoid exposure of the Luciferase Assay Reagent to multiple freeze-thaw cycles by dispensing the reconstituted reagent into working aliquots. Store any unused Luciferase Assay Reagent at –20°C for up to 1 month or –70°C for up to one year. Thaw Luciferase Assay Reagent at temperatures below 25°C and mix well before use.

## 4.3 CTG assay

### 4.3.1 Chemicals CTG assay

- CellTiter-Glo® Luminescent Cell Viability Assay (Promega, cat # G757)
- Anhydrous DMSO, e.g. Sigma Cat# 276855 or equivalent
- Iscove's modified Dulbecco's medium (1×) buffered with $NaHCO_3$ (Gibco Life Technologies, cat # 21056-023 or equivalent)
- Fetal Bovine Serum (Gibco Life Technologies, cat # 10270-098 or equivalent)
- Penicillin-streptomycin (Cytiva Hyclone, cat # SV30010 or equivalent)
- Geneticin selective antibiotics (G-418 sulfate) (Gibco Life Technologies cat # 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 $Ca^{2+,}$ $Mg^{2+}$ (GE Healthcare Hyclone, cat # SH30028.02 or equivalent)

### 4.3.2 Reference and Control Items CTG assay

Reference item:

- 2,3-dichloro-1,4-napthoquinione (DCNQ), CAS 117-80-6 (Acros Organics Cat # 113480250 or equivalent)

Control Item(s):

- Positive control (PC): 2,3-dichloro-1,4-napthoquinione (DCNQ), CAS 117-80-6 (Acros Organics Cat # 113480250 or equivalent)
- Negative control (NC): 2-Hydroxy-4-methoxy-benzophenone (BP3), CAS 131-57-7 (Sigma (Merck) Cat # H36206 or equivalent)

Vehicle Control (VC): Maximal viability/activity, with solvent at same level as test items in complete assay medium

Blank (BC): cell-free wells, otherwise same content as vehicle control – gives the background signal in the assay

### 4.3.3 Reagent preparation CTG assay

#### 4.3.3.1 Complete assay medium 1× FTC CTG

Iscove's modified Dulbecco's medium (1×) buffered with NaHCO3 supplemented with:

- 5 % Fetal Bovine Serum
- 100 IU/ml penicillin and 100 µg/ml streptomycin (from stock solution 100×)

#### 4.3.3.2 CellTiter-Glo® reagent

Transfer the appropriate volume (10ml for Cat.# G7570 and G7571, or 100ml for Cat.# G7572 and G7573) of CellTiter-Glo® Buffer into the amber bottle containing CellTiter-Glo® Substrate to reconstitute the lyophilized enzyme/substrate mixture. This forms the CellTiter-Glo® Reagent. Mix by gently vortexing, swirling or inverting the contents to obtain a homogeneous solution. The CellTiter-Glo® Substrate should go into solution easily in less than 1 minute.

According to the technical bulletin, reconstituted CellTiter-Glo® Reagent can be stored at room temperature for up to 8 hours with <10% loss of activity, at 4°C for 48 hours with ~5% loss of activity, at 4°C for 4 days with ~20% loss of activity or at –20°C for 21 weeks with ~3% loss of activity. The reagent is stable for up to ten freeze-thaw cycles, with less than 10% loss of activity.

#### 4.3.4 Cells

FTC-238 cell line (human follicular thyroid carcinoma, ECACC 94060902).

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

## 5 Qualification of TPO extracts

TPO from FTC-238/hrTPO cells is prepared as a whole cell extract according to SOP "Thyroperoxidase (TPO) extract preparation". For long-term storage, keep cell lysate at -80°C.

Before using a TPO extract for testing of test items with the AUR-TPO assay, the extracts must be qualified for use according to the following procedure:
Measure the TPO efficiency of the extract by performing the AUR-TPO assay according to section 6.2.using only the reference item MMI, vehicle control, BC1, and BC2. For the TPO extract to be qualified for use with the AUR-TPO assay, the following criteria must be met, c.f section 9:

- The MMI $AC_{50}$ must pass acceptance criteria.
- The TPO efficiency of the extract must be greater than 3-fold the TPO-free control (BC2).

## 6 Method

An overview of the method is given in Figure 1. The first step is to determine the solubility of the test item(s), both in the solvent and in the work solution (AUR-TPO assay only), c.f. SOP "Solubility determination by visual inspection". The highest soluble concentration of the test item is further diluted (10× dilutions) and a range-finding experiment is performed (dose-response, 7 concentrations) with the AUR-TPO assay (section 6.2). If the TPO is inhibited by more than or equal to 20% at any concentration, the main experiment is performed; if the test item does not inhibit TPO, the range-finding experiment is repeated to confirm the absence of TPO inhibition. The main experiment is performed (with 8 concentrations selected to cover the response) with the AUR-TPO assay and the two control assays, the QuantiLum® inhibition (QLI) assay and the CellTiter-Glo® (CTG) cytotoxicity assay, after which a classification of the test item can be made.

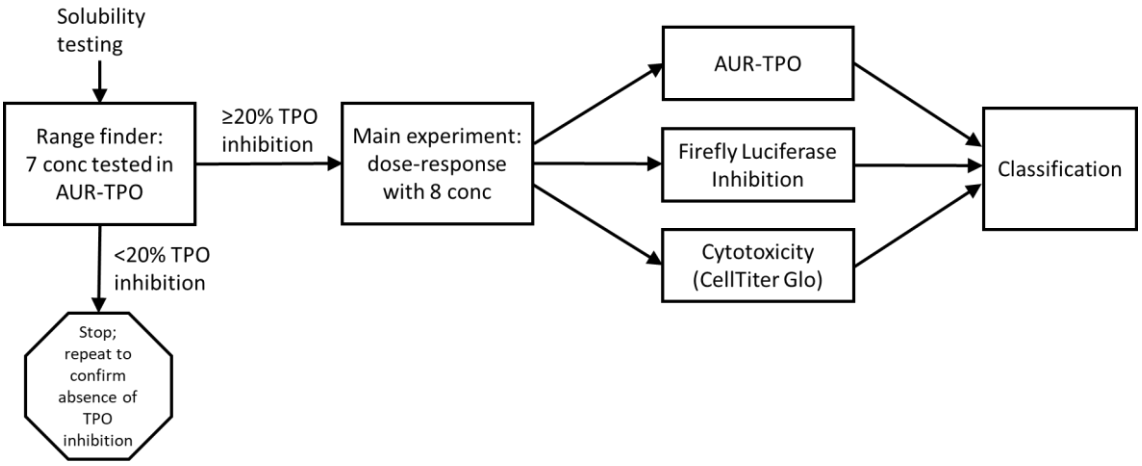


**Figure 1.** Overview of the work flow of the AUR-TPO assay

Several test items can be evaluated simultaneously using multiple plates, constituting one experiment, where the reference and control items are included only on the first plate in the series. The subsequent plates must, however, be prepared from the same dilutions of reagents and same reservoir with diluted TPO extract (AUR-TPO assay), recombinant luciferase (QLI assay) or cell suspension (CTG assay). The vehicle control and reference item maximal concentration (C8) must also be from the same preparation on all plates in the experiment. The reagent stability, and hence the time frame during which a series of plates must be completed, determines the number of plates that can be performed in one experiment. This should be determined during method implementation in the test facility.

## 6.1 Preparation of test, reference and control item solutions for range finding or main experiment

This section describes the preparation of stock and working solutions for range-finding experiments and the main experiment in the AUR-TPO assay as well as the two control assays. Before this is performed, the solvent, mode of preparation, and maximum soluble concentration (in the chosen solvent and in the work solution for the AUR-TPO assay), should be determined for each test item according to SOP “Solubility determination by visual inspection”.

DMSO is the first choice of solvent to use (below the instructions are assuming DMSO is used), the 2nd choice is $H_2O$. However, if other solvent than DMSO is used:

- Always ensure that both solvents (DMSO of reference and control items and the solvent of the test item) are present at the same concentration in all work solutions. E.g. if the test item is dissolved in $H_2O$, use medium that already contains 0.2% DMSO to prepare the test item work solutions. Similarly, the work solutions of the reference and control items should be prepared using medium that is supplemented with the chosen solvent at 0.2% (not if the solvent is $H_2O$).
- The effect of another solvent used must be examined and only concentrations that do not interfere with the AUR-TPO assay and the two control assays may be used.
- Test items that are dissolved in a solvent different from DMSO shall be tested in a separate run with the appropriately prepared work solutions of the reference and control items.

