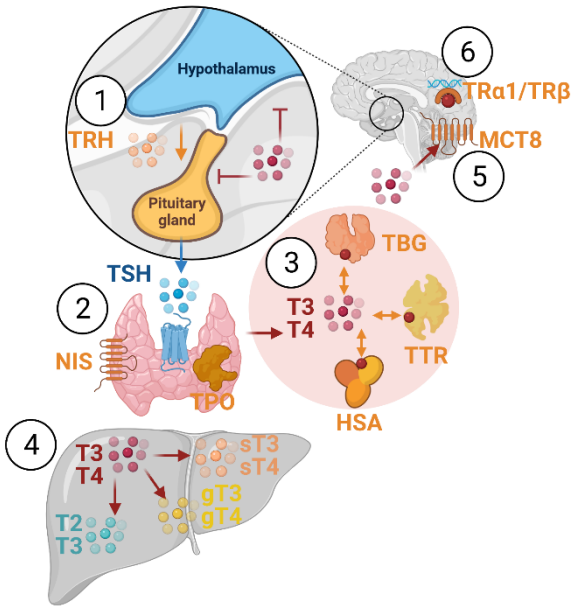
European Commission

STUDY REPORT

for the thyroperoxidase activity assay with Amplex UltraRed (AUR-TPO) – Part 1

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 study report 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 study report describes the experimental design and includes data generated in Part 1 of the validation study. The method was developed by US EPA and subsequently implemented by the EU-NETVAL test facility RISE (Sweden) within the validation study.

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REPORT

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Robustness and reliability assessment of the Thyroperoxidase Activity Assay with Amplex UltraRed (AUR-TPO)

(4 appendices)

Purpose and applicability

The purpose of this study was to assess the robustness and reliability of the AUR-TPO *in vitro* method by generating data in five valid runs for 6 test items provided by the sponsor, together with reference and control items. The method is described in SOP KM 20077 "Thyroperoxidase activity assay with Amplex UltraRed (AUR-TPO)". The method was developed for the detection of compounds with ability to inhibit the enzyme thyroperoxidase, thus acting as thyroid disruptors.

Study ID: 8P06603:A

Assignment

Generation of experimental data for 6 test items according to the AUR-TPO *in vitro* method described in SOP KM 20077 "Thyroperoxidase activity assay with Amplex UltraRed (AUR-TPO)". The testing was further specified at RISE in standard operating procedures SOP KM 19207 "Culture of FTC 238 and FTC 238/hrTPO cells", SOP KM 20168 "Thyroperoxidase (TPO) extract preparation", and SOP KM 17783 "Solubility determination by visual inspection".

Test description

Cellular extracts of recombinant FTC-238 cells overexpressing human thyroperoxidase (TPO) are exposed to the test items in presence of the fluorogenic substrate Amplex® UltraRed (AUR) and an excess of hydrogen peroxide. Functional thyroperoxidase converts Amplex® UltraRed to fluorescent Amplex® Ultroxred, which can be measured with a fluorimeter. Test items with an endocrine disruptive effect, able to impair thyroperoxidase function, will give rise to a decrease in signal in the assay. In addition to this assay, test items that show an effect are further evaluated in a separate control assay utilising recombinant luciferin and measuring light emitted by luciferase, to verify that the inhibition shown in the AUR-TPO assay is specific to thyroperoxidase and not a general enzyme inhibiting effect.

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Study dates

Study initiation date: 2021-03-22
Experimental starting date: 2021-03-22
Experimental completion date: 2021-07-01

Test items

Information regarding the test items is summarized in Table 1.

Table 1. Test item details.

Chemical name; RISE ID	Lot /batch number	Purity	Storage	Expiry date	Physical state	Hazard label
MMI; 8P06603:1	WXBC8588V	99.7 %	RT	No exp date – responsibility of sponsor	Solid	H317, H361
BP2; 8P06603:2	MKBN6515V	98.6 %	RT	No exp date – responsibility of sponsor	Solid	H302, H315, H319, H335
BP3; 8P06603:3	WXBC6458V	99.8 %	RT	No exp date – responsibility of sponsor	Solid	H315, H319, H335
Diethyl phthalate; 8P06603:4	BCBW5091	99.7 %	RT	No exp date – responsibility of sponsor	Liquid	None
Genistein; 8P06603:5	REIYE	99.3 %	2-8°C	No exp date – responsibility of sponsor	Solid	None
PTU; 8P06603:6	BCBR8708V	99.2 %	RT	No exp date – responsibility of sponsor	Solid	H302, H351

Date of test items arrival at RISE: 2019-01-22 (all except PTU), 2020-05-26 (PTU)

Disposal of test items: Test items will be kept until the completion of the study. Thereafter all materials will be destroyed unless return is requested by the sponsor.

Materials

Reference and control items

Reference item for the AUR-TPO in vitro method is methimazole (MMI). Positive control for the assay is propylthiouracil (PTU) and negative control is benzophenone-3 (BP3). Reference item for the control assay Quantilum inhibition (QLI) is luciferase inhibitor II (Lucinh2). Positive control for the QLI control assay is luciferase inhibitor I (Lucinh1) and negative control is benzophenone-3 (BP3). Reference item and positive control for the cytotoxicity control assay is dicyano-1,4-naphthoquinone (DCNQ). Negative control is benzophenone-3 (BP3). The chemicals are provided by the sponsor, except PTU and DCNQ which were purchased by RISE. Details regarding the chemicals are listed in Table 2.

Table 2. Reference and control item details for AUR-TPO and associated control assays.

Chemical name	Lot /batch number	Purity	Storage	Expiry date	Physical state	Hazard label
MMI	WXBC8588V	99.7 %	RT	No exp date – responsibility of sponsor	Solid	H317, H361
LUCINH2	3445953 3492236, 3574625	97.93 %	2-8°C	07 NOV 2023	Solid	None
DCNQ	A0397754	99.9 %	RT	06/2023	Solid	H302, H315, H319
PTU	BCBR8708V	99.2 %	RT	Release date 06 APR 2016	Solid	H302, H351
BP3	WXBC6458V	99.8 %	RT	No exp date – responsibility of sponsor	Solid	H315, H319, H335
LUCINH1	3386894, 3482937	98.11 %	2-8°C	21 OCT 2027	Solid	None

Reagents

- Amplex UltraRed reagent (Thermo Fisher cat # A36006)
- KH_2PO_4, CAS 7778-77-0, Potassium phosphate monobasic, 99% (Sigma (Merck) cat # P5379)
- K_2HPO_4, CAS 7758-11-4, Potassium phosphate dibasic, 98% (Sigma (Merck) cat # P3786)
- Anhydrous DMSO (Sigma (Merck) cat # 276855)
- Hydrogen peroxide solution, 30% (w/w) in H2O, CAS 7722-84-1, (Sigma (Merck) cat # H1009)
- Sodium deoxycholate, CAS 302-95-4 (Thermo Fisher Scientific Cat # 89904)
- Luciferase assay reagent (Promega cat # E1501)
- QuantiLum® Recombinant Luciferase (Promega, cat # E170)
- Bovine serum albumin (BSA) (GE Healthcare Life Sciences Hyclone Laboratories, cat # SH30574.01)
- CellTiter-Glo® Luminescent Cell Viability Assay (Promega, cat # G757)
- Iscove's modified Dulbecco's medium (1×) buffered with $NaHCO_3$ (Gibco Life Technologies, cat # 21056-023)
- Fetal Bovine Serum (Gibco Life Technologies, cat # 10270-098)
- Penicillin-streptomycin (Cytiva Hyclone, cat # SV30010)
- Geneticin (G-418 sulfate) (Gibco Life Technologies cat # 10131-035)
- TrypLE-EDTA (Gibco Life Technologies cat. no. A12177 and Gibco Life Technologies cat. no. 15040033)
- DPBS without Ca_{2+}, Mg_{2+} (GE Healthcare Hyclone, cat # SH30028.02)
- Cell culture grade Dimethyl sulfoxide (DMSO), Sigma Aldrich, Cat. No. D2650 (for cryopreservation of cells)
- Deionized water

For preparation of TPO extracts (outside of this study):

- Hematin (Sigma Aldrich, cat no. H3281)
- Cell culture grade water (e.g. Cytiva Hyclone, cat. no. SH30529.02)
- Sodium hydroxide solution to dissolve hematin (Merck KGaA, Cat. No. 1.09959.0001)
- Bovine serum albumin (BSA) standard (if not included in the testing kit; Thermo Scientific cat. no. 23209)
- Pierce™ BCA Protein Assay Kit (Thermo Scientific cat. no. 23225 and 23227)

5.5 Important equipment and disposables

- 96-well compound storage plates, Corning® 96 Well Storage Microplates, Corning Costar cat # 3365, for long-term storage of DMSO stock solutions and efficient preparation of dilution series, equipped with sealing mat (Corning Costar cat # 3080).
- Black solid 96-well plates, Corning Costar cat # 3356 (AUR-TPO assay)
- White solid 96-well plates, Corning Costar cat # 3912 (QLI assay)
- White 96-well plates with transparent bottom, Corning Costar cat # 3610 (cytotoxicity assay)
- Luminometer/Absorbance/Fluorescence plate reader Synergy 2 SLFAD (plate reader with dual dispense modules), BioTek Instruments Inc.

Test system description

Test system description

The test system in this assay is a whole cell extract of recombinant follicular thyroid carcinoma cells (FTC-238) expressing human TPO. In the implementation of the study, a whole cell extract of the wildtype cells was evaluated and found to have no activity in the assay, hence the activity measured is attributable to the recombinant TPO protein. The cells used to produce the test system were originally constructed by Prof J. Köhrle at Charité, Berlin, using the vector pCDNA3.1 with G418 resistance gene and human thyroid peroxidase as insert and transfected using Lipofectamine Plus (Invitrogen). The cells were provided to the RISE test facility by the sponsor. The sponsor has characterised the cells as being free from cross-contamination with mouse, rat, Syrian hamster, or Chinese hamster, cells; to be free from mycoplasma; to be free from HIV-1, HIV-2, Hepatitis B, and Hepatitis C, virus; and to have an STR profile matching the original wildtype cell line. At RISE, the cells have been expanded to master and working cells banks that each have been confirmed to be free from mycoplasma and free from other contaminations. The cell culture was performed according to SOP KM 19207 "Culture of FTC-238 and FTC-238/hrTPO cells". Whole cell extracts were prepared according to SOP KM 20168 "Thyroperoxidase (TPO) extract preparation" in several batches that were being stored at -80°C. Before use in experiments, each batch of extracts was verified to meet the acceptance criteria in the assay ("TPO efficiency" > 3 and expected AC_{50} of reference item MMI). Further, the continued activity ("TPO efficiency") of the TPO protein was verified to meet the acceptance criteria on each plate in each experimental round.

For the CTG control assay, that evaluates whether the test item causes cytotoxicity, wildtype FTC-238 cells were employed. These were provided by the sponsor from ECACC (catalogue number 94060902) and were cultured according to SOP KM 19207 "Culture of FTC-238 and FTC-238/hrTPO cells".

Test system management

Aliquots of whole cell extracts were stored at -80 °C and were thawed on ice for each experimental round of the assay. The test system was kept cold for as long as possible during the assay, and the work was performed swiftly after that the test system could no longer be kept on ice.

All work with cells was performed in a biological safety cabinet class II inside an ISO class 7 environment using aseptic techniques. Work with cells was required for the CTG control assay. No additional production of TPO test system was required during the study.

Test system quality control

After completion of the study, the cells that were in culture for cytotoxicity evaluation (wildtype FTC-238 cells) were frozen down at a passage number higher than their highest passage number used, for each vial of cells that was initiated from the cell bank. $4*10^6$ cells of each type will be sent to the sponsor for quality control. In addition , one vial from the working cell bank of TPO-transfected cells (FTC-238/hrTPO) used to generate TPO extracts in the study will also be sent to the sponsor.

Method

The evaluation of the endocrine disrupting capacity of the test items was performed according to SOP KM 20077. The testing is outlined briefly below.

Test procedure

The whole cell extract of cells overexpressing thyroperoxidase was stored at -80 °C and were thawed on ice for each experimental round of the assay. At the start of the test, 12.5 ng of protein per well was added to a black 96 well plate. Test items/controls were added, followed by the Amplex® UltraRed reagent and the reaction was then initiated by adding an excess of H_2O_2. The plate was incubated for 30 minutes at 37 °C before measuring fluorescence at ex/em wavelengths 540/600 nm with dichroic mirror 570 nm.

For the specificity control assay, QuantiLum inhibition (QLI), 6 ng of recombinant Quantilum luciferase were added to each well of a white 96 well plate. Test items/controls were added and the plate was incubated for 30 minutes at 37 °C. Thereafter luciferin reagent (Luciferase assay system) was added to the plate one well at a time using one of the dispensers in the plate reader, and luminescence was detected for 1 second for each well immediately after dispensing.

For the cytotoxicity control assay, FTC-238 cells were seeded in white, clear-bottomed 96 well plates at a density of 8000 cells/well. Test items/controls were added and the plate was incubated for 24 h at 37 °C. At the end of the incubation, plates were examined under a phase contrast microscope and evaluated for cytotoxic effects, whereafter Cell-Titer Glo reagent was added to the whole plate and luminescence was detected using a plate reader.

For the AUR-TPO assay, range-finding experiments were carried out to:

1) Confirm that the test item was soluble in stock and work solutions at certain concentrations
2) Determine if the test item displayed inhibition of enzyme or no response.
3) Select the concentration (C8, the highest concentration) and dilution factor that most likely would provide a full dose-response curve (for a test item showing a full or partial response).

An experiment was considered valid when all acceptance criteria outlined in SOP KM 20077 had been met. For each experiment, test items were weighed out and dissolved independently, and a biologically independent batch of TPO extract was used.

Test items that did not show a response were continued to be tested with the range-finding dilution range 1:10. The data set was considered complete when 4 additional valid range-finding experiments had been performed.

In case the inhibition response was ≥ 20 % from solvent control in the range-finding assay, a main test was performed in the next experimental round to enable calculation of all dose-response curve parameters. Five valid main assay runs were obtained per test chemical showing inhibitory response.

Cytotoxicity was evaluated for four of the five independent preparations of test items.

Before the start of the test, the maximum solubility of the test items was determined according to SOP KM 17783. The highest concentration evaluated was 100 mM for all test items. The obtained maximum solubility is presented in the Results section.

Evaluation of Results

The analysis of the inhibitory effect was performed as described in SOP KM 20077 using the Microsoft Excel data analysis forms AUR_TPO_main_210114, AUR_TPO_range_210114, QLI_210603, CTG_210316, and GraphPad Prism 6. The data analysis forms have inbuilt functions to check whether the acceptance criteria in SOP KM 20077 are met:

AUR-TPO assay:

- TPO efficiency (ratio between VC and BC2): >3
- Reference item MMI AC_{50}: Historical mean ± 2SD, control chart not yet finished at start of study
- Inhibition (%) for PC PTU 25 µM: > 50
- Inhibition (%) for NC BP3 100 µM: < 10
- Z-factor for MMI C8: ≥ 0.5
- Plate dynamic range (ratio between VC and BC1): >3
- Standard deviation of Inhibition (%) for each replicate of vehicle control, blanks, reference, control or test items on each plate: ≤ 20%

QLI assay:

- Reference item LUCINH2 AC_{50}: 2.0*10-9 – $2.0*10^{-8}$ M
- Inhibition (%) for PC LUCINH1: > 80
- Inhibition (%) for NC BP3 10 µM: < 20
- Z-factor for LUCINH2 C8: ≥ 0.5
- Standard deviation of Inhibition (%) for each replicate of vehicle control, blank, reference, control or test items on each plate: ≤ 20%

Cytotoxicity control assay:

- Reference item DCNQ AC_{50}: 1.5*10-6 – $1.05*10^{-5}$
- Cytotoxicity (%) for PC DCNQ 10 µM: > 70
- Cytotoxicity (%) for NC BP3 100 µM: < 20
- Z-factor for DCNQ C8: ≥ 0.5
- Standard deviation of Inhibition (%) for each replicate of vehicle control, blank, reference, control or test items on each plate: ≤ 20%

Manually checked additional acceptance criteria were that: 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).

Results

Tables 3, 4 and 5 give an overview of all valid and invalid range-finding ("RANGE") and main ("MAIN") experiments, carried out for each test item, for the AUR-TPO assay and for the two control assays QLI and CTG. Invalid runs are indicated in italics and the reason for invalidity is indicated in footnotes below the table in each case.

Table 3. Overview of all experimental runs with the AUR-TPO assay carried out in Study 1. Invalid runs are indicated in italics, and explained below the table. Unusual occurrences in valid runs are also explained below the table.

Chemical name; RISE Test item ID	**Run 1**	**Run 2**	**Run 3**	**Run 4**	**Run 5**	**Run 6**	**Run 7**
MMI; 8P06603:1	RANGE	*MAIN*[1]	*MAIN*[2]	MAIN[4]	MAIN	MAIN	*MAIN*[5]
BP2; 8P06603:2	RANGE	*MAIN*[1]	MAIN	MAIN[4]	MAIN	MAIN	MAIN
BP3; 8P06603:3	RANGE	*RANGE*[1]	RANGE	RANGE[4]	RANGE	RANGE	RANGE
Diethyl phthalate; 8P06603:4	RANGE	*RANGE*[1]	*RANGE*[3]	RANGE[4]	RANGE	RANGE	RANGE
Genistein; 8P06603:5	RANGE	*MAIN*[1]	*MAIN*[3]	MAIN[4]	MAIN	MAIN	MAIN
PTU; 8P06603:6	RANGE	*MAIN*[1]	*MAIN*[3]	MAIN[4]	MAIN	MAIN	MAIN

1. Study personnel forgot to add stock solution MMI C8 to working solution.
2. The plate dynamic range was just below the cut-off (2.75; cut-off 3), however the TPO efficiency was acceptable (3.67, cut-off 3).
3. Slightly too high variability in VC caused the z factor for the plate to be too low (0.45; < 0.5)
4. There was a pipetting error in one of the negative control wells, so the negative control was only calculated from two wells. The results were accepted.
5. The plate dynamic range was just below the cut-off (2.92; cut-off 3), however the TPO efficiency was acceptable (5.86, cut-off 3).

Table 4. Overview of all experimental runs with the QLI assay carried out in Study 1. Invalid runs are indicated in italics, and explained below the table.

Chemical name; RISE Test item ID	**Run 1**	**Run 2**	**Run 3**	**Run 4**	**Run 5**	**Run 6**	**Run 7**	**Run 8**
MMI; 8P06603:1	*MAIN*[1]	*MAIN*[2]	MAIN	MAIN	*MAIN*[3]	*MAIN*[4]	MAIN	MAIN
BP2; 8P06603:2	*MAIN*[1]	*MAIN*[2]	MAIN	MAIN	*MAIN*[3]	*MAIN*[4]	MAIN	MAIN
BP3; 8P06603:3	*RANGE*[1]	*RANGE*[2]	RANGE	RANGE	*RANGE*[3]	*RANGE*[4]	RANGE	RANGE
Genistein; 8P06603:5	*MAIN*[1]	*MAIN*[2]	MAIN	MAIN	*MAIN*[3]	*MAIN*[4]	MAIN	MAIN
PTU; 8P06603:6	*MAIN*[1]	*MAIN*[2]	MAIN	MAIN	*MAIN*[3]	*MAIN*[4]	MAIN	MAIN

1. Pipetting errors: Too high standard deviation for reference item C8 and for negative control.
2. Pipetting errors: Too high standard deviation for positive control, and positive control outside range; reference item did not display sigmoidal behaviour.
3. Uneven signal from plate causing multiple errors, probably due to uneven dispensing of test system.
4. Uneven signal from plate causing multiple errors, probably due to uneven dispensing of test system.

Table 5. Overview of all experimental runs with the cytotoxicity assay CTG carried out in Study 1. Invalid runs are indicated in italics, and explained below the table.

Chemical name; RISE Test item ID	Run 1	Run 2	Run 3	Run 4	Run 5
MMI; 8P06603:1	MAIN	*MAIN*[1]	MAIN	*MAIN*[2]	MAIN
BP2; 8P06603:2	MAIN	*MAIN*[1]	MAIN	*MAIN*[2]	MAIN
BP3; 8P06603:3	PRE	*PRE*[1]	PRE	*PRE*[2]	PRE
Diethyl phthalate; 8P06603:4	PRE	*PRE*[1]	PRE	*PRE*[2]	PRE
Genistein; 8P06603:5	MAIN	*MAIN*[1]	MAIN	*MAIN*[2]	MAIN
PTU; 8P06603:6	MAIN	*MAIN*[1]	MAIN	*MAIN*[2]	MAIN

1. NC too high (20.7)
2. NC too high (31.6)

Reference and control items

The obtained dose-response curves for AUR-TPO reference item MMI are shown in Figure 1, the dose-response curves obtained for QLI reference item Luciferase inhibitor II are shown in Figure 2, and the dose-response curves obtained for CTG reference item DCNQ are shown in Figure 3. Curve labels refer to the ID of the aliquot of chemical and the 96-well plate used for the experiment.

Graphs for calculated parameters (plate dynamic ranges, Z-factors, MMI AC_{50} and CV for the vehicle control) for the AUR-TPO assay are presented in figures 4-7. The determined relative induction for negative and positive control items are presented in figures 8-9. The corresponding data is presented in tables in appendix 3. Graphs for calculated parameters (plate induction factors, Z-factors, and reference item IC_{50} for the QLI control assay are presented in figures 10-12. The determined relative induction for negative and positive control items are presented in figures 13-14. The corresponding data is presented in tables in appendix 4. Graphs for calculated parameters (plate induction factors, Z-factors, and reference item IC_{50} for the CTG control assay are presented in figures 15-17. The determined relative induction for negative and positive control items are presented in figures 18-19. The corresponding data is presented appendix 5.

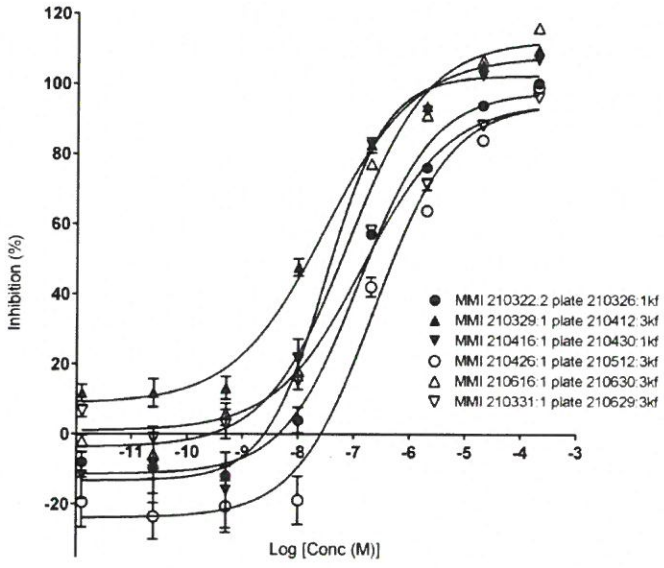


Figure 1. Obtained dose-response curves for AUR-TPO reference item MMI.

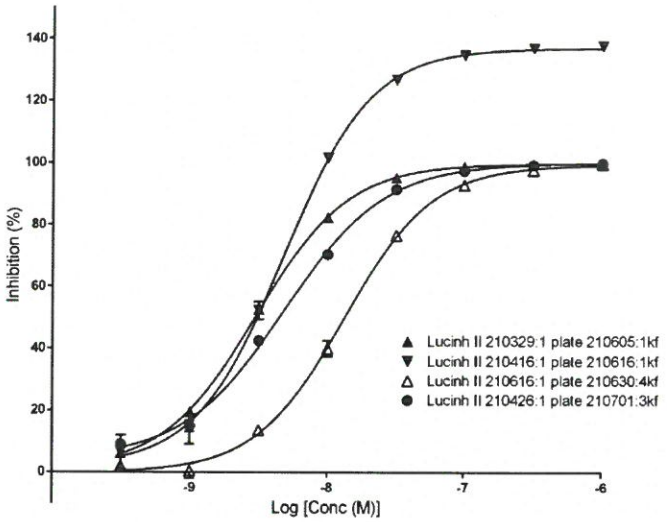


Figure 2. Obtained dose-response curves for QLI reference item luciferase inhibitor II.

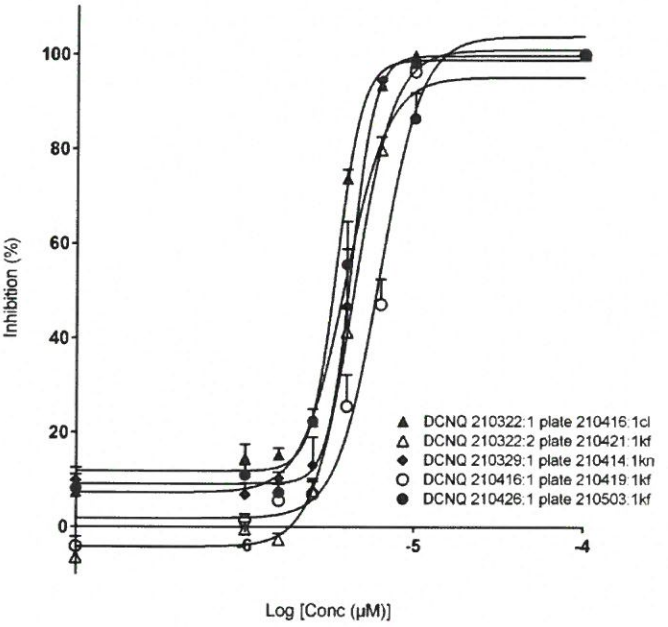


Figure 3. Obtained dose-response curves for CTG reference item DCNQ.