For general instructions on the handling of hazardous test, reference and control items, please refer to facility specific instructions .

### 6.1.1 Preparation of test, reference and control item stock solutions

Preparations of DMSO stock solutions and dilution series may be stored in glass vials or in polypropylene compound storage plates in a desiccator for up to 1 year at -80ºC and up to 2 months in a desiccator at room temperature.

Use a glass pipette for the preparation of a vial with solvent DMSO from which to take aliquots with plastic pipette tips.

For test items, a new preparation of stock solution should be performed for each biological replicate.

1. Weigh between 10 and 20 mg (suggested amount) of test, reference and control items into clear glass vials. Keep containers closed as much as possible and clean the bench surface between weighing of substances to avoid cross-contamination. Record the weighing of test, reference and control items.
2. Prepare the required stock solution concentration (i.e. stock solution A) according to Table *1* using the formula V = m / (M*c).
    - Reference and control items are dissolved in DMSO.
    - For the test item the first choice of solvent is DMSO, see discussion above. Use the solvent and maximum soluble concentration determined with SOP “Solubility determination by visual inspection”.

Table 1. Overview of stock solutions.

| | Molecular weight (g/mole) | Concentration Stock solution A |
|---|---|---|
| Test item | | 100 mM or, for unknown test items, 50 mg/ml, or maximum soluble concentration |
| MMI (reference item AUR-TPO assay) | 114.17 | 100 mM |
| PTU (PC AUR-TPO assay) | 170.23 | 100 mM |
| LUCINH2 (reference item QLI assay) | 254.35 | 2 mM |
| LUCINH1 (PC QLI assay) | 253.26 | 100 mM |
| DCNQ (reference item and PC CTG assay) | 227.04 | 100 mM |
| BP3 (NC all three assays) | 228.24 | 100 mM |

3. After that the procedure determined necessary according to SOP “Solubility determination by visual inspection” to fully dissolve the particular test item is complete, vortex mix for 1 minute. For the reference and control items vortex mixing for 1-3 minutes is sufficient to dissolve the respective chemicals, except for LucInh2 which also requires ultrasonication for 15 minutes for complete solubility.
4. Ensure complete solubility by visual (macroscopic) inspection.
5. *For the main experiment:* If needed, further dilute the stock solution of a test item to the highest dilution to be tested using the same solvent as used to prepare the stock solution. This dilution is indicated as the C8 dilution.

6. Where necessary, further dilute positive and negative control items in DMSO to create stock solutions B, see Table 2.

Table 2. Concentrations and suggested volumes for preparation of stock solutions B for control items.

| | Volume of stock solution A | Volume DMSO | Concentration Stock solution B | Final test concentration |
|---|---|---|---|---|
| PTU (PC AUR-TPO assay) | 10 µl | 70 µl | 12.5 mM | 25 µM |
| BP3 (NC AUR-TPO assay) | 50 µl | 50 µl | 50mM | 100 µM |
| LUCINH1 (PC QLI assay) | 2 µl | 198 µl | 1 mM | 2 µM |
| BP3 (NC QLI assay) | 5 µl | 95 µl | 5 mM | 10 µM |
| DCNQ (PC CTG assay) | 5 µl | 95 µl | 5 mM | 10 µM |
| BP3 (NC CTG assay) | 50 µl | 50 µl | 50mM | 100 µM |

7. Prepare serial dilution series of the reference item and the test item(s) in polypropylene compound storage plates or glass vials/tubes using the same solvent as used to prepare the stock solution, starting with the highest concentration of test item (r-C7 for the range-finding experiment and C8 for the main experiment).
   - If dilutions are performed in compound storage plates, transfer the appropriate volume of compound from one column to the next, change tips to minimize carry over, and mix by pipetting up and down 12 times.
   - If dilutions are performed in glass vials/tubes, each vial/tube should be vortex mixed between consecutive dilutions. Pipette tips shall be changed between each concentration.
   - **Range-finding experiment**: for test items the dilution factor to use is 10× between consecutive concentrations, starting with the maximum soluble concentration, and 7 test concentrations. As an example: for a test item soluble at 100 mM, the following concentrations should be prepared: 100 mM, 10 mM, 1 mM, 100 µM, 10 µM, 1 µM and 100 nM. Record the preparation of dilution series of test items.
   - **Main experiment:** The dilution factor and highest concentration (C8) shall be selected according to the instructions in section 7, and a dilution series with 8 test concentrations shall be prepared. The highest test concentration and/or dilution factor might need to be adjusted in order to capture the entire response. It is important to both capture the maximum activity and to test low enough concentrations for the test item to be inactive. Record the preparation of dilution series of test items.
   - Suggested dilution schemes and pipetting volumes for reference items are presented below: in Table 3 for reference item MMI (AUR-TPO assay), Table *4* for reference item LUCINH2 (QLI assay) and Table *5* for reference item DCNQ (CTG assay). Record the preparation of dilution series.
     - For the AUR-TPO reference item at least 5 MMI concentrations that range from 0.001µM to 100 µM, with at least two concentrations that are below 1 µM, must be included (DMSO stock solution concentration, resulting in concentrations in well ranging from $2*10^{-6}$ µM to 0.2 µM). Higher concentrations can be evaluated.
8. Label vials/plates appropriately to avoid mixing-up dilutions.

Record the weight, solvent, volume added and final concentration of the test item, reference item and control items stock solutions.

Table 3. Dilution scheme with concentrations and suggested volumes of stock solutions of reference item MMI for the AUR-TPO assay, and final test concentrations in the AUR-TPO assay.

| Reference item | DMSO stock solutions | | | Concentration in AUR-TPO assay |
|---|---|---|---|---|
| | Concentration | Reference item volume | Volume DMSO | |
| C8 MMI | $1.0\times10^{-1}$ M | 200µl Stock solution A MMI | 0µl | 200 µM |
| C7 MMI | $1.0\times10^{-2}$ M | 20 µl C8 MMI | 180 µl | 20.0 µM |
| C6 MMI | $1.0\times10^{-3}$ M | 20 µl C7 MMI | 180 µl | 2.00 µM |
| C5 MMI | $1.0\times10^{-4}$ M | 20 µl C6 MMI | 180 µl | 0.2 µM |
| C4 MMI | $5.0\times10^{-6}$ M | 10 µl C5 MMI | 190 µl | 0.010 µM |
| C3 MMI | $2.5\times10^{-7}$ M | 10 µl C4 MMI | 190 µl | 0.0005 µM |
| C2 MMI | $1.25\times10^{-8}$ M | 10 µl C3 MMI | 190 µl | $2.5\times10^{-5}$ µM |
| C1 MMI | $6.25\times10^{-10}$ M | 10 µl C2 MMI | 190 µl | $1.25\times10^{-6}$ µM |

Table 4. Dilution scheme with concentrations and suggested volumes of stock solutions of reference item LUCINH2 for the QuantiLum® Luciferase inhibition assay.

| Reference item | DMSO stock solutions | | | Concentration in QLI assay |
|---|---|---|---|---|
| | Concentration | Reference item volume | Volume DMSO | |
| C8 LUCINH2 | $5\times10^{-4}$ M | 25 µl Stock solution A LUCINH2 | 75µl- | 1.00 µM |
| C7 LUCINH2 | $1.58\times10^{-4}$ M | 37 µl C8 LUCINH2 | 80 µl | 0.316 µM |
| C6 LUCINH2 | $5\times10^{-5}$ M | 37 µl C7 LUCINH2 | 80 µl | 0.100 µM |
| C5 LUCINH2 | $1.58\times10^{-5}$ M | 37 µl C6 LUCINH2 | 80 µl | 0.0316 µM |
| C4 LUCINH2 | $5\times10^{-6}$ M | 37 µl C5 LUCINH2 | 80 µl | 0.0100 µM |
| C3 LUCINH2 | $1.58\times10^{-6}$ M | 37 µl C4 LUCINH2 | 80 µl | 0.00316 µM |
| C2 LUCINH2 | $5\times10^{-7}$ M | 37 µl C3 LUCINH2 | 80 µl | 0.00100 µM |
| C1 LUCINH2 | $1.58\times10^{-7}$ M | 37 µl C2 LUCINH2 | 80 µl | 0.000316 µM |

Table 5. Dilution scheme with concentrations and suggested volumes of stock solutions of reference item DCNQ for the CTG cytotoxicity assay.

| Reference item | DMSO stock solutions | | | Concentration in CTG assay |
|---|---|---|---|---|
| | Concentration | Reference item volume | Volume DMSO | |
| C8 DCNQ | $50.0\times10^{-2}$ M | 100 µl Stock solution A DCNQ | 100 µl | 100 µM |
| C7 DCNQ | $5.00\times10^{-2}$ M | 20 µl C8 DCNQ | 180 µl | 10.0 µM |
| C6 DCNQ | $3.15\times10^{-3}$ M | 106 µl C7 DCNQ | 62 µl | 6.31 µM |
| C5 DCNQ | $1.99\times10^{-3}$ M | 106 µl C6 DCNQ | 62 µl | 3.98 µM |
| C4 DCNQ | $1.26\times10^{-3}$ M | 106 µl C5 DCNQ | 62 µl | 2.51 µM |
| C3 DCNQ | $7.92\times10^{-4}$ M | 106 µl C4 DCNQ | 62 µl | 1.58 µM |
| C2 DCNQ | $5.00\times10^{-4}$ M | 106 µl C3 DCNQ | 62 µl | 1.00 µM |
| C1 DCNQ | $5.00\times10^{-5}$ M | 20 µl C2 DCNQ | 180 µl | 0.10 µM |

### 6.1.2 Preparation of work solutions

The dilution of the DMSO stock solution is performed in two steps: 1) an intermediate dilution in the assay medium, referred to as the "work solution" and 2) a final dilution when the work solution is added to the plate. Work solutions should be prepared fresh for each assay. In the suggested pipetting scheme (see step 1 for each assay in section 6.2, 6.3 and 6.4, respectively), the DMSO-concentration is the same for all three assays (0.2%), allowing the same DMSO dilution series to be used for all three assays. The final concentration of the test, reference or control item in the assay plate will therefore be 500× lower than the DMSO stock concentration.

## 6.2 AUR TPO Assay

1. Prepare work solutions (62.5× dilution) of test, reference and control items, vehicle control and blanks in 0.2M potassium phosphate buffer, suggested volumes to prepare are presented in Table 6.
   - Avoid bubble formation as far as possible and ensure proper mixing.
   - It is highly important that the VC work solution (used for both VC wells and blank wells, see below) and "MMI C8" work solution is made in sufficiently large amount to be used for all plates in the experiment.

   The work solutions will be further diluted 8× when added to plate (step 9 below), for a final DMSO concentration of 0.2% in plate.

Table 6. Suggestions for volumes of work solutions to prepare, sufficient for testing of 8 test items (3 plates).

| **Test, reference or control ID** | **Amount buffer (µl)** | **Amount DMSO stock (µl)** | **Total volume (µl)** |
|---|---|---|---|
| VC (incl BC1 & BC2) | 1476 µl | 24 µl | 1500 µl |
| MMI C1-C7 | 123 µl | 2 µl | 125 µl |
| MMI C8 | 492 µl | 8 µl | 500 µl |
| PC | 123 µl | 2 µl | 125 µl |
| NC | 123 µl | 2 µl | 125 µl |
| Test item C1 to C8 or r-C1 to r-C6 | 123 µl | 2 µl | 125 µl |
| Test item r-C7 | 246 µl | 4 µl | 250 µl |
| | | **Total (8 test items/3 plates)** | **11125 µl** |

2. For the TPO-free wells (c.f. the plate layout in Figure 2 (range-finding experiment) or Figure 3 (main experiment)), prepare potassium phosphate buffer supplemented with

0.1% sodium deoxycholate in the same concentration as in the TPO-containing wells (c.f step 5 below). Dispense 75µl to each well. Use black solid 96-well plates.

3. Prepare the Amplex UltraRed reagent and 2.4 mM $H_2O_2$ solution (fresh) according to section 4.1.3.3 above. Protect the Amplex UltraRed reagent from light.
4. Thaw TPO extracts and keep cold (< 8°C).
5. *Recommended:* Dilute the TPO extract to 1 µg/µl in 0.1% sodium deoxycholate in DPBS (used for preparation of the TPO extract and prepared according to section 4.1.2 in SOP "Thyroperoxidase (TPO) extract preparation". This strives to make the conditions of the assay more similar between different batches of TPO extracts, since both the sodium deoxycholate content and the ionic strength affects the AUR signal. This dilution step can optionally be performed before cryopreservation of extracts.
6. Dilute the TPO extract in 0.2M potassium phosphate buffer to 0.167 µg/µl (12.5 µg/75 µl per well is required for the assay). E.g., a full plate 1 according to the plate layouts in Figure 2 and Figure 3 will require 6.6 ml and 7.0 ml, respectively, of diluted TPO extract.
7. Dispense AUR working solution, TPO extract diluted in potassium phosphate buffer, and $H_2O_2$ into labelled reagent reservoirs.
8. Use a multichannel (electronic) pipette to dispense 75 µl of diluted TPO extract to all grey-colored wells, according to the plate layout in Figure 2 (range-finding experiment) or Figure 3 (main experiment).
    - Row H in the range-finding experiment does not contain TPO for the test items, in order to check for test item interference (e.g. TPO-independent oxidation of AUR in the presence of $H_2O_2$ or autofluorescence) with the assay reagent.
    - If a test item has demonstrated an interference (Activity(%) > 10 %) in the range-finding experiment, the next experiment (repeat range-finding experiment or main experiment) shall be carried out in duplicate on the same plate: once with TPO and once without TPO (replace another test item on the plate with the experiment without TPO present, in order to evaluate the magnitude of the interference for each test item concentration). In this case, the data needs to be corrected for the interference, c.f. section 8.1.4.
9. Use a multichannel (electronic) or single-channel pipette to dispense 25µl of test and reference item, VC, BC1 or BC2 work solutions per well, according to the plate layout in Figure 2 (range-finding experiment) or Figure 3 (main experiment). Each test or reference item concentration is tested in triplicates.
    - Record test item location on plates and tested concentrations.
10. Use a multichannel (electronic) pipette to dispense 75µl of AUR working solutions to all wells used for the assay.
11. Use a multichannel (electronic) pipette to dispense 25 µl of $H_2O_2$ working solution to each well (except BC1) to initiate the reaction.
12. Incubate the plate for 30 min at 37°C either in the plate reader or in an incubator. Record the date and time of incubation.
13. Read the plate (top read) on the fluorescence plate reader after 30 minutes with an excitation/emission filter set and dichroic mirror suitable for detection of the Amplex UltraRed reagent.
14. Export the data, as appropriate for the equipment/software, and perform data analysis and verification of acceptance criteria according to sections 8 and 9

An overview of the content to be added to each well of the 96-well plate is provided in Table *7*.

Table 7. Content per well of the 96-well plate for the AUR-TPO assay.

| | Amount | Final concentration/amount in well |
|---|---|---|
| Diluted TPO extract | 75µl | 12.5 µg protein |
| Test item, reference item, control item, vehicle control, or blank, work solution | 25µl | 0.2% DMSO in well |
| 67 mM AUR work solution | 75 µl | 25 mM |
| 2.4 mM $H_2O_2$ | 25 µl | 0.3 mM |
| **Total volume of the assay** | **200 µl** | |

| Plate 1 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | MMI C1 | MMI C1 | MMI C1 | TI1 r-C1 | TI1 r-C1 | TI1 r-C1 | TI2 r-C1 | TI2 r-C1 | TI2 r-C1 | VC | VC | BC1 |
| B | MMI C2 | MMI C2 | MMI C2 | TI1 r-C2 | TI1 r-C2 | TI1 r-C2 | TI2 r-C2 | TI2 r-C2 | TI2 r-C2 | VC | VC | BC1 |
| C | MMI C3 | MMI C3 | MMI C3 | TI1 r-C3 | TI1 r-C3 | TI1 r-C3 | TI2 r-C3 | TI2 r-C3 | TI2 r-C3 | VC | VC | BC1 |
| D | MMI C4 | MMI C4 | MMI C4 | TI1 r-C4 | TI1 r-C4 | TI1 r-C4 | TI2 r-C4 | TI2 r-C4 | TI2 r-C4 | VC | VC | BC1 |
| E | MMI C5 | MMI C5 | MMI C5 | TI1 r-C5 | TI1 r-C5 | TI1 r-C5 | TI2 r-C5 | TI2 r-C5 | TI2 r-C5 | NC | PC | BC1 |
| F | MMI C6 | MMI C6 | MMI C6 | TI1 r-C6 | TI1 r-C6 | TI1 r-C6 | TI2 r-C6 | TI2 r-C6 | TI2 r-C6 | NC | PC | BC1 |
| G | MMI C7 | MMI C7 | MMI C7 | TI1 r-C7 | TI1 r-C7 | TI1 r-C7 | TI2 r-C7 | TI2 r-C7 | TI2 r-C7 | NC | PC | BC1 |
| H | MMI C8 | MMI C8 | MMI C8 | TI1 r-C7 | TI1 r-C7 | TI1 r-C7 | TI2 r-C7 | TI2 r-C7 | TI2 r-C7 | BC2 | BC2 | BC2 |