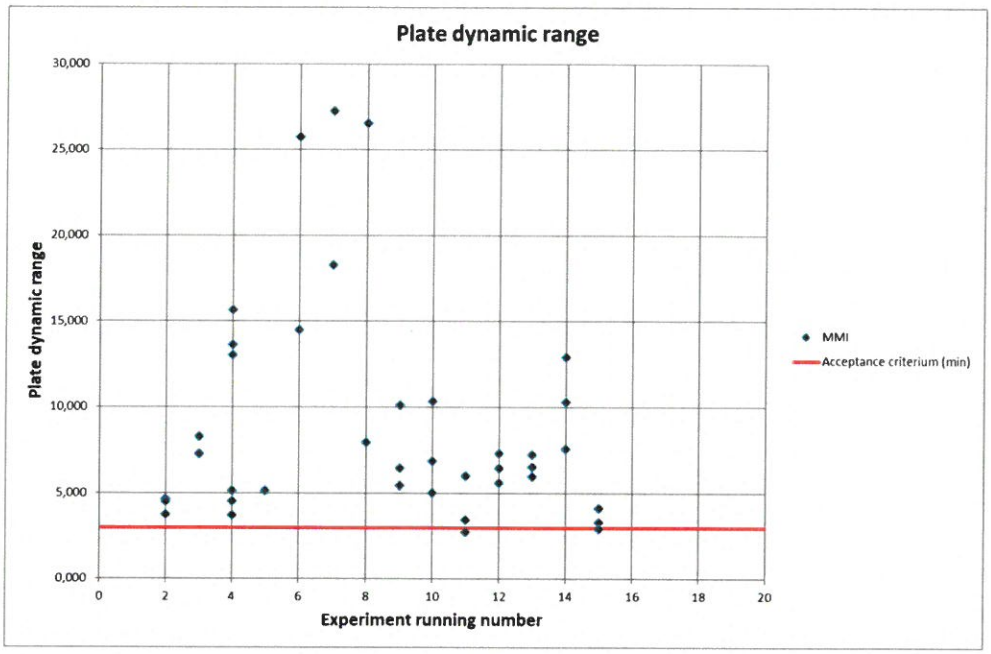


Figure 4. Plate dynamic ranges all plates in the AUR-TPO assay, for the runs in the study (#9-#15) and for the runs performed during the implementation of the assay.

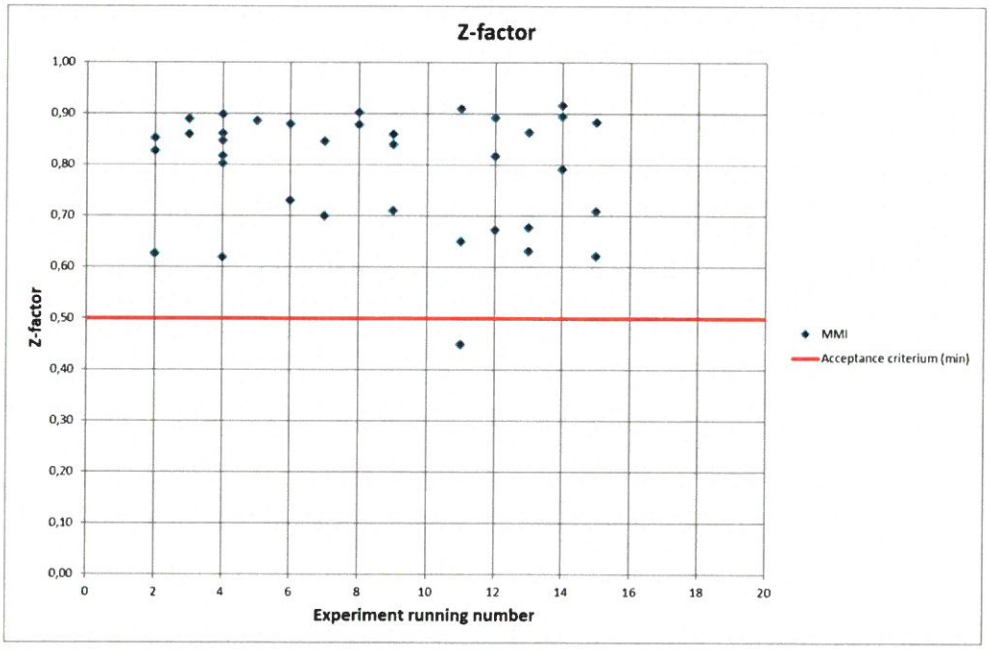


Figure 5. Z-factors for all plates in the AUR-TPO assay, for the runs in the study (#9-#15) and for the runs performed during the implementation of the assay.

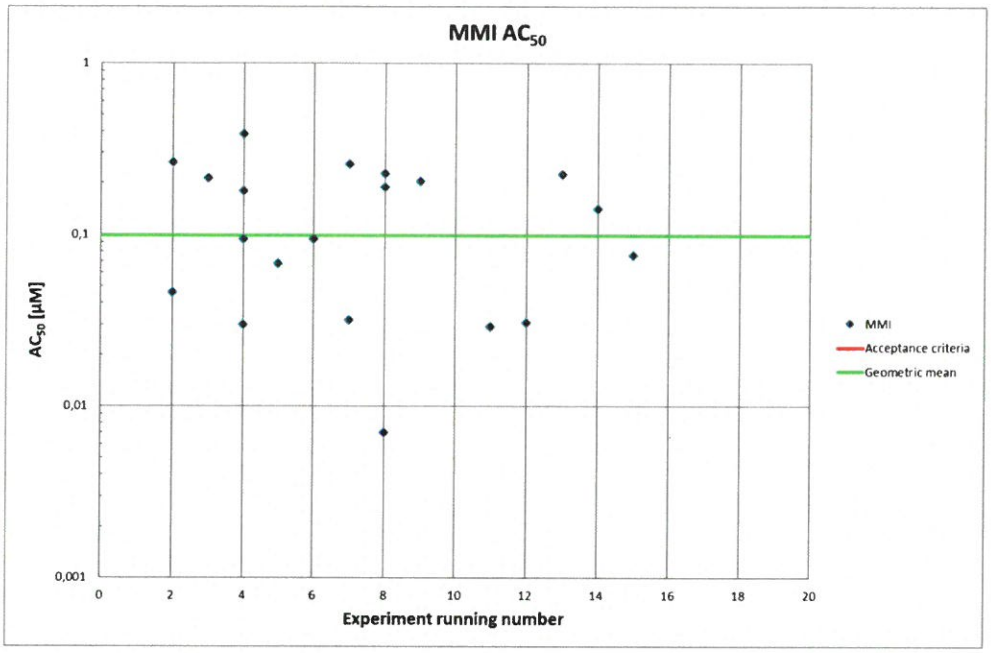


Figure 6. AC_{50} [µM] values for reference item MMI for the AUR-TPO assay, for the runs in the study (#9-#15) and for the runs performed during the implementation of the assay.

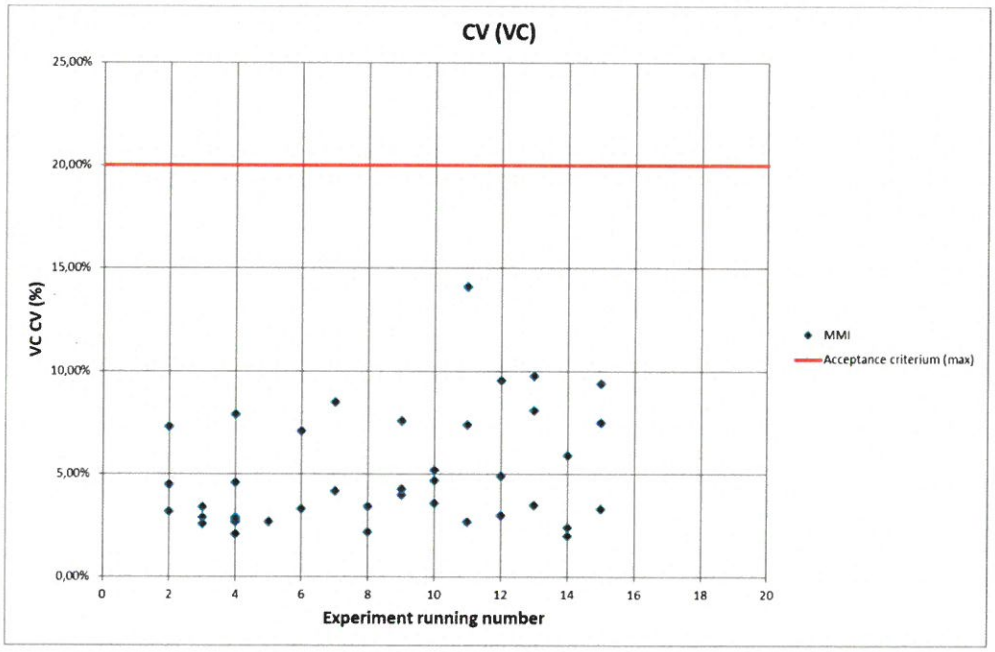


Figure 7. CV for the vehicle control (VC) for all plates in the AUR-TPO assay, for the runs in the study (#9-#15) and for the runs performed during the implementation of the assay.

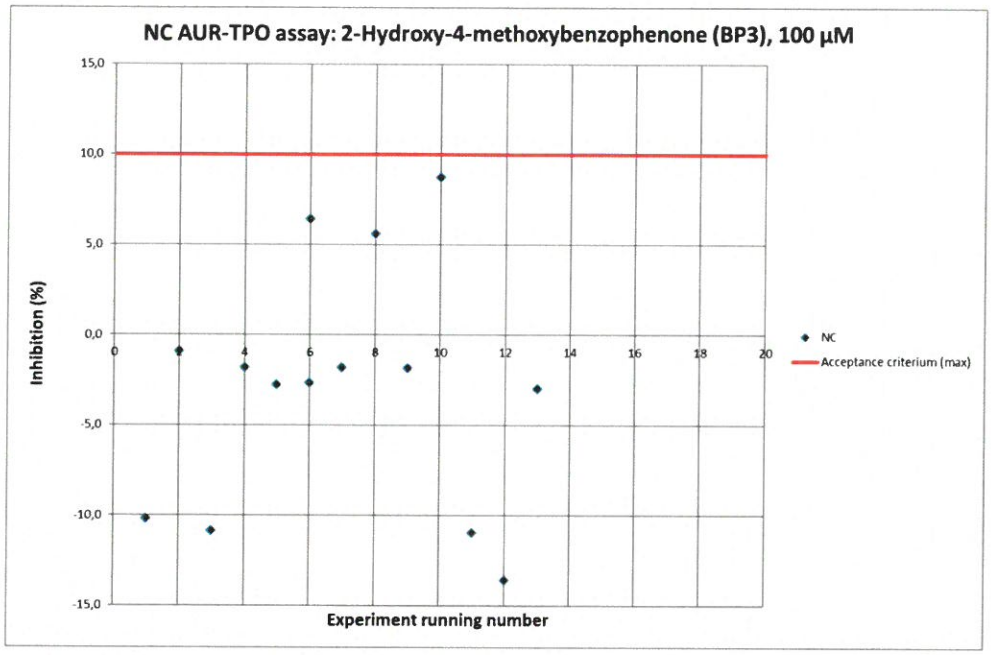


Figure 8. Relative inhibition (%) for negative control item BP3 in the AUR-TPO assay, for the runs in the study (#9-#15) and for the runs performed during the implementation of the assay.

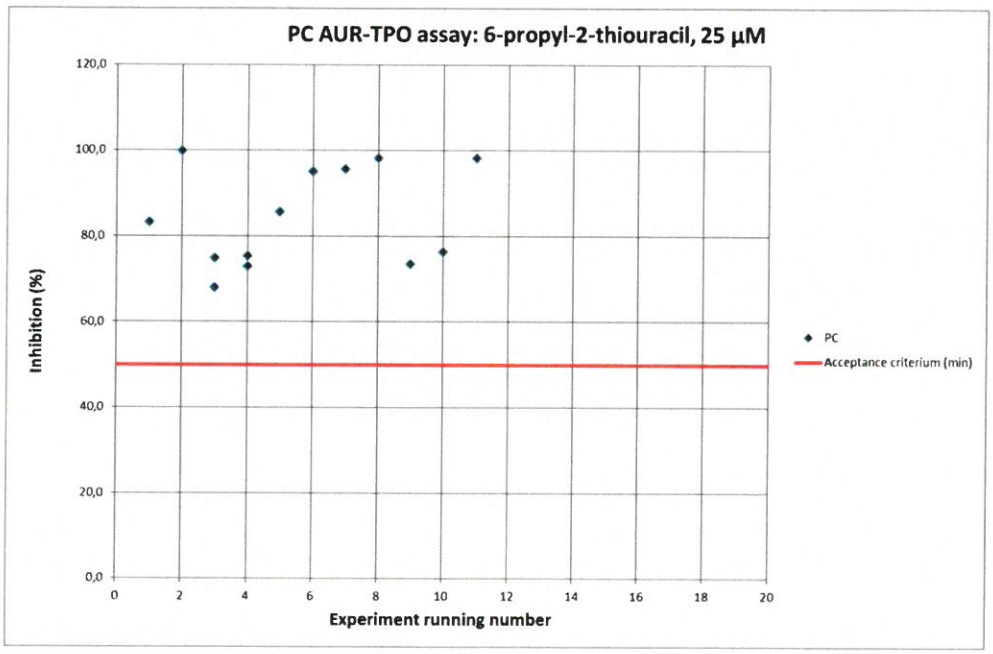


Figure 9. Relative inhibition (%) for positive control item PTU in the AUR-TPO assay, for the runs in the study (#5-#11) and for the runs performed during the implementation of the assay.