| Plate 2-X | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | TI3 r-C1 | TI3 r-C1 | TI3 r-C1 | TI4 r-C1 | TI4 r-C1 | TI4 r-C1 | TI5 r-C1 | TI5 r-C1 | TI5 r-C1 | VC | MMI C8 | BC1 |
| B | TI3 r-C2 | TI3 r-C2 | TI3 r-C2 | TI4 r-C2 | TI4 r-C2 | TI4 r-C2 | TI5 r-C2 | TI5 r-C2 | TI5 r-C2 | VC | MMI C8 | BC1 |
| C | TI3 r-C3 | TI3 r-C3 | TI3 r-C3 | TI4 r-C3 | TI4 r-C3 | TI4 r-C3 | TI5 r-C3 | TI5 r-C3 | TI5 r-C3 | VC | MMI C8 | BC1 |
| D | TI3 r-C4 | TI3 r-C4 | TI3 r-C4 | TI4 r-C4 | TI4 r-C4 | TI4 r-C4 | TI5 r-C4 | TI5 r-C4 | TI5 r-C4 | VC | MMI C8 | BC1 |
| E | TI3 r-C5 | TI3 r-C5 | TI3 r-C5 | TI4 r-C5 | TI4 r-C5 | TI4 r-C5 | TI5 r-C5 | TI5 r-C5 | TI5 r-C5 | VC | MMI C8 | BC1 |
| F | TI3 r-C6 | TI3 r-C6 | TI3 r-C6 | TI4 r-C6 | TI4 r-C6 | TI4 r-C6 | TI5 r-C6 | TI5 r-C6 | TI5 r-C6 | VC | MMI C8 | BC1 |
| G | TI3 r-C7 | TI3 r-C7 | TI3 r-C7 | TI4 r-C7 | TI4 r-C7 | TI4 r-C7 | TI5 r-C7 | TI5 r-C7 | TI5 r-C7 | VC | MMI C8 | BC1 |
| H | TI3 r-C7 | TI3 r-C7 | TI3 r-C7 | TI4 r-C7 | TI4 r-C7 | TI4 r-C7 | TI5 r-C7 | TI5 r-C7 | TI5 r-C7 | BC2 | BC2 | BC2 |

Figure 2. Plate layout for the AUR-TPO assay, **range finding experiments**. VC: Vehicle control, NC: negative control BC3, PC: positive control PTU, BC1: H2O2 free wells, BC2: TPO-free wells, TIx r-Cy: test item x at 7 different range-finding concentrations, MMI C#: reference item at different concentrations. Grey cells contain the test system (TPO), white cells are without test system

| Plate 1 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | MMI C1 | MMI C1 | MMI C1 | TI1 C1 | TI1 C1 | TI1 C1 | TI2 C1 | TI2 C1 | TI2 C1 | VC | VC | BC1 |
| B | MMI C2 | MMI C2 | MMI C2 | TI1 C2 | TI1 C2 | TI1 C2 | TI2 C2 | TI2 C2 | TI2 C2 | VC | VC | BC1 |
| C | MMI C3 | MMI C3 | MMI C3 | TI1 C3 | TI1 C3 | TI1 C3 | TI2 C3 | TI2 C3 | TI2 C3 | VC | VC | BC1 |
| D | MMI C4 | MMI C4 | MMI C4 | TI1 C4 | TI1 C4 | TI1 C4 | TI2 C4 | TI2 C4 | TI2 C4 | VC | VC | BC1 |
| E | MMI C5 | MMI C5 | MMI C5 | TI1 C5 | TI1 C5 | TI1 C5 | TI2 C5 | TI2 C5 | TI2 C5 | NC | PC | BC1 |
| F | MMI C6 | MMI C6 | MMI C6 | TI1 C6 | TI1 C6 | TI1 C6 | TI2 C6 | TI2 C6 | TI2 C6 | NC | PC | BC1 |
| G | MMI C7 | MMI C7 | MMI C7 | TI1 C7 | TI1 C7 | TI1 C7 | TI2 C7 | TI2 C7 | TI2 C7 | NC | PC | BC1 |
| H | MMI C8 | MMI C8 | MMI C8 | TI1 C8 | TI1 C8 | TI1 C8 | TI2 C8 | TI2 C8 | TI2 C8 | BC2 | BC2 | BC2 |

| Plate 2-X | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | TI3 C1 | TI3 C1 | TI3 C1 | TI4 C1 | TI4 C1 | TI4 C1 | TI5 C1 | TI5 C1 | TI5 C1 | VC | MMI C8 | BC1 |
| B | TI3 C2 | TI3 C2 | TI3 C2 | TI4 C2 | TI4 C2 | TI4 C2 | TI5 C2 | TI5 C2 | TI5 C2 | VC | MMI C8 | BC1 |
| C | TI3 C3 | TI3 C3 | TI3 C3 | TI4 C3 | TI4 C3 | TI4 C3 | TI5 C3 | TI5 C3 | TI5 C3 | VC | MMI C8 | BC1 |
| D | TI3 C4 | TI3 C4 | TI3 C4 | TI4 C4 | TI4 C4 | TI4 C4 | TI5 C4 | TI5 C4 | TI5 C4 | VC | MMI C8 | BC1 |
| E | TI3 C5 | TI3 C5 | TI3 C5 | TI4 C5 | TI4 C5 | TI4 C5 | TI5 C5 | TI5 C5 | TI5 C5 | VC | MMI C8 | BC1 |
| F | TI3 C6 | TI3 C6 | TI3 C6 | TI4 C6 | TI4 C6 | TI4 C6 | TI5 C6 | TI5 C6 | TI5 C6 | VC | MMI C8 | BC1 |
| G | TI3 C7 | TI3 C7 | TI3 C7 | TI4 C7 | TI4 C7 | TI4 C7 | TI5 C7 | TI5 C7 | TI5 C7 | VC | MMI C8 | BC1 |
| H | TI3 C8 | TI3 C8 | TI3 C8 | TI4 C8 | TI4 C8 | TI4 C8 | TI5 C8 | TI5 C8 | TI5 C8 | BC2 | BC2 | BC2 |

Figure 3. Plate layout for the AUR-TPO assay, **main experiment**. If more than 5 test items are tested, plate 3 and subsequent plates are prepared with the same layout as plate 2. VC: Vehicle control, NC: negative control, PC: positive control, BC1: $H_2O_2$ free wells, BC2: TPO-free wells, TIx Cy: test item x at 8 different concentrations selected to cover the AUR-TPO assay response, MMI Cy: reference item at 8 different concentrations.

## 6.3 QLI assay

For the QLI assay, QuantiLum® recombinant luciferase (Promega, cat # E170) is used. The QuantiLum® recombinant luciferase is a 61 kDa monomeric protein that catalyses the oxidation of beetle luciferin with the concomitant production of light.

This assay is performed in white solid 96-well plates. Each test or reference item concentration is tested in triplicates. The highest test concentration of the test item is also tested without luciferase, in order to check for interference with luciferase assay reagent. In case interference is observed, it is highly recommended to perform a control experiment with the full concentration range of the test item and determine case-by-case whether the data can be used with correction for background interference or if the test item shall be reported as incompatible with the QLI control assay.

1. Table 8.
   - Avoid bubble formation as far as possible and ensure proper mixing.
   - It is highly important that the VC work solution (used for both VC wells and blank wells, see below) and "LUCINH2 C8" work solution is made in sufficiently large amount to be used for all plates in the experiment.

   The work solutions will be further diluted 10× when added to plate (step 5 below), for a final DMSO concentration of 0.2% in plate.
2. Thaw QuantiLum® recombinant luciferase on ice. Do not vortex.
3. Dilute the recombinant luciferase to 66.7 ng/ml in 0.2 M PO4 buffer, supplemented with 1mg/ml BSA (c.f. section 4.2.3.1 above).
   - Equilibrate the luciferase to room temperature for 20 minutes before performing an assay. Typically, it is sufficient to ensure that the buffer used for dilution holds room temperature as the dilution from enzyme stock solution is very high.
   - Do not vortex.

Table 8. Suggestions for volumes of work solutions to prepare, sufficient for testing of 8 test items (3 plates) in the QLI and the CTG assays.

| **Test, reference or control ID** | **Amount buffer/ assay medium (µl)** | **Amount DMSO stock (µl)** | **Total volume (µl)** |
|---|---|---|---|
| VC (incl BC) | 490 µl | 10 µl | 500 µl |
| REF C1-C7 | 98 µl | 2 µl | 100 µl |
| REF C8 | 147 µl | 3 µl | 150 µl |
| PC | 98 µl | 2 µl | 100 µl |
| NC | 98 µl | 2 µl | 100 µl |
| Test item C1 to C8 | 98 µl | 2 µl | 100 µl |
| | | **Total (8 test items/3 plates)** | **7950µl** |

4. Use a multichannel (electronic or manual) pipette to dispense 90 µl of the diluted recombinant luciferase per well (6 ng recombinant luciferase) or 0.2M potassium phosphate buffer supplemented with 1 mg/ml BSA only, according to the plate layout in Figure 4. Use white solid 96-well plates.
5. Use a multichannel or single-channel pipette to dispense 10 µl of test and reference item, PC, NC, VC and BC work solutions per well, according to the plate layout in Figure 4. Each test or reference item concentration is tested in triplicates. The highest test concentration of the test item is also tested without luciferase, in order to check for interference with luciferase assay reagent.
   - Record test item location on plates and tested concentrations.

6. Incubate the plate at 37°C in the dark for 25 min and then 5 minutes at room temperature, to mimic the AUR-TPO assay conditions, but still allow for cooling to room temperature where the luminescence signal is under more optimal conditions. Record the date and time of incubation .
7. Prepare the Luciferase Assay reagent according to section 4.2.3.2 above. Make sure the reagent is equilibrated to room temperature before use. Prime the dispenser of the plate reader with the luciferase assay reagent.
8. Read the plate without lid on the luminescence plate reader after 30 minutes total incubation. The read should be performed well by well by first injecting 50 µl of the assay reagent to the well, followed by measurement of emitted light (integration time depends on the instrument).
9. Export the data, as appropriate for the equipment/software, and perform data analysis and verification of acceptance criteria according to sections 8 and 9.

| Plate 1 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | LUCINH2 C1 | LUCINH2 C1 | LUCINH2 C1 | TI1 C1 | TI1 C1 | TI1 C1 | TI2 C1 | TI2 C1 | TI2 C1 | VC | VC | VC |
| B | LUCINH2 C2 | LUCINH2 C2 | LUCINH2 C2 | TI1 C2 | TI1 C2 | TI1 C2 | TI2 C2 | TI2 C2 | TI2 C2 | VC | VC | VC |
| C | LUCINH2 C3 | LUCINH2 C3 | LUCINH2 C3 | TI1 C3 | TI1 C3 | TI1 C3 | TI2 C3 | TI2 C3 | TI2 C3 | NC | NC | NC |
| D | LUCINH2 C4 | LUCINH2 C4 | LUCINH2 C4 | TI1 C4 | TI1 C4 | TI1 C4 | TI2 C4 | TI2 C4 | TI2 C4 | PC | PC | PC |
| E | LUCINH2 C5 | LUCINH2 C5 | LUCINH2 C5 | TI1 C5 | TI1 C5 | TI1 C5 | TI2 C5 | TI2 C5 | TI2 C5 | BC | BC | BC |
| F | LUCINH2 C6 | LUCINH2 C6 | LUCINH2 C6 | TI1 C6 | TI1 C6 | TI1 C6 | TI2 C6 | TI2 C6 | TI2 C6 | BC | BC | BC |
| G | LUCINH2 C7 | LUCINH2 C7 | LUCINH2 C7 | TI1 C7 | TI1 C7 | TI1 C7 | TI2 C7 | TI2 C7 | TI2 C7 | TI1 C8 | TI1 C8 | TI1 C8 |
| H | LUCINH2 C8 | LUCINH2 C8 | LUCINH2 C8 | TI1 C8 | TI1 C8 | TI1 C8 | TI2 C8 | TI2 C8 | TI2 C8 | TI2 C8 | TI2 C8 | TI2 C8 |

| Plate 2-X | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | TI3 C1 | TI3 C1 | TI3 C1 | TI4 C1 | TI4 C1 | TI4 C1 | TI5 C1 | TI5 C1 | TI5 C1 | VC | VC | VC |
| B | TI3 C2 | TI3 C2 | TI3 C2 | TI4 C2 | TI4 C2 | TI4 C2 | TI5 C2 | TI5 C2 | TI5 C2 | VC | VC | VC |
| C | TI3 C3 | TI3 C3 | TI3 C3 | TI4 C3 | TI4 C3 | TI4 C3 | TI5 C3 | TI5 C3 | TI5 C3 | LUCINH2 C8 | LUCINH2 C8 | LUCINH2 C8 |
| D | TI3 C4 | TI3 C4 | TI3 C4 | TI4 C4 | TI4 C4 | TI4 C4 | TI5 C4 | TI5 C4 | TI5 C4 | BC | BC | BC |
| E | TI3 C5 | TI3 C5 | TI3 C5 | TI4 C5 | TI4 C5 | TI4 C5 | TI5 C5 | TI5 C5 | TI5 C5 | BC | BC | BC |
| F | TI3 C6 | TI3 C6 | TI3 C6 | TI4 C6 | TI4 C6 | TI4 C6 | TI5 C6 | TI5 C6 | TI5 C6 | TI3 C8 | TI3 C8 | TI3 C8 |
| G | TI3 C7 | TI3 C7 | TI3 C7 | TI4 C7 | TI4 C7 | TI4 C7 | TI5 C7 | TI5 C7 | TI5 C7 | TI4 C8 | TI4 C8 | TI4 C8 |
| H | TI3 C8 | TI3 C8 | TI3 C8 | TI4 C8 | TI4 C8 | TI4 C8 | TI5 C8 | TI5 C8 | TI5 C8 | TI5 C8 | TI5 C8 | TI5 C8 |

Figure 4. Plate layout for the QLI. If more than 5 test items are tested, plate 3 and subsequent plates are prepared with the same layout as plate 2. VC: Vehicle control, NC: negative control, PC: positive control, BC: blank, i.e. test system-free wells, TIx Cy: test item x at 8 different concentrations, REF Cx: Reference item (LUCINH2) at 8 different concentrations. Grey wells contain the test system (luciferase), white cells are without the test system (contain buffer instead).

## 6.4 CTG assay

This assay is performed in white 96-well plates with clear bottom. Each test or reference item concentration is tested in triplicates. The highest test concentration of the test item is also tested with only assay medium and no cells present, in order to check for interference with the assay reagent. In case interference is observed, it is highly recommended to perform a control experiment with the full concentration range of the test item with and without cells, and determine case-by-case whether the data can be used with correction for background interference, or if the cytotoxicity of the test item shall only be evaluated through microscopic observation of each well. Similarly, if cytotoxicity is not observed by microscopic evaluation but a decrease in luminescence signal is observed, this might also be due to interference of the test item with the test system. In that case, it shall be determined on a case-by-case basis whether absence of cytotoxicity can be determined only by microscopic evaluation or whether another cytotoxicity assay with a different readout than luminescence shall be employed for the test item and the reference item in parallel. In such case this should be clearly described in the report. Interference with the luciferase reagent can be verified in a control experiment using ATP, c.f. the manufacturer's manual for the CellTiter-Glo® reagent.

The plate layout suggested below uses all wells of the 96-well plate, which requires that edge effects are absent. This shall be verified during assay implementation, e.g. by seeding a full plate with cells and exposure to the vehicle control only, and comparing the luminescence from the surrounding wells and the inner wells.