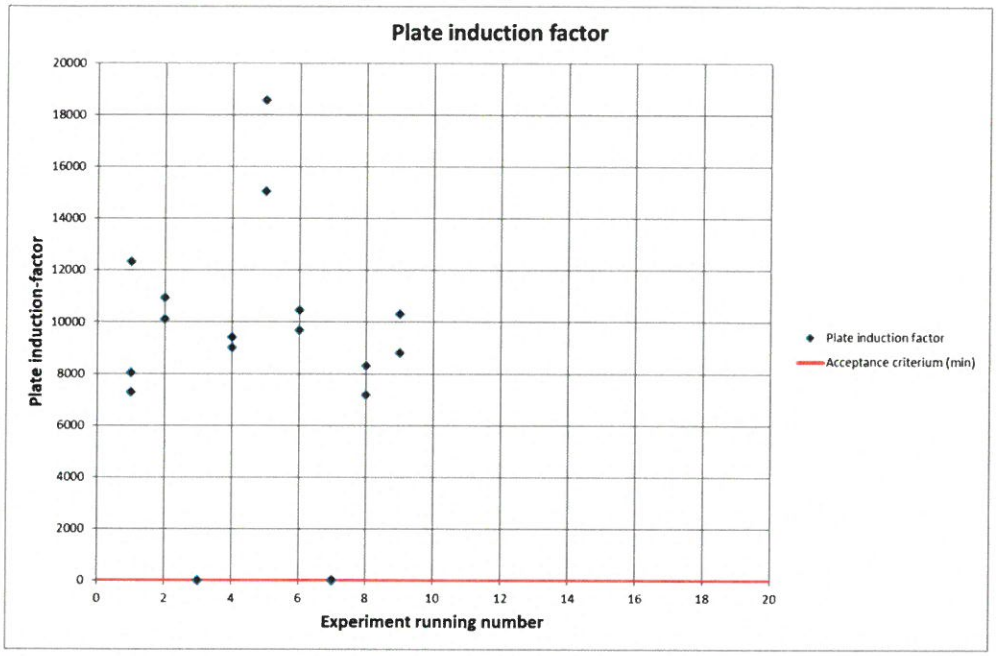


Figure 10. Plate dynamic ranges all plates in the QLI assay, for the valid runs in the study (#6-#9) and for the runs performed during the implementation of the assay.

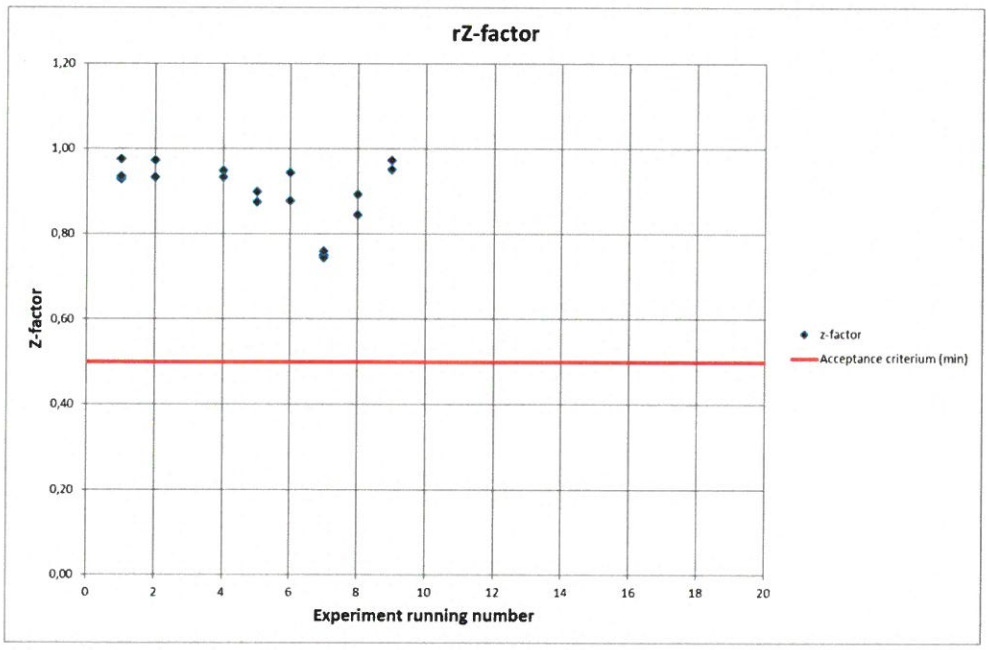


Figure 11. Z-factors for all plates in the QLI assay, for the valid runs in the study (#6 - #9) and for the runs performed during the implementation of the assay.

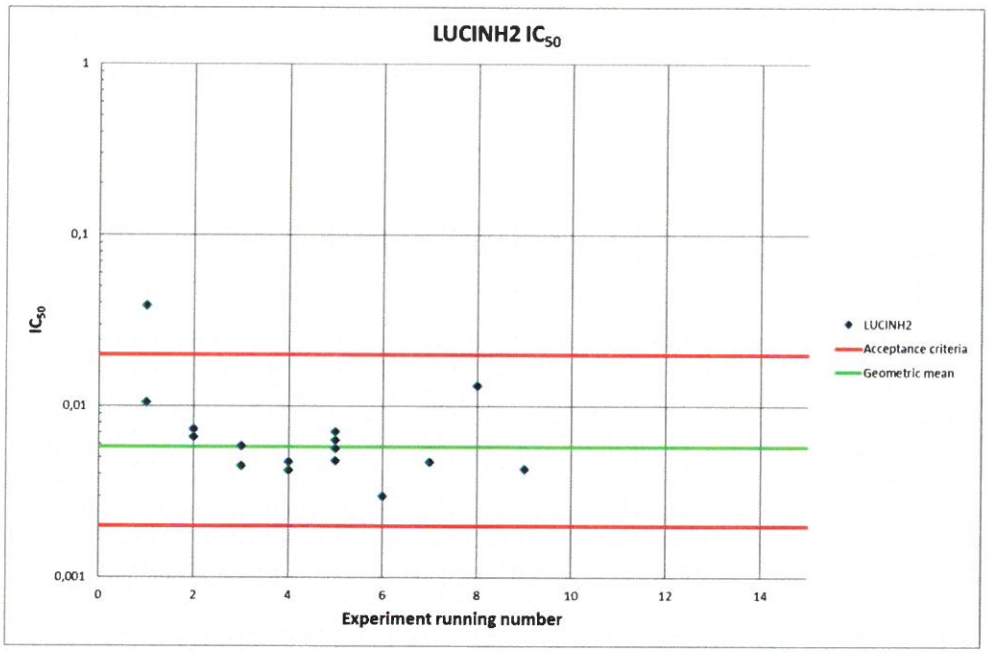


Figure 12. AC_{50} [µM] values for reference item Luciferase inhibitor II for the QLI assay, for the valid runs in the study (#6 - #9) and for the runs performed during the implementation of the assay.

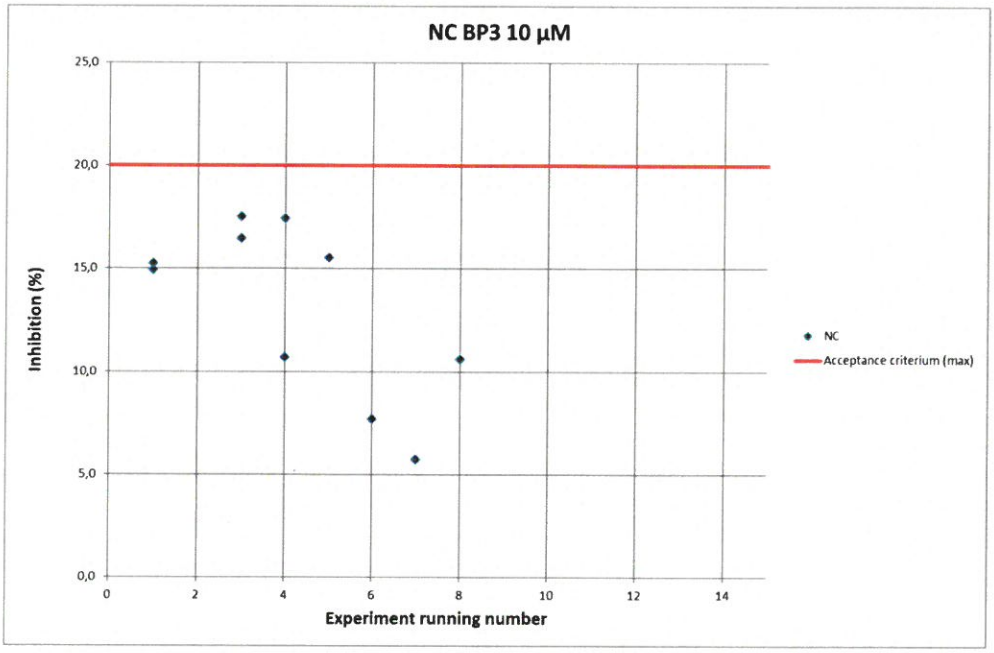


Figure 13. Relative inhibition (%) for negative control item BP3 in the QLI assay, for the runs in the study (#5 - #8) and for the runs performed during the implementation of the assay

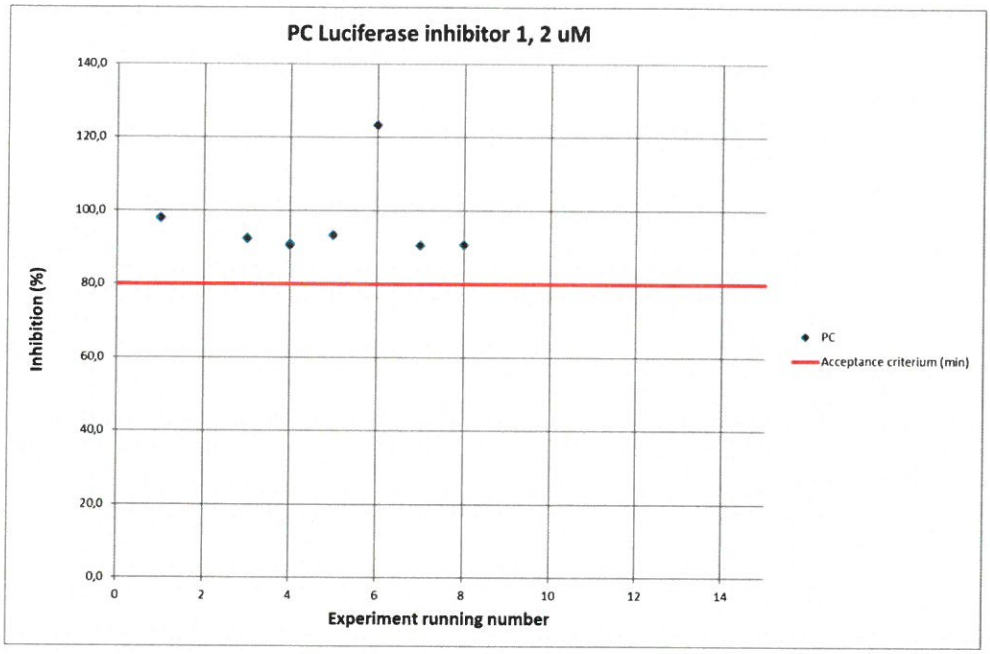


Figure 14. Relative inhibition (%) for positive control item Luciferase inhibitor I in the QLI assay, for the valid runs in the study (#5 - #8) and for the runs performed during the implementation of the assay.

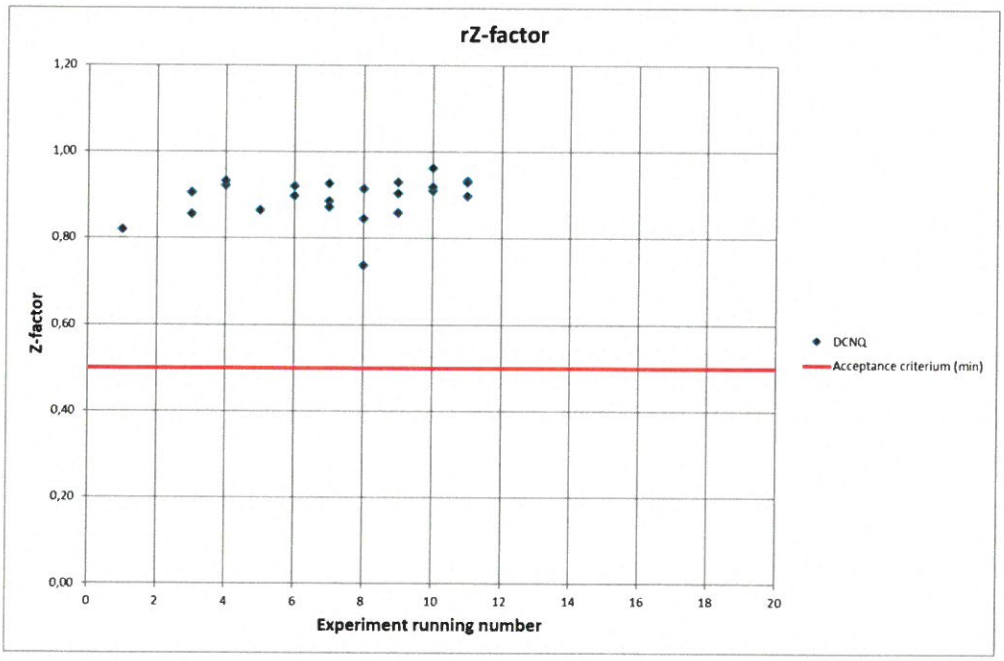


Figure 15. Z factor for all plates in the CTG assay, for all runs in the study (#7-#11) and for all plates in the runs performed during the implementation of the assay.

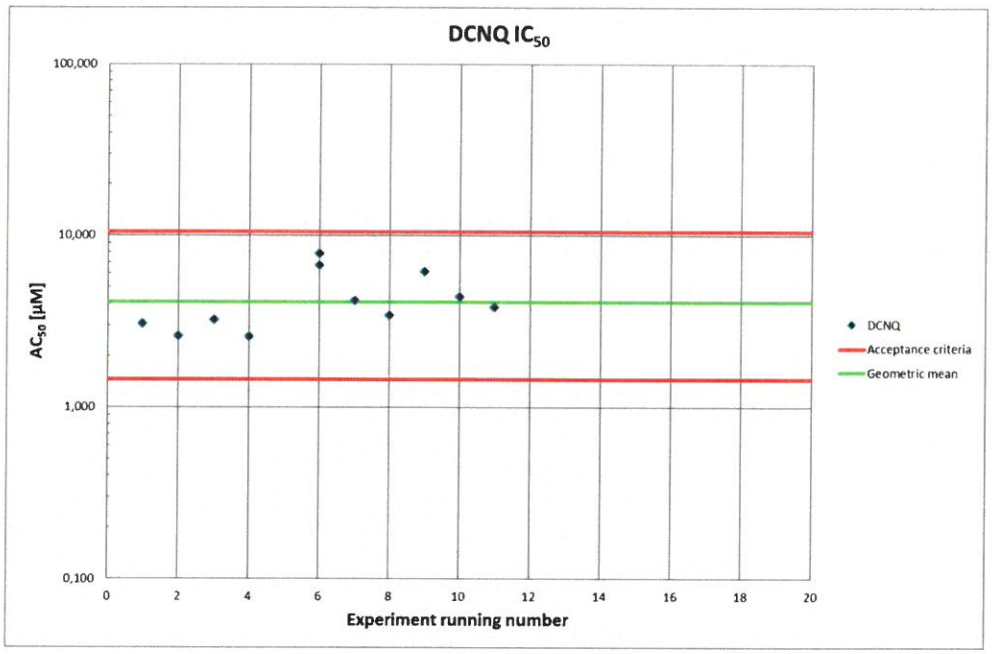


Figure 16. AC_{50} [µM] values for reference item DCNQ for the CTG assay, for the runs in the study (#7-#11) and for the runs performed during the implementation of the assay.

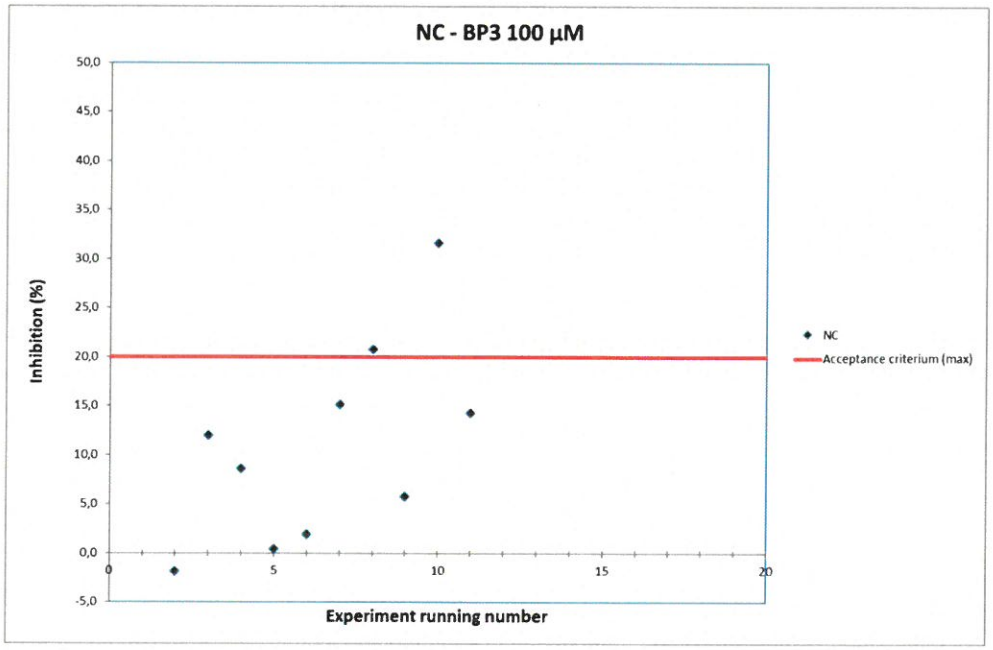


Figure 17. Relative inhibition (%) for negative control item BP3 for the CTG assay, for the runs in the study (#7-#11) and for the runs performed during the implementation of the assay.

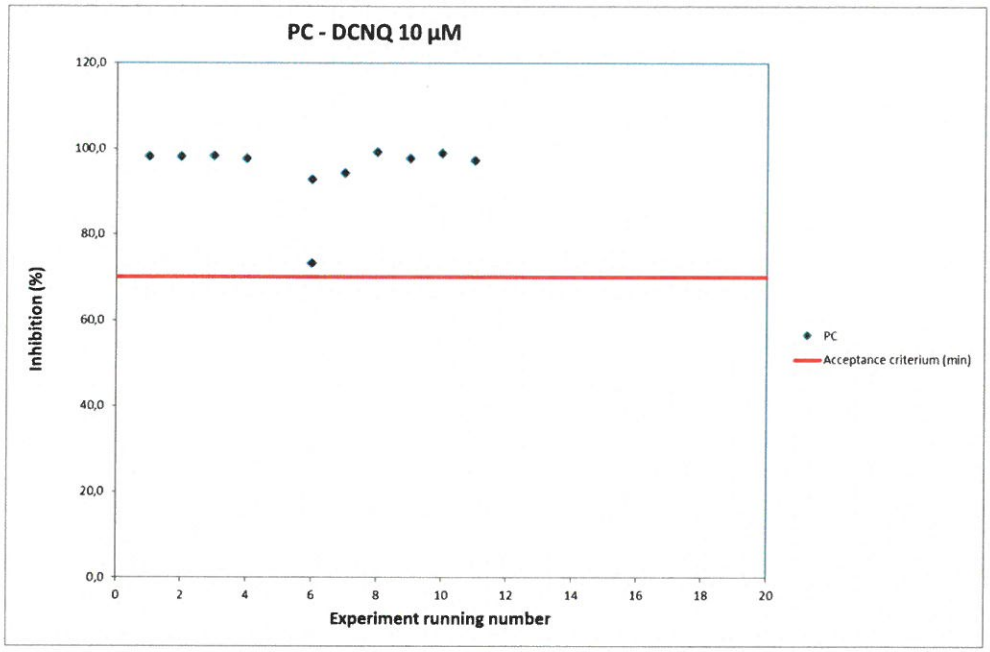


Figure 18. Relative inhibition (%) for positive control item DCNQ for the CTG assay, for the runs in the study (#7-#11) and for the runs performed during the implementation of the assay.

Test items

Solubility evaluation

All test items dissolved in DMSO. The procedure followed to obtain full solubility and the soluble concentrations in DMSO are given in Table 6, together with the determined starting concentrations (effective solubility) for range finding tests.

Table 6. Results from solubility testing according to SOP KM 17783.

Chemical name; RISE Test item ID	Solubility in DMSO (mM)	Procedure followed to obtain solubility in DMSO	Observations on evaluated concentrations	Effective solubility (mM)
MMI; 8P06603:1	100	Vortexing 1min	C100, C30 and C10 soluble in DMSO and in buffer/medium, by microscopy and centrifugation	100
BP2; 8P06603:2	100	Vortexing 1min	C100, C30 and C10 soluble in DMSO and in buffer/medium, by microscopy and centrifugation	100
BP3; 8P06603:3	100	Vortexing 1min	C100 soluble in DMSO, by microscopy and centrifugation, however the intermediate dilution was not soluble in buffer or medium.C100 final concentration was soluble in buffer/medium. C30 and C10 soluble.	100
Diethyl phthalate; 8P06603:4	100	Vortexing 1min	C100, C30 and C10 soluble in DMSO and in buffer/medium, by microscopy and centrifugation	100

Chemical name; RISE Test item ID	Solubility in DMSO (mM)	Procedure followed to obtain solubility in DMSO	Observations on evaluated concentrations	Effective solubility (mM)
Genistein; 8P06603:5	100	Vortexing 1min	C100 soluble in DMSO but not in medium, by microscopy and centrifugation; C30 soluble in DMSO and in buffer/medium, the intermediate dilution was not soluble in buffer or medium.C30 final concentration was soluble in buffer/medium. C10 soluble.	30
PTU; 8P06603:6	100	Vortexing 1min	C100, C30 and C10 soluble in DMSO and in buffer/medium, by microscopy and centrifugation	100

Concentration selection for main assay/continued range finding tests

Table 7 shows the selected concentrations and dilution factors for the main assay, in case of found effect, or for continued range finding tests in case no response was detected. Justification for the selection(s) is also given in each case.

Table 7. Concentration selection for main assay/continued range finding testing.

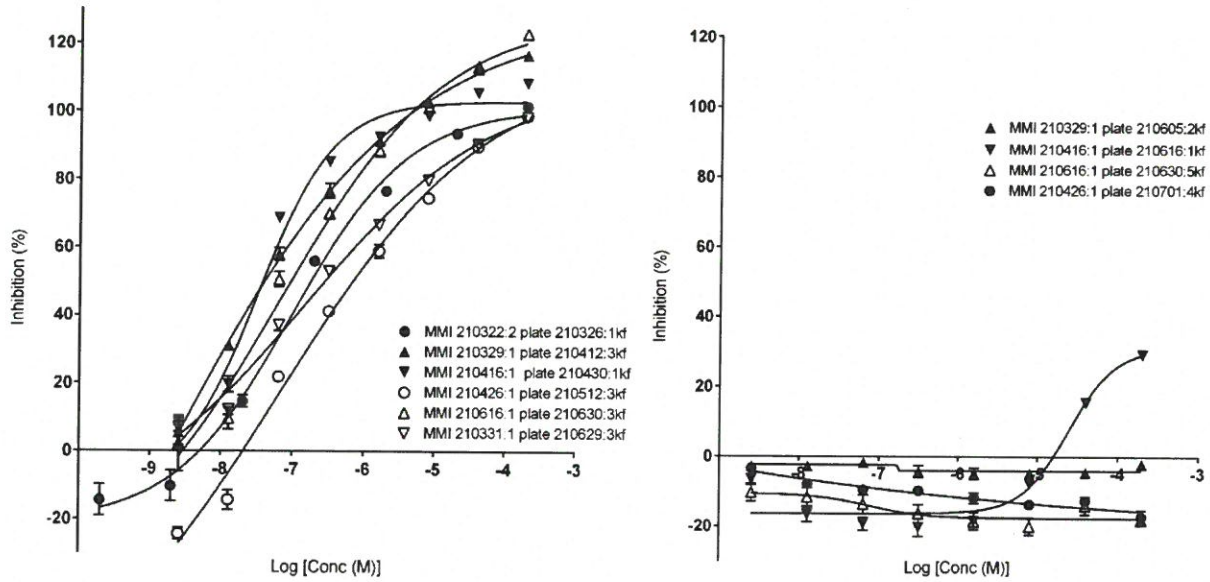
Chemical name; RISE Test item ID	[C8] and dilution factor selected for further testing	Reason for concentration selection
MMI; 8P06603:1	200 µM, dilution factor 5	Inhibitory response with max effect at 200 µM; at least one concentration >70% inhibition, dilution factor 4 suspected to not cover the full response so increased to 5.
BP2; 8P06603:2	80 µM, dilution factor 4	Inhibitory response, start at 20 µM*dilution factor; at least one concentration >70% inhibition
BP3; 8P06603:3	200 µM, dilution factor 10	No effect
Diethyl phthalate; 8P06603:4	200 µM, dilution factor 10	No effect
Genistein; 8P06603:5	80 µM, dilution factor 4	Inhibitory response, start at highest soluble concentration; at least one concentration >70% inhibition
PTU; 8P06603:6	200 µM, dilution factor 4	Inhibitory response with max effect at 200 µM; at least one concentration >70% inhibition

TPO inhibition of test items

An overview of all runs performed was given in Table 3-5, and the selected concentrations for main experiments were presented in Table 7. Below the results of evaluation of TPO inhibitory effect are shown for each test item, together with results from the control assays QLI (unspecific enzyme inhibition) and CTG (cytotoxicity).

8P06603:1, MMI

The test item showed an inhibitory effect on TPO, see Figure 19. The inhibition was specific to TPO as the control assay QLI was mostly negative. The test item was not cytotoxic to FTC-238 cells at any of the evaluated concentrations. The parameters describing inhibitory properties for the valid AUR-TPO runs are presented in Table 8, and the parameters for the QLI runs are presented in Table 9.



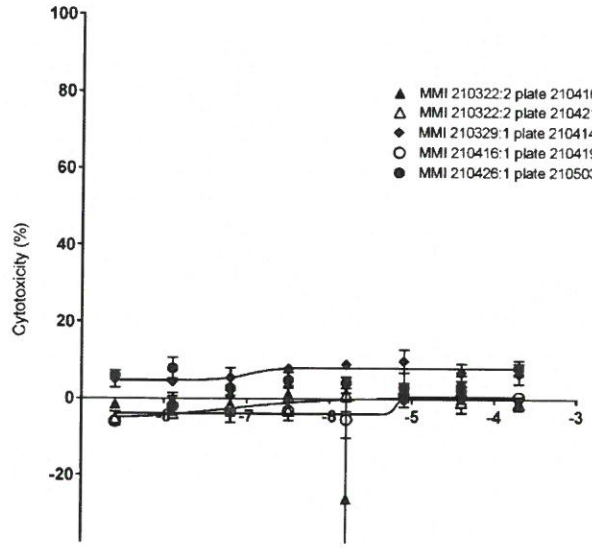


Figure 19. Top left: TPO inhibition dose-response curves; top right: QLI control assay data, and bottorm: CTG control assay data, for test item 8P06603:1, MMI. Error bars represent the standard deviation (n=3).