Table 9. CTG cytotoxicity test work flow - overview.

| | |
|---|---|
| **Day 1** | • Prepare work solutions of test, reference and control items as well as vehicle control and blank.<br>• Seed 96-well plates: $8\times10^3$ cells/90µl assay medium per well<br>• Add work solutions (10 µl/well) to the 96-well plates according to specified plate layout .<br>• Incubate (37°C, 5% $CO_2$) for 24±0.5h |
| **Day 2** | • Microscopic evaluation of cell growth<br>• Add Cell Titer glow reagent (100 µl) to the 96-well plates<br>• Detect luminescence using a plate reader |

### 6.4.1 Before the test – grow up FTC-238 cells from stock

Details regarding maintenance and handling of FTC-238 cells are found in .

After thawing from stock, subcultivate the cells using complete culture medium 2 to 7 times before using the cells in the test). Verify that the cell growth, viability and morphology is as expected before using the cells in the test. The cell cultures may not be overgrown.

### 6.4.2 1st day after growing up the cells from frozen stock

1. Prepare work solutions (50× dilution) of test, reference and control items, vehicle control and blanks in Complete assay medium 1× FTC CTG (c.f. section 4.3.3.1 above), suggested volumes to prepare are presented in
2. Table *8*.
    - Avoid bubble formation as far as possible and ensure proper mixing.
    - It is highly important that the VC work solution (used for both VC wells and blank wells, see below) and "DCNQ C8" work solution are made in sufficiently large amounts to be used for all plates in the experiment.

    The work solutions will be further diluted 10× when added to plate (step 13 below), for a final DMSO concentration of 0.2% in plate.
3. Check the culture macroscopically and microscopically for microbial contamination.
4. Evaluate cell morphology with the microscope and record observations .
5. 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.
6. Briefly rinse the cell layer with sterile DPBS (e.g. 20-30 ml for a T150 flask) to remove all traces of serum.
7. 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.
8. Add complete assay medium (or 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.
9. Collect cells by gentle centrifugation (200 g, 3 min).

10. Resuspend the cells Complete assay medium 1× FTC CTG (e.g. for a T150 flask, 5 ml is usually suitable).
11. Remove ~100µl for counting and adjust the density to 8000 viable cells/well (88889 cells/ml) in Complete assay medium 1× FTC CTG.
12. Using a multichannel pipette, dispense 90 µl of cell suspension or Complete assay medium 1× FTC CTG only in white 96-well plates with transparent bottom according to Figure 5
13. Use a multichannel or single-channel pipette to dispense 10µl of test and reference item, PC, NC, VC and BC work solutions per well, according to the plate layout in Figure 5. Each test or reference item concentration is tested in triplicate. The highest test concentration of the test item is also tested without cells (assay medium only), in order to check for interference with the CellTiter-Glo® Reagent.
    - Record test item location on plates and tested concentrations.
14. Culture cells for 24±2 h (5±1 % $CO_2$, 37±1 °C, > 90 % humidity). Record the date and time of incubation.

| Plate 1 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | DCNQ C1 | DCNQ C1 | DCNQ C1 | TI1 C1 | TI1 C1 | TI1 C1 | TI2 C1 | TI2 C1 | TI2 C1 | VC | VC | VC |
| B | DCNQ C2 | DCNQ C2 | DCNQ C2 | TI1 C2 | TI1 C2 | TI1 C2 | TI2 C2 | TI2 C2 | TI2 C2 | VC | VC | VC |
| C | DCNQ C3 | DCNQ C3 | DCNQ C3 | TI1 C3 | TI1 C3 | TI1 C3 | TI2 C3 | TI2 C3 | TI2 C3 | NC | NC | NC |
| D | DCNQ C4 | DCNQ C4 | DCNQ C4 | TI1 C4 | TI1 C4 | TI1 C4 | TI2 C4 | TI2 C4 | TI2 C4 | PC | PC | PC |
| E | DCNQ C5 | DCNQ C5 | DCNQ C5 | TI1 C5 | TI1 C5 | TI1 C5 | TI2 C5 | TI2 C5 | TI2 C5 | BC | BC | BC |
| F | DCNQ C6 | DCNQ C6 | DCNQ C6 | TI1 C6 | TI1 C6 | TI1 C6 | TI2 C6 | TI2 C6 | TI2 C6 | BC | BC | BC |
| G | DCNQ C7 | DCNQ C7 | DCNQ C7 | TI1 C7 | TI1 C7 | TI1 C7 | TI2 C7 | TI2 C7 | TI2 C7 | TI1 C8 | TI1 C8 | TI1 C8 |
| H | DCNQ C8 | DCNQ C8 | DCNQ C8 | TI1 C8 | TI1 C8 | TI1 C8 | TI2 C8 | TI2 C8 | TI2 C8 | TI2 C8 | TI2 C8 | TI2 C8 |

| Plate 2-X | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | TI3 C1 | TI3 C1 | TI3 C1 | TI4 C1 | TI4 C1 | TI4 C1 | TI5 C1 | TI5 C1 | TI5 C1 | VC | VC | VC |
| B | TI3 C2 | TI3 C2 | TI3 C2 | TI4 C2 | TI4 C2 | TI4 C2 | TI5 C2 | TI5 C2 | TI5 C2 | VC | VC | VC |
| C | TI3 C3 | TI3 C3 | TI3 C3 | TI4 C3 | TI4 C3 | TI4 C3 | TI5 C3 | TI5 C3 | TI5 C3 | DCNQ C8 | DCNQ C8 | DCNQ C8 |
| D | TI3 C4 | TI3 C4 | TI3 C4 | TI4 C4 | TI4 C4 | TI4 C4 | TI5 C4 | TI5 C4 | TI5 C4 | BC | BC | BC |
| E | TI3 C5 | TI3 C5 | TI3 C5 | TI4 C5 | TI4 C5 | TI4 C5 | TI5 C5 | TI5 C5 | TI5 C5 | BC | BC | BC |
| F | TI3 C6 | TI3 C6 | TI3 C6 | TI4 C6 | TI4 C6 | TI4 C6 | TI5 C6 | TI5 C6 | TI5 C6 | TI3 C8 | TI3 C8 | TI3 C8 |
| G | TI3 C7 | TI3 C7 | TI3 C7 | TI4 C7 | TI4 C7 | TI4 C7 | TI5 C7 | TI5 C7 | TI5 C7 | TI4 C8 | TI4 C8 | TI4 C8 |
| H | TI3 C8 | TI3 C8 | TI3 C8 | TI4 C8 | TI4 C8 | TI4 C8 | TI5 C8 | TI5 C8 | TI5 C8 | TI5 C8 | TI5 C8 | TI5 C8 |

Figure 5. Plate layout for the CTG assay. If more than 5 test items are tested, plate 3 and subsequent plates are prepared with the same layout as plate 2. VC: Vehicle control, NC: negative control, PC: positive control, BC: blank, i.e. test system-free wells, TIx Cy: test item x at 8 different concentrations, DCNQ Cx: Reference item DCNQ at 8 different concentrations. Grey wells contain the test system (FTC-238 cells), white cells are without the test system (contain assay medium instead).

### 6.4.3 2nd day – cytotoxicity determination

1. Thaw the CellTiter-Glo® Buffer and equilibrate to room temperature. For convenience the CellTiter-Glo® Buffer may be thawed and stored at room temperature for up to 48 hours prior to use.
2. Prepare the CellTiter-Glo® Reagent, according to section 4.3.3.2.
3. After 24±0.5h culture, examine each plate under an inverted phase contrast microscope to identify systematic cell seeding errors and growth characteristics of cells. Look for changes in morphology of the cells due to cytotoxic effects of the test item. Also note whether cytotoxic effects are absent from the test item, in order to rule out cases where the test item interferes with the luciferase reagent causing loss of signal that is not related to cytotoxicity. Undesirable growth characteristics of cells exposed to vehicle control and reference/control items can indicate experimental error and can be cause for rejection of the assay. Also check for cloudy wells as an indicator of contamination and verify solubility of the test item. In case of contamination, the whole plate should be disregarded, and in case of insolubility of the test item all test results for this concentration should be disregarded.
    - Record visual signs of cytotoxicity, contamination, insolubility or operator errors.
    - For test items, record presence or absence of cytotoxicity.
4. Let the plates reach room temperature (approx. 30 min outside incubator) and then add 100 µl of the Cell titer glow® solution to each test well.
5. Shake the microtiter plate on a microtiter plate shaker until complete cell lysis, 4 minutes at a setting of 130 rpm is usually sufficient.
6. Allow the plates to incubate (in dark) at room temperature for 10 minutes to stabilize luminescent signal.
10. Measure luminescence with the plate reader (without lid). Integration time depends on the instrument.
11. Export the data, as appropriate for the equipment/software, for data analysis and verification of acceptance criteria according to sections 8 and 9.