Table 8. Reporting of TPO inhibition data, test item 8P06603:1, MMI.

Run number and plate number	Plate dynamic range	IC_{50} (µM]	CV (Log IC_{50}) (%)	IC_{20} (µM]	Classification & # concentrations > 20%
Run 1, Plate 210326:1kf	10.1	9.64E-02	1.04%	7.08E-03	Positive, 4 (range finding)
Run 3, Plate 210412:3kf	2.8	1.41E-03	6.84%	7.25E-06	Positive, 7
Run 4, Plate 210430:3kf	5.6	3.24E-02	1.42%	6.00E-03	Positive, 7
Run 5, Plate 210512:3kf	6.0	6.35E-02	4.60%	6.35E-04	Positive, 6
Run 6, Plate 210629:3kf	7.6	1.45E-01	2.86%	2.24E-03	Positive, 6
Run 7, Plate 210630:3kf	2.9	6.41E-02	4.22%	1.62E-03	Positive, 6
Mean*	**NR**	**3.71E-02**	**NR**	**9.44E-04**	**NR**

** Geometric mean for IC_{50} and IC_{20},. NR = not relevant.*

Table 9. Reporting of QuantiLum inhibition data, test item 8P06603:1, MMI.

Run number and plate number	Plate dynamic range	IC_{50} (µM]	CV (Log IC_{50}) (%)	IC_{20} (µM]	Classification & # concentrations > 20%
Run 3, Plate 210605:2kf	10466	N/A	N/A	N/A	Negative, 0
Run 4, Plate 210616:2kf	4	2.42E01	2.0	9.56E00	Positive, 1
Run 7, Plate 210630:5kf	8312	N/A	N/A	N/A	Negative, 0
Run 8, Plate 210701:2kf	8837	N/A	N/A	N/A	Negative, 0

8P06603:2, BP2

The test item showed an inhibitory effect on TPO, see Figure 20. The inhibition was specific to TPO although the control assay QLI showed an effect at the two highest concentrations. The shift in determined IC50 values between the two assays is several orders of magnitude (cf Tables 10 and 11). The test item was not cytotoxic to FTC-238 cells at any of the evaluated concentrations. The parameters describing inhibitory properties for the valid AUR-TPO runs are presented in Table 10, and the parameters for the QLI runs are presented in Table 11.

Table 10. Reporting of TPO inhibition data, test item 8P06603:2, BP2.

Run number and plate number	Plate dynamic range	IC_{50} (µM]	CV (Log IC_{50}) (%)	IC_{20} (µM]	Classification & # concentrations > 20%
Run 1, Plate 210326:1kf	10.1	1.51 E-01	0.41%	3.58E-02	Positive, 4 (range finding)
Run 3, Plate 210412:1kf	6.0	1.91 E-01	0.24%	4.56E-02	Positive, 6
Run 4, Plate 210430:1kf	7.3	1.54 E-01	0.40%	3.84E-02	Positive, 6
Run 5, Plate 210512:1kf	7.3	1.97E-01	0.66%	3.82E-02	Positive, 6
Run 6, Plate 210629:1kf	12.9	2.41E-01	0.45%	5.15E-02	Positive, 6
Run 7, Plate 210630:1kf	4.1	1.58 E-01	0.62%	2.72E-02	Positive, 6
Mean*	**NR**	**1.79E-01**	**NR**	**3.87E-02**	**NR**

** Geometric mean for IC_{50} and IC_{20},. NR = not relevant.*

Table 11. Reporting of QuantiLum inhibition data, test item 8P06603:2, BP2. Ambiguos curve fits are written in italic.

Run number and plate number	Plate dynamic range	IC_{50} (µM]	CV (Log IC_{50}) (%)	IC_{20} (µM]	Classification & # concentrations > 20%
Run 3, Plate 210605:1kf	9680	4.86E01	2.5	1.79E01	Positive, 1
Run 4, Plate 210616:2kf	4	3.11E01	8.7	1.52E01	Positive, 2
Run 7, Plate 210630:4kf	7190	*2.34E01*	*299*	*1.93E01*	Positive, 1
Run 8, Plate 210701:1kf	9049	2.58E01	9.6	1.82E01	Positive, 1
Mean*	**NR**	**3.39E+01**	**NR**	**1.71E+01**	**NR**

** Geometric mean for IC_{50}, IC_{20}, PCI_{50} and PCI_{20}. NR = not relevant.*

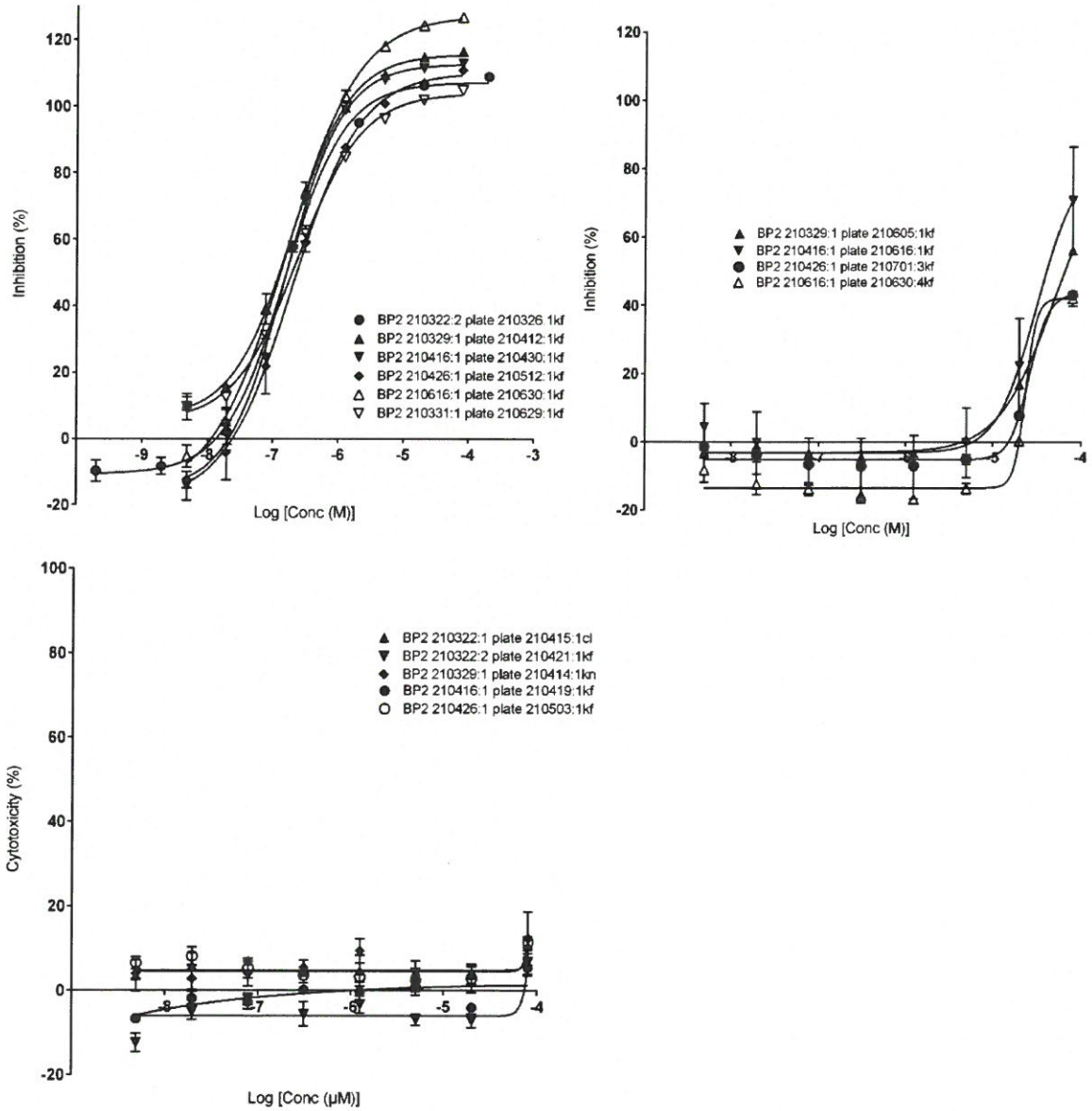
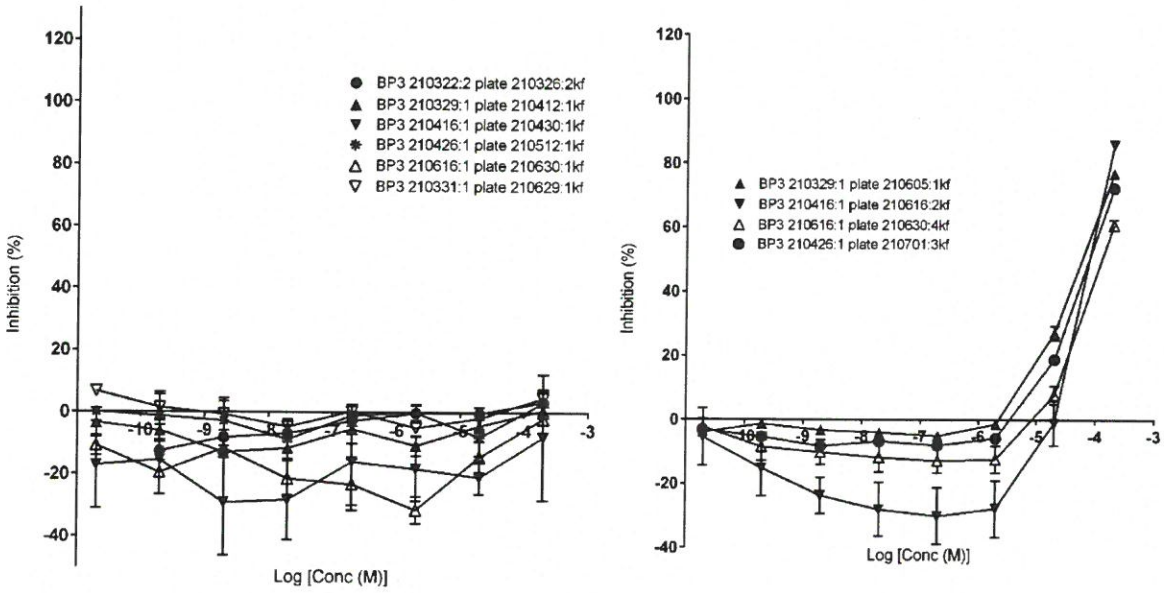


Figure 20. Top left: TPO inhibition dose-response curves; top right: QLI control assay data, and bottorm: CTG control assay data, for test item 8P06603:2, BP2. Error bars represent the standard deviation (n=3).

8P06603:3, BP3

The test item showed no inhibitory effect in the AUR-TPO assay, see figure 21. Since the test item was chosen as negative control for all three assays, data sets for the QLI assay was nevertheless generated and the test item has an effect at higher concentrations. The test item was cytotoxic to FTC-238 cells at the highest evaluated concentration, 200 µM. Parameters for the QLI assay are shown in Table 12.



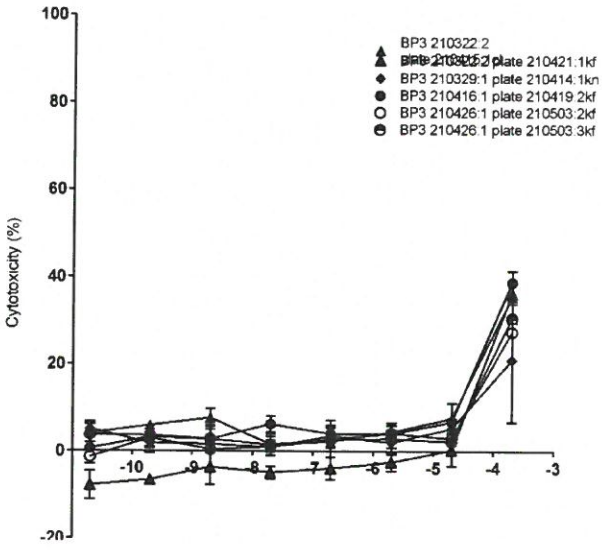


Figure 21. Top left: TPO inhibition dose-response curves; top right: QLI control assay data, and bottorm: CTG control assay data, for test item 8P06603:3, BP3. Error bars represent the standard deviation (n=3).

Table 12. Reporting of QuantiLum inhibition data, test item 8P06603:3, BP3. Ambiguos curve fits are written in italic.

Run number and plate number	Plate dynamic range	IC_{50} (µM]	CV (Log IC_{50}) (%)	IC_{20} (µM]	Classification & # concentrations > 20%
Run 3, Plate 210605:1kf	9680	3.12E+01	1.1	1.12 E+01	Positive, 2
Run 4, Plate 210616:2kf	4	*2.62 E+01*	*11366*	*2.01 E+01*	Positive, 1
Run 7, Plate 210630:4kf	7190	*2.46 E+01*	*4997*	*1.91 E+01*	Positive, 1
Run 8, Plate 210701:1kf	9049	3.05 E+01	2.75	1.46 E+01	Positive, 1
Mean*	**NR**	**3.08E+01**	**NR**	**1.28E+01**	**NR**

** Geometric mean for IC_{50}, IC_{20}, PCI_{50} and PCI_{20}. NR = not relevant.*

8P06603:4, DEP

The test item showed no effect in the AUR-TPO assay (Figure 22). The test item was not cytotoxic to FTC-238 cells at any of the tested concentrations.

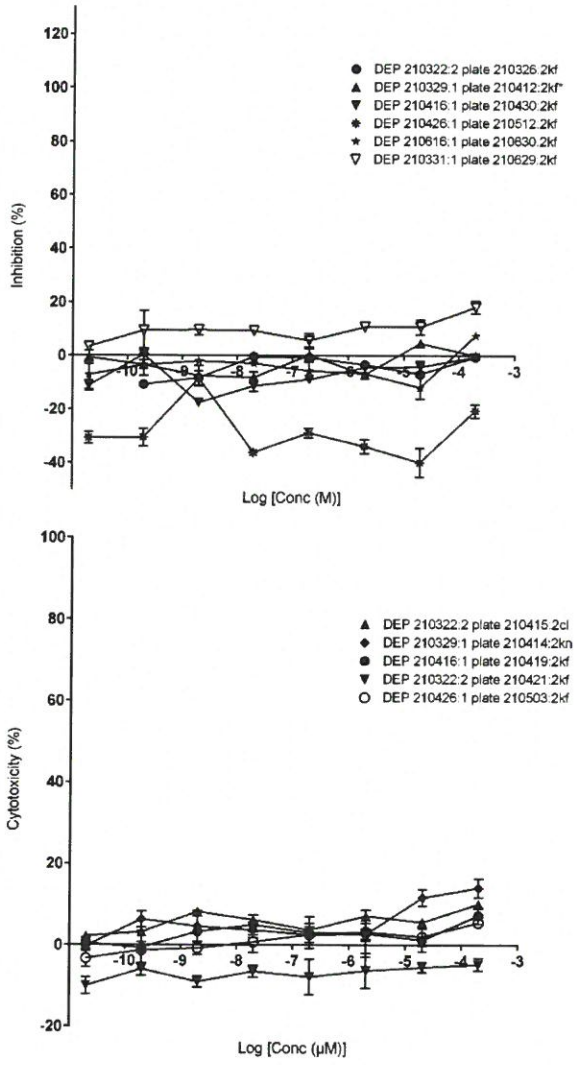


Figure 22. Top: TPO inhibition dose-response curves; bottorm: CTG control assay data, for test item 8P06603:4, DEP. Error bars represent the standard deviation (n=3).

8P06603:5, Genistein

The test item showed an inhibitory effect on TPO, see Figure 23. The test item also showed a clear effect in the QLI control assay. The shift in determined IC50 values between the two assays is very small (cf Tables 13 and 14), indicating that the test item is a false positive for TPO inhibition. The selectivity value for the AUR-TPO assay is calculated in Table 17. The test item was cytotoxic to FTC-238 cells at the highest evaluated concentration, 60 µM, in most of the runs. The parameters describing inhibitory properties for the valid AUR-TPO runs are presented in Table 13, and the parameters for the QLI runs are presented in Table 14.

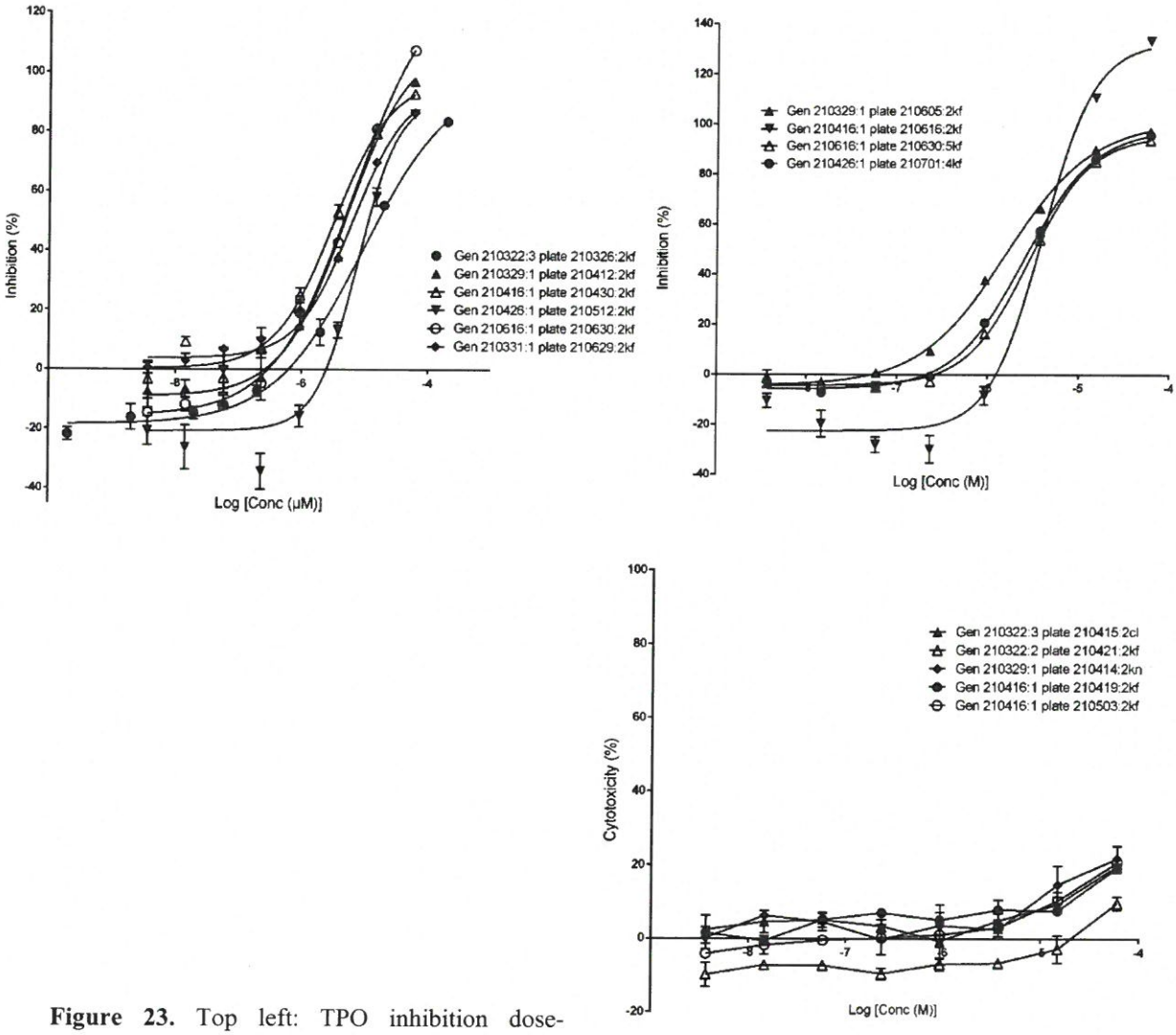


Figure 23. Top left: TPO inhibition dose-response curves; top right: QLI control assay data, and bottorm: CTG control assay data, for test item 8P06603:5, Genistein. Error bars represent the standard deviation (n=3).

Table 13. Reporting of TPO inhibition data, test item 8P06603:5, Genistein.

Run number and plate number	Plate dynamic range	IC_{50} (µM]	CV (Log IC_{50}) (%)	IC_{20} (µM]	Classification & # concentrations > 20%
Run 1, Plate 210326:2kf	6.4	9.16 E+00	1.77%	9.86E-01	Positive, 2 (range finding)
Run 3, Plate 210412:2kf	3.5	4.91 E+00	1.73%	9.78E-01	Positive, 4
Run 4, Plate 210430:2kf	6.5	3.14 E+00	1.30%	7.32E-01	Positive, 4
Run 5, Plate 210512:2kf	6.5	7.51 E+00	2.14%	2.69E+00	Positive, 2
Run 6, Plate 210629:2kf	10.3	6.18 E+00	0.92%	1.58 E+00	Positive, 3
Run 7, Plate 210630:2kf	3.3	6.44 E+00	1.56%	8.82E-01	Positive, 3
Mean*	**NR**	**5.90E+00**	**NR**	**1.18E+00**	**NR**

** Geometric mean for IC_{50} and IC_{20},. NR = not relevant.*

Table 14. Reporting of QuantiLum inhibition data, test item 8P06603:5, Genistein

Run number and plate number	Plate dynamic range	IC_{50} (µM]	CV (Log IC_{50}) (%)	IC_{20} (µM]	Classification & # concentrations > 20%
Run 3, Plate 210605:2kf	10466	1.59E+00	0.29	3.51E-01	Positive, 4
Run 4, Plate 210616:2kf	3	3.98E+00	0.71	1.62E+00	Positive, 3
Run 7, Plate 210630:5kf	8312	2.85E+00	0.35	9.69E-01	Positive, 3
Run 8, Plate 210701:2kf	8837	2.52E+00	0.32	7.48E-01	Positive, 4
Mean*	**NR**	**2.60E+00**	**NR**	**8.01E-01**	**NR**

** Geometric mean for IC_{50}, IC_{20}, PCI_{50} and PCI_{20}. NR = not relevant.*

8P06603:6, PTU

The test item showed an inhibitory effect on TPO, see Figure 24. The inhibition was specific to TPO as the control assay QLI was mostly negative. The test item was not cytotoxic to FTC-238 cells at any of the evaluated concentrations. The parameters describing inhibitory properties for the valid AUR-TPO runs are presented in Table 15, and the parameters for the QLI runs are presented in Table 16.

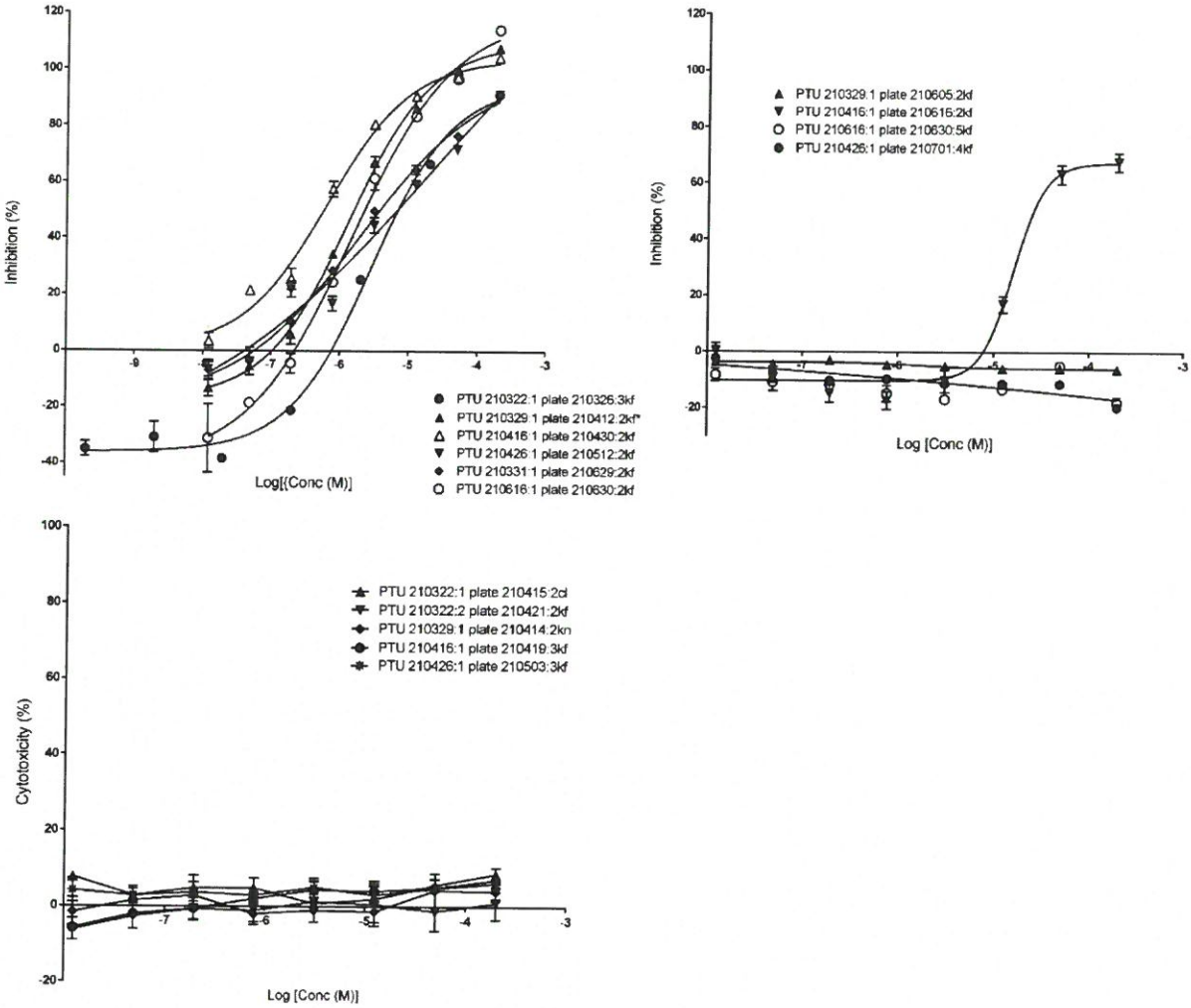


Figure 24. Top left: TPO inhibition dose-response curves; top right: QLI control assay data, and bottorm: CTG control assay data, for test item 8P06603:6, PTU. Error bars represent the standard deviation (n=3).

Table 15. . Reporting of TPO inhibition data, test item 8P06603:6, PTU.