# 7 Selection of concentrations for main experiment

Following measurement of the fluorescence signal, calculation of Activity (%), Inhibition (%), and verification of acceptance criteria of the range finding AUR-TPO experiment(s), the highest concentration of the test item (C8), to be used for the main testing, shall be selected.

- Concentrations showing insolubility during the preparation of stock or work solutions for range-finding experiments, as determined by visual inspection, shall not be selected for further testing.
- Verify the dose response. When the test item does not show a full dose response curve in the lower concentration range, with at least two concentrations resulting in signals at the lowest level, repeat the range-finding experiment with a lower C8 concentration or alternatively a larger dilution factor.

1. If there is at least 1 concentration (that is not suspected to be an outlier) where the mean Inhibition (%) ≥ 20%, identify the concentration giving maximum inhibition (Figure 6).
    i. Select this concentration multiplied by the dilution factor (selected in step ii below) to be used as the highest concentration in the main experiment (=C8).
        - If the resulting C8 concentration is not soluble, choose the highest verified soluble concentration as C8.
        - If the concentration giving maximum inhibition is the maximum test concentration 200 µM, choose 200 µM as C8.
    ii. If there is at least one concentration where the mean Inhibition (%) ≥ 70%, the suggested dilution factor (DF) to use is 4; if < 70% DF=2 is suggested. Verify that the suggested dilution factor will capture as much as possible of the dose response curve (zero level is reached for at least two concentrations) and sufficient data points are in the region of interest (the slope of the Hill curve) in the main experiment, otherwise adjust the dilution factor, e.g.:
        - If the Inhibition(%) dose-response curve will not reach zero-level for at least two concentrations; increase the dilution factor.
        - If the Inhibition(%) dose-response curve is very steep in a narrow concentration range, consider using different dilution factors in different parts of the curve.
2. If Inhibition (%) is < 20% for all tested concentrations AND the assay interference control (highest test item concentration r-C7 tested without the test system) has an Activity (%) ≤ 10 %, repeat the range-finding experiment.
3. If Inhibition (%) is < 20% for all tested concentrations but the assay interference control (highest test item concentration r-C7 tested without the test system) has an Activity (%) > 10 % correct the Activity (%) and Inhibition (%) for r-C7 according to section 8.1.4.
    i. If the $\text{Inhibition}_{\text{corr}}$ (%) for r-C7 is ≥20%, select the C8 concentration and dilution factor according to step 1 above and perform a main experiment. In addition, test the entire dilution series without the test system being present.
    ii. If the corrected Inhibition (%) for r-C7 is <20%, repeat the range-finding experiment in duplicate: with and without the test system.

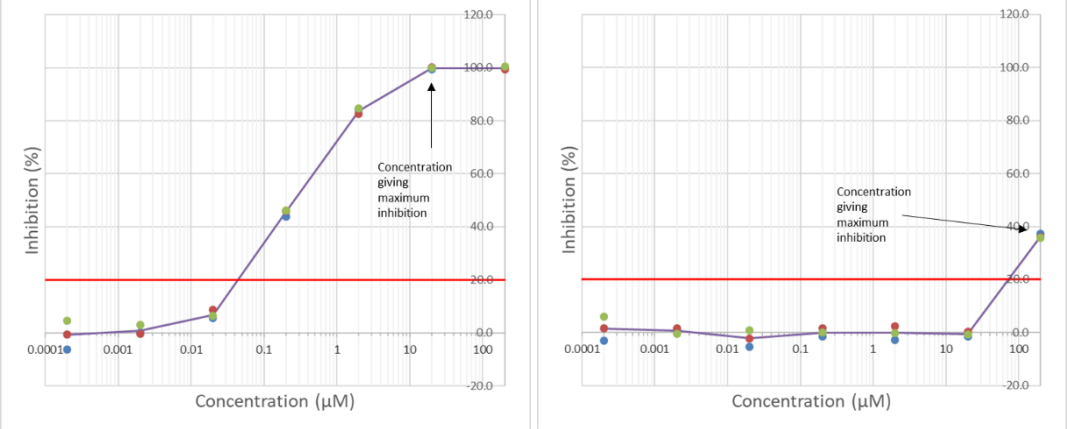


Figure 6. Graphs illustrating identification of concentrations giving maximum inhibition and selection of dilution factors. For the left experiment the dilution factor shall be 4, since the maximum induction is ≥ 70%, and the C8-concentration for the main experiment shall be chosen as 4× the concentration giving maximum induction. For the right experiment, the r-C7 (200 µM) concentration shall be selected as C8 in the main experiment, with a dilution factor of 2, since the maximum induction is < 70%.

# 8 Data analysis

## 8.1 AUR TPO Assay

### 8.1.1 Dose-response modelling

The Activity (%) for each well on the plate is calculated according to:

$$Activity\ (\%) = 100 \times \frac{(RFU_x - \overline{RFU_{BC1}})}{\overline{RFU_{VC}} - \overline{RFU_{BC1}}}, \quad (1)$$

Where $RFU_x$ is the raw fluorescence value for well x (containing e.g. the test, reference or control item), and $\overline{RFU_{VC}}$ and $\overline{RFU_{BC1}}$ are the corresponding mean values for replicate wells of the VC (vehicle control) and BC1 (lacking $H_2O_2$) wells, respectively, on the same plate.

The Inhibition (%) is then calculated as:

$$Inhibition\ (\%) = 100 - Activity\ (\%) \quad (2)$$

From the Inhibition (%) values the mean and standard deviation of all replicates shall be calculated for each test, reference or control item concentration, as well as blanks and vehicle controls.

For a main experiment a variable slope Hill model with four parameters should be fitted to the Inhibition (%) data for each test and reference item, using e.g. GraphPad Prism, R or other suitable software:

$$y = Bottom + \frac{Top - Bottom}{1 + 10^{(\log AC50 - x)HillSlope}} \quad (3)$$

where *x* is the log concentration, *Top* is the maximum inhibition, Bottom is the minimum inhibition, logAC50 is the logarithm (base 10) of the $AC_{50}$, i.e. the concentration where the test/reference item reaches 50% of its maximum response (AC = activity concentration), and *HillSlope* is the Hill coefficient.

### 8.1.2 Parameters for the test item

The following point-of-departure (POD) estimates should be determined for each of the three assays:

$AC_{50}$ (one of the fitted model parameters)
$AC_{20}$ (calculated from the fitted model parameters and equation), i.e. the activity concentration at cutoff in µM

In addition, the maximum mean inhibition among the different test concentrations:

$$Inhibition\_max = \max(\overline{Inhibition(\%)_{Ci}}), \quad (4)$$

where i = 1-8.

### 8.1.3 Quality parameters

Assay quality parameters are calculated for each plate.

The TPO efficiency for the extracts used for the AUR-TPO assay is calculated separately for each plate as:

$$TPO\ efficiency = \frac{\overline{RFU_{VC}}}{\overline{RFU_{BC2}}}, \quad (5)$$

Where $RFU_{BC2}$ are the raw fluorescence values of the BC2 (TPO-free wells) and the mean is taken over all replicates on the same plate.

Similarly, the plate dynamic range is calculated as:

$$Plate\ dynamic\ range = \frac{\overline{RFU_{VC}}}{\overline{RFU_{BC1}}}. \quad (6)$$

The Z factor is calculated for each plate using the Inhibition (%) data according to:

$$Z = 1 - \frac{3(\sigma_{REF\ C8} + \sigma_{VC})}{\left|\overline{Inhibition(\%)_{REF\ C8}} - \overline{Inhibition(\%)_{VC}}\right|} \quad (7)$$

where $\sigma_{REF\ C8}$ is the standard deviation of the reference item at the highest test concentration (C8) and $\sigma_{VC}$ is the corresponding value for the VC, $\overline{Inhibition(\%)_{REF\ C8}}$ is the mean of the reference item at the highest concentration (C8), and $\overline{Inhibition(\%)_{VC}}$ is the mean of the vehicle control (per definition =0).