Run number and plate number	Plate dynamic range	IC_{50} (µM]	CV (Log IC_{50}) (%)	IC_{20} (µM]	Classification & # concentrations > 20%
Run 1, Plate 210326:3kf	5.5	2.83E+00	1.24%	4.29E-01	Positive, 3 (range finding)
Run 3, Plate 210412:2kf	3.5	1.33E+00	0.53%	1.96E-01	Positive, 5
Run 4, Plate 210430:2kf	6.5	6.43E-01	1.21%	9.15E-02	Positive, 7
Run 5, Plate 210512:2kf	6.5	2.14E+01	27.90%	7.13E-02	Positive, 5
Run 6, Plate 210629:2kf	10.3	1.97E+00	1.35%	1.07E-01	Positive, 5
Run 7, Plate 210630:2kf	3.3	1.44E+00	1.21%	1.33E-01	Positive, 5
Mean*	**NR**	**2.29E+00**	**NR**	**1.41E-01**	**NR**

** Geometric mean for IC_{50}, IC_{20}, PCI_{50} and PCI_{20}. NR = not relevant.*

Table 16. Reporting of QuantiLum inhibition data, test item 8P06603:6, PTU.

Run number and plate number	Plate dynamic range	IC_{50} (µM]	CV (Log IC_{50}) (%)	IC_{20} (µM]	Classification & # concentrations > 20%
Run 3, Plate 210605:2kf	10466	N/A	N/A	N/A	Negative, 0
Run 4, Plate 210616:2kf	3	1.58E01	1.1	9.34E00	Positive, 2
Run 7, Plate 210630:5kf	8312	N/A	N/A	N/A	Negative, 0
Run 8, Plate 210701:2kf	8837	N/A	N/A	N/A	Negative, 0

Classification of test items

A summary of the findings is presented in tables 17 and 18. The selectivity value used for classification is calculated according to

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

and is presented in table 17. A test item with inhibition ≥ 20% for any tested concentration and Selectivity > 0 is classified as positive in the AUR-TPO assay, and test items with inhibition ≥ 20% but Selectivity ≤ 0 is a false positive. All other results are classified as negative.

Table 17. Determination of the selectivity value.

RISE ID, identity	AC_{20} AUR-TPO assay (µM)	AC_{20} QLI assay (µM)	AC_{20} CTG assay (µM)	Selectivity value[1]
8P06603:1, MMI	9.44E-04	N/A[2]	N/A	12
8P06603:2, BP2	3.87E-02	1.71E+01[2]	N/A	10
8P06603:3, BP3	N/A	1.28E+01[3]	N/A[4]	N/A
8P06603:4, DEP	N/A	Not evaluated	N/A	N/A
8P06603:5, Genistein	1.18E+00	8.01E-01	N/A	-0.17
8P06603:6, PTU	1.41E-01	N/A[3]	N/A	6.9

1. The selectivity value is calculated using the logarithm of the AC_{20} values expressed in M, not in µM.
2. The test item showed an effect in one of four runs, with an AC_{20} of 9.6 µM. It was negative in all other runs.
3. The test item showed an effect in one of four runs, with an AC_{20} of 9.3 µM. It was negative in all other runs.

Table 18. Summary of found TPO inhibitory properties. Ambiguous curve fits are indicated by a "~" sign before a calculated value. Negative results are indicated by "N/A".

RISE ID, identity	AC_{50} AUR-TPO assay (µM)	AC_{50} QLI assay (µM)	AC_{50} CTG assay (µM)	Classification
8P06603:1, MMI	3.71E-02	N/A[1]	N/A	Positive
8P06603:2, BP2	1.79E-01	3.08E+01[2]	N/A	Positive
8P06603:3, BP3	N/A	3.39E+01[3]	N/A[4]	Negative
8P06603:4, DEP	N/A	Not evaluated	N/A	Negative
8P06603:5, Genistein	5.90E+00	2.6E+00	N/A	False positive-
8P06603:6, PTU	2.29E+00	N/A[5]	N/A	Positive

4. The test item showed an effect in one of four runs, with an AC_{50} of 24.2 µM. It was negative in all other runs.
5. Ambiguous curve fit for one of four runs, which might affect this value to be lower than it should be.
6. Ambiguous curve fit for two of four runs, which might affect this value to be lower than it should be.
7. The test item showed a response >20% for the highest tested concentration, but no curves were fit to the data.
8. The test item showed an effect in one of four runs, with an AC_{50} of 15.8 µM. It was negative in all other runs.

Conclusion

Valid data sets with at least five runs were obtained for all test items submitted. A summary of the results is shown in Table 18.

Quality assurance

The quality assurance statement for this study is found in appendix 2.

The test facility has registration number 7983 and is approved according to the OECD Principles on Good Laboratory Practice (GLP) to perform *in vitro* toxicity studies with cell systems and tissues. This study was not performed under GLP. The quality of the study and of generated data was ensured by applying the following measures:

- Consistent documentation
- Internal QA review of produced data
- Change control of the applied SOPs, data analysis forms, study log templates, data recording files for the plate reader, etc.
- Validated templates for assisting work in the laboratory, e.g. study logs that perform calculations for dilution series and preparation of reagents
- Validated data analysis forms
- Calibrated and fit-for-purpose equipment and facilities
- Qualified personnel properly trained for the method according to facility routines

Records

All documents concerning this assignment will be archived at RISE for 10 years after the study completion date. After 10 years the documentation will be subject to destruction unless specific and written instruction to return them to the sponsor has been submitted to RISE.

Remaining test. reference and control items from this study will be kept until the end of the validation study (i.e. until after study 3). and. if so requested by the sponsor. returned to the sponsor. At the end of the validation study. all items will be disposed of unless return is requested.

References

- OECD (1998a). Organisation for Economic Co-operation and Development series on Principles of Good Laboratory Practice and Compliance Monitoring. Number 1. OECD Principles on Good Laboratory Practice (as revised in 1997). OECD Environmental Health and Safety Publications. Environment Directorate: ENV/MC/CHEM(98)17. Paris. France: OECD.
- 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.

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Chemistry and Materials - Medical Device Technology

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Appendices

1. Quality Assurance Statement
2. AUR-TPO assay, data for reference and control items
3. QLI assay, data for reference and control items
4. CTG assay, data for reference and control items

Appendix 1

Quality Assurance Statement

Inspections have been performed according to the quality assurance program specified in SOP KM 12003, these are summarized below.

	Study-based Inspection(s) of 8P06603:A
Phases included:	Analysis, data, and control charts.
Date of inspection(s):	2021-09-06--23
Date of inspection report:	2021-09-23

The study report of 8P06603:A is complete and accurately reflects the conduct and raw data of the study.

RISE Research Institutes of Sweden AB
Chemistry and Materials - Medical Device Technology

Henrik Bäckdahl
Quality Assurance

2021-09-24
Date

Appendix 2

AUR-TPO assay, data for reference and control items

Data for reference item MMI, negative control item BP3 and positive control item PTU are presented in tables A3.1-A3.3 below. The data corresponds to figures 4-9 in the report.

Table A3.1. Data for reference item MMI, corresponding to figures 4-7 in the report.

Exp #	Plate ID	TPO efficiency	Plate dynamic range	Z-factor	AC_{50} (M)	Experiment accepted?	Plate accepted?
1	210326:1kf	21.8	10.1	0.86	2.04E-01	yes	yes
1	210326:2kf	13.0	6.4	0.84			yes
1	210326:3kf	8.61	5.5	0.71			yes
2	210407:1kf	18.2	10.4		N/A	No	no
2	210407:2kf	9.74	6.9				no
2	210407:3kf	7.24	5.0				no
3	210412:1kf	20.5	6.0	0.91	2.90E-02	yes	yes
3	210412:2kf	6.42	3.5	0.45			t.b.d, z factor too low
3	210412:3kf	3.67	2.7	0.65			yes, despite plate dynamic range too low
4	210430:1kf	21.6	7.3	0.89	3.07E-02	yes	Yes
4	210430:2kf	15.5	6.5	0.67			Yes
4	210430:3kf	10.9	5.6	0.82			Yes
5	210512:1kf	20.6	7.3	0.86	2.24E-01	yes	Yes
5	210512:2kf	15.9	6.5	0.6			Yes
5	210512:3kf	11.0	6.0	0.7			Yes
6	210629:1kf	17.8	12.9	0.8	0.1405	yes	Yes
6	210629:2kf	13.0	10.3	0.9			Yes
6	210629:3kf	9.25	7.6	0.9			Yes
7	210630:1kf	17.1	4.1	0.9	7.58E-02	yes	Yes
7	210630:2kf	8.81	3.3	0.7			Yes
7	210630:3kf	5.87	2.9	0.6			yes,despite plate dynamic range too low

Table A3.2. Data for negative control item BP3, corresponding to figure 8 in the report.

Exp #	Plate ID	Relative inhibition (%)	Experiment accepted?
1	210326:1kf	-1.8	yes
2	210407:1cl	5.6	no (not due to NC)
3	210412:1kf	-1.8	Yes
4	210430:1kf	8.7	yes
5	210512:1kf	-11.0	yes
6	210629:1kf	-13.6	yes
7	210630:1kf	-3.0	Yes

Appendix 2

Table A3.3. Data for positive control item PTU, corresponding to figure 9 in the report.

Exp #	Plate ID	Relative inhibition (%)	Experiment accepted?
1	210326:1kf	85.6	Yes
2	210407:1cl	95.1	no (not due to PC)
3	210412:1kf	95.7	Yes
4	210430:1kf	98.2	yes
5	210512:1kf	73.5	yes
6	210629:1kf	76.3	yes
7	210630:1kf	98.3	yes

Appendix 3

QLI control assay, data for reference and control items

Data for reference item luciferase inhibitor II, negative control item BP3 and positive control item luciferase inhibitor I are presented in tables A4.1-A4.3 below. The data corresponds to figures 10-14 in the report.

Table A4.1. Data for reference item luciferase inhibitor II, corresponding to figures 10-12 in the report.

Exp #	Plate ID	Plate dynamic range	Z-factor	AC_{50} (M)	Experiment accepted?	Plate accepted?
1	210605:1kf	9680	0.94	2.99E-03	yes	yes
1	210605:2kf	10466	0.88			yes
2	210616:1kf	4	0.75	4.73E-03	Yes	yes
2	210616:2kf	3	0.74			yes
2	210616:3kf	34	0.76			yes
3	210630:4kf	7190	0.85	1.32E-02	Yes	yes
3	210630:5kf	8312	0.89			yes
4	210701:1kf	8811	0.97	4.29E-3	Yes	yes
4	210701:2kf	10303	0.95			yes

Table A4.2. Data for negative control item BP3, corresponding to figure 13 in the report.

Exp #	Plate ID	Relative inhibition (%)	Experiment accepted?
1	210605:1kf	15.5	Yes
2	210616:1kf	7.7	Yes
3	210630:4kf	5.8	Yes
4	210701:1kf	10.6	Yes

Table A4.3. Data for positive control item luciferase inhibitor I, corresponding to figure 14 in the report.

Exp #	Plate ID	Relative inhibition (%)	Experiment accepted?
1	210605:1kf	93.3	yes
2	210616:1kf	123.4	Yes
3	210630:4kf	90.4	Yes
4	210701:1kf	90.7	yes

Appendix 4

CTG control assay, data for reference and control items

Data for reference item DCNQ, negative control item BP3 and positive control item DCNQ are presented in tables A5.1-A5.3 below. The data corresponds to figures 15-19 in the report.

Table A5.1. Data for reference item DCNQ, corresponding to figures 15-17 in the report.

Exp #	Plate ID	Plate dynamic range	Z-factor	AC_{50} (M)	Experiment accepted?	Plate accepted?
1	210414:1kn	173.3	0.89	4.19E+00	yes	yes
1	210414:2kn	160.2	0.87			yes
1	210414:3kn	790.9	0.93			yes
2	210415:1cl	175.1	0.91	3.45E+00	Yes	yes
2	210415:2cl	176.6	0.84			yes
2	210415:3cl	573.8	0.74			yes
3	210419:1kf	172.7	0.93	6.16E+00	Yes	Yes
3	210419:2kf	176.9	0.90			yes
3	210419:3kf	811.2	0.86			yes
4	210421:1kf	173.5	0.91	4.40E+00	Yes	yes
4	210421:2kf	165.9	0.92			yes
4	210421:3kf	639.4	0.96			yes
5	210503:1kf	196.4	0.93	3.86E+00	Yes	yes
5	210503:2kf	185.2	0.93			yes
5	210503:3kf	526.6	0.90			yes

Table A5.2. Data for negative control item BP3, corresponding to figure 18 in the report.

Exp #	Plate ID	Relative inhibition (%)	Experiment accepted?
1	210414:1kn	15.1	Yes
2	210415:1cl	20.7	Yes*
3	210419:1kf	5.8	Yes
4	210421:1kf	31.6	Yes*
5	210503:1kf	14.3	Yes

* The negative control was outside of the acceptance criteria in the SOP. These might have been set too tight at assay implementation in the lab. Very few data points were the basis for the limits and thus limits might need to be adjusted for further experiments.

Table A5.3. Data for positive control item DCNQ, corresponding to figure 19 in the report.

Exp #	Plate ID	Relative inhibition (%)	Experiment accepted?
1	210414:1kn	94.3	yes
2	210415:1cl	99.3	Yes
3	210419:1kf	97.7	Yes
4	210421:1kf	99.0	yes
5	210503:1kf	97.2	yes

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