### 8.1.4 Correction for assay interference

If the assay interference control in the AUR-TPO range-finder experiment (highest test item concentration r-C7 tested without the test system) has an Activity (%) > 10, the Activity (%) and Inhibition (%) needs to be corrected for the interference, both for the r-C7 concentration in the range-finding experiment and for all test item concentrations in subsequent experiments:

1. Calculate the corrected test item Activity (%) for each well of each concentration $C_i$ ($i$=1-8), according to the following formula:

$$Activity_{corr}(\%)_{C_i,x} = Activity(\%)_{C_i,x} - \left(\overline{Activity(\%)_{C_i,no\ TPO}} - \overline{Activity(\%)_{BC2}}\right) \quad (8)$$

   Where $Activity(\%)_{C_i,x}$ is the activity calculated according to Equation (1), and $\overline{Activity(\%)_{C_i,no\ TPO}}$ is the mean activity of the three corresponding wells with test item at the same concentration but without TPO present, and $\overline{Activity(\%)_{BC2}}$ is the mean activity of BC2. Set all corrected Activities (%) ≥ 100% to 100%. Calculate the mean Activity_corr (%) for each test item concentration.
2. Calculate the corrected Inhibition (%) for each well of test item at concentration $C_i$ according to

$$Inhibition_{corr}(\%)_{C_i,x} = 100 - Activity_{corr}(\%)_{C_i,x} \quad (9)$$

   Use the corrected Inhibition (%) for curve-fitting and calculation of parameters for the test item.

### 8.2 QLI and CTG assays

For the QLI and CTG assays, the raw luminescence units (RLUs) are normalized in the same manner as for the AUR-TPO assay (BC corresponds to 100% inhibition/cytotoxicity and VC is the zero level). Further, inhibition/cytotoxicity is fitted using the same Hill model as described for the AUR-TPO assay, and corresponding parameters (except for TPO activity) are calculated. Plate dynamic range and Z-factors are also calculated for each plate.

## 9 Acceptance criteria

Table 9 summarizes the acceptance criteria for the AUR-TPO assay, while tables 10-11 summarizes the acceptance criteria for the control assays QLI and CTG, respectively.

For the AUR-TPO assay, each newly prepared TPO extract must pass acceptance criteria number 1 and 2 before the TPO extract is used in a real experiment (c.f. section 5). Acceptance criteria number 1-7 must be met for the first plate of each experiment, for the entire experiment to be considered valid. In addition, acceptance criteria number 5-7 must be met for each individual plate of the experiment for the plate to be considered valid (individual test items that does not fulfill criterium 7 will be considered invalid, without rejecting the entire plate).

Similarly acceptance criteria 1-5 must be met for the first plate in the experiment, for the QLI and CTG assays, respectively, and each plate must fulfill acceptance criteria 4 and 5 for a plate to considered valid (number 5 may exclude just a test item, not the entire plate).

**Table 9.** Acceptance criteria for the AUR-TPO assay.

| Number | Acceptance criteria | |
|---|---|---|
| | Parameter/description | Requirement |
| 1 | TPO efficiency (ratio between VC and BC2) | >3 |
| 2 | MMI $AC_{50}$ | $8.2*10^{-9}$ – $1.4*10^{-6}$ M |
| 3 | Inhibition (%) for PC PTU 25 µM | > 50 |
| 4 | Inhibition (%) for NC BP3 100 µM | < 10 |
| 5 | Z-factor for MMI C8 | ≥ 0.5 |
| 6 | Plate dynamic range (ratio between VC and BC1) | >2 |
| 7 | Standard deviation of Inhibition (%) for each replicate of vehicle control, blanks, reference, control or test items on each plate | ≤ 20% |

**Table 10.** Acceptance criteria for the QLI assay.

| Number | Acceptance criteria | |
|---|---|---|
| | Parameter/description | Requirement |
| 1 | LUCINH2 $AC_{50}$ | $2.0*10^{-9}$ – $2.0*10^{-8}$ M |
| 2 | Inhibition (%) for PC LUCINH1 | > 80 |
| 3 | Inhibition (%) for NC BP3 10 µM | < 20 |
| 4 | Z for LUCINH2 C8 | ≥ 0.5 |
| 5 | Standard deviation of Inhibition (%) for each replicate of vehicle control, blank, reference, control or test items on each plate | ≤ 20% |

**Table 11.** Acceptance criteria for the CTG assay.

| Number | Acceptance criteria | |
|---|---|---|
| | Parameter/description | Requirement |
| 1 | DCNQ $AC_{50}$ | $1.5*10^{-6}$ – $1.05*10^{-5}$ |
| 2 | Cytotoxicity (%) for PC DCNQ 10 µM | > 70 |
| 3 | Cytotoxicity (%) for NC BP3 100 µM | < 20 |
| 4 | Z factor for DCNQ C8 | ≥ 0.5 |
| 5 | Standard deviation of Inhibition (%) for each replicate of vehicle control, blank, reference, control or test items on each plate | ≤ 20% |

Additional acceptance criterion for individual test items (in any of the assays):

- Maximum two concentrations may be excluded from the test item or reference item dilution series, on basis of operator errors or other information (including requirement for standard deviation of triplicates).

If the mean Inhibition (%) at the lowest concentration C1 for of the test or reference item is > 20% (and the sigmoid seems to have reached the bottom level), try to identify the cause and its impact on the data and calculation of parameters.

# 10 Classification of test items

Provided that two independent concordant experiments have been performed (two range-finding experiments or one range-finding experiment followed by one main experiment):

The test item is classified as Negative (not a TPO inhibitor) if:

- The Inhibition (%) (or $Inhibition_{corr}$ (%), in case of a test item interference with the assay) is < 20% for all tested concentrations, in two concordant range-finding experiments.

If Inhibition (%) (or $Inhibition_{corr}$ (%), in case of a test item interference with the assay) is ≥ 20% for any test item concentration, a selectivity value for each test is calculated from the main experiment according to

$$Selectivity = \min\left(\log\left(AC_{20,CTG}\right), \log\left(AC_{20,QLI}\right), 3\right) - \log\left(AC_{20,AUR}\right)$$

where the $AC_{20}$ concentrations are given in units of µM.

The test item is classified as Positive (a TPO inhibitor) if Selectivity > 0, Negative (unspecific inhibitor) otherwise.

# 11 Record keeping and reporting

At a minimum, records should be kept for

- Chemicals, media and reagents used
- Equipment and consumables used
- Solubility information, weighing and dilution of test, reference and control items
- AUR-TPO experiments:
  - TPO extract batch number
  - Test, reference and control items location on plates and tested concentrations
  - Date and time of incubation
  - Documentation of fluorescence measurements and subsequent calculations
  - Fulfilment of acceptance criteria
- QLI experiments:
  - Test, reference and control items location on plates and tested concentrations
  - Date and time of incubation
  - Documentation of luminescence measurements and subsequent calculations
  - Fulfilment of acceptance criteria
- CTG experiments:
  - 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
  - Test, reference and control items location on plates and tested concentrations
  - Date and time of incubation
  - Documentation of luminescence measurements and subsequent calculations
  - Fulfilment of acceptance criteria

Forms and templates available are listed below, for reference.

Documentation of test system handling, logging of raw data and analysis, use of equipment, usage of reagents and consumables, etc.. should be performed according to the test facility's internal procedures. The following forms and templates are available to ensure that study specific details of the experimental work are well documented:

- AUR-TPO study log

  This Microsoft Excel template is used to document most of the details regarding the study. It contains 4 tabs: "test item preparation" where details regarding identity, weighing and dissolution of test, reference and control items are recorded; "AUR-TPO" where all steps of the study are logged and which automatically calculates required volumes for e.g. stock solutions; "QLI", where the details of the QuantiLum® luciferase inhibition assay are recorded; and "CTG", where all details of the Cell Titer Glo cytotoxicity test are recorded.

# 12 Revision history of SOP

| Edition* | Date | Author | Short information about the changes |
|---|---|---|---|
| 1.0 | 2021-10-20 | KF | • Changed volumes (but not concentrations) in Table 3, to be able to use pipettes in their higher volume range where they are more accurate.<br>• Corrected calculations of total volumes in Tables 6 and 8.<br>• Under "Classification of test items": changed "false positive" to "unspecific inhibitor"<br>• Changed acceptance criteria #6, plate dynamic range, from >3 to >2.<br>• Fixed acceptance criteria #2, MMI $AC_{50}$, to $8.2*10^{-9}$ – $1.4*10^{-6}$ M, corresponding to historical mean ± 3 SD based on data from implementation and Study 1 at RISE.<br>• Under "Selection of concentrations for main experiment", added requirement that zero level should be reached for at least two concentrations, to enable determination of baseline level and accurate curve fitting.<br>• Minor spell checks and correction of typos. |

* 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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