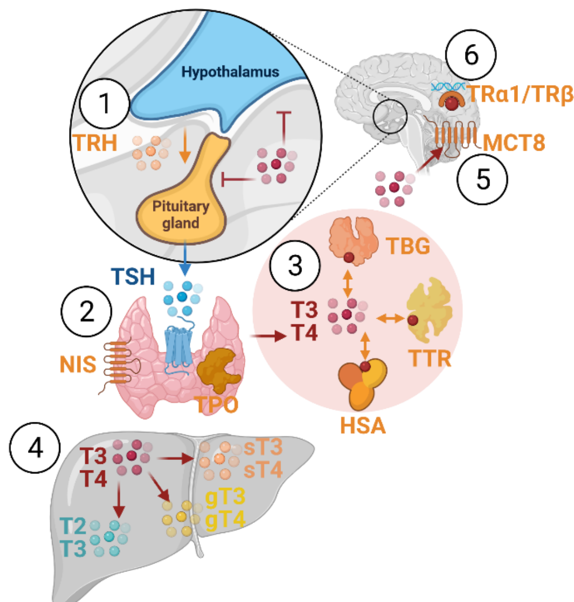
European Commission

STANDARD OPERATING PROCEDURE

for the human thyroid hormone receptor alpha (TRα) and beta (TRβ) reporter genes transactivation assay measuring agonist activity - version 02

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

Caviola, E. and Sherf, B.



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 describes the experimental procedure to run the "*human thyroid hormone receptor alpha (TRα) and beta (TRβ) reporter genes transactivation assay measuring agonist activity*"'. The method was developed by INDIGO Biosciences, Inc. and subsequently implemented by the EU-NETVAL test facility Vitroscreen S.r.l. (Italy) within the validation study. The SOP was approved the 13th of May 2022 and used during part 2 of the validation study.

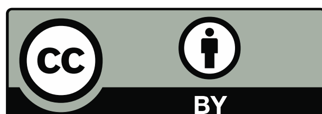
Contact information
Method developer:
INDIGO Biosciences, Inc
Bruce Sherf, PhD
3006 Research Drive
Suite A1
State College, PA 16801 USA
www.Indigobiosciences.com

EU-NETVAL facility
VitroScreen Srl
Marisa Meloni, CEO
Via Mosè Bianchi, 103
20149 Milan (IT)
www.vitroscreen.com

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

JRC132776

Ispra: European Commission, 2023
© European Union, 2023



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

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

How to cite this report: Caviola, E. and Sherf, B., *Standard operating procedure for the human thyroid hormone receptor alpha (TRα) and beta (TRβ) reporter gene transactivation assay measuring agonist activity, approved 13 May 2022 and applied in Part 2 of the EURL ECVAM thyroid validation study,* JRC132776, European Commission, Ispra, 2023.

STANDARD OPERATING PROCEDURES
Human thyroid Hormone Receptor Alpha and Beta Reporter Assays

Index

1 Contents

1 General Information 2
2 Scope of the SOP 2
2.1 SOP steps 2
2.2 SOP changes from previous version 3
3 Principles of the assays 3
3.1 INDIGO's Thyroid Hormone Receptors Assays 3
3.2 INDIGO's Live Cell Multiplex Assay (LCMA) 3
4 Proprietary and/or Confidentiality Issues 4
5 Health and Safety Issues 4
6 Definitions and Abbreviations 4
7 Assay Test Kits, Reagents, Consumable Materials, Equipment and Reagents 5
7.1 Assay Test Kits to be acquired by the testing lab 5
7.2 Reagents and Materials Provided with the INDIGO Assay Kits 6
7.3 Reference and control items 6
7.4 INDIGO Assay Kit Storage 6
7.5 Reagents to be provided by the testing lab 7
7.6 Equipment Requirements of the testing lab 7
7.7 Consumable Materials to be provided by the testing lab 7
8 Preparation of test item Stock Solutions and confirming TI solubility in Assay and Treatment Media 7
8.1 Preparing test item stocks 8
8.2 Test item solubility 8
8.3 Test item solubility in Treatment Medium (CSM) 9
8.4 Test item insolubility in CSM 9
9 Dose-range finding 10
9.1 Preparation of the assay plates and exposure of the TR reporter cells 10
9.2 Quantification of luminescence and fluorescence 13
9.3 DATA ANALYSES Dose-range finding 13
9.3.1 Data analysis Dose-range finding TRα and TRβ Assays 13
9.3.2 ACCEPTANCE CRITERIA dose-range finding TRα and TRβ Assays 14
9.3.3 ACTVITY THRESHOLD ASSESSMENT_TI activities 15

9.3.4 Data analyses cytotoxicity Live Cell Miltiplex Assay. 15
9.3.5 ACCEPTANCE CRITERIA Cytotoxicity Live Cell Multiplex Assay....16
9.3.6 CYTOTOXICITY THRESHOLD ASSESSMENT_TI treatment concentrations. 16
10 TR Activity Assessment 17
10.1 Preparation of the assay plates and exposure of the TR reporter cells 18
10.2 Quantification of luminescence and fluorescence 20
10.3 DATA ANALYSES TR activity assessment....21
10.3.1 10.3.1 Data analysis for TRa and TRb assays 21
10.3.2 ACCEPTANCE CRITERIA for TRα and TRβ Assays....22
10.3.3 Determine TR assay metrics for TI treatments. 23
10.3.4 LCMA Data analyses. 23
10.3.5 ACCEPTANCE CRITERIA_LCM Assays....24
10.3.6 ACCEPTANCE CRITERIA for TR data based on CYTOTOXICITY ASSESMENT 24
11 Bibliography 25

1 General Information

This SOP incorporates steps for running INDIGO Biosciences' Human Thyroid Hormone Receptors Alpha and Beta Reporter Assay Systems, as well as the Live Cell Multiplex Assay.

Corresponding Autor(s):
Elisa Caviola, Vitroscreen srl (Milan, Italy), elisa.caviola@vitroscreen.com
Bruce Sherf, INDIGO Biosciences, Inc. (State College, PA, USA), bhserf@indigobiosciences.com

Date of SOP creation: 29-02-2020
Last Update: version 2, 13-05-2022 (Vitroscreen rev)

2 Scope of the SOP

2.1 SOP steps

This SOP encompasses three discreet steps, as follows.

- Solubility testing: The specific aims of solubility testing are to *i.)* generate stock solutions of Test Items (TI) that will be diluted 500 times in the assays, using DMSO as the preferred solvent, and *ii.)* determine the solubility of the TIs in assay treatment medium.

- Dose range finding: The specific aims of dose-range finding are to *i.)* conduct a preliminary determination of TI agonist activities against TRa and TRb, and *ii.)* establish the respective cytotoxicity thresholds of those TIs determined to exhibit 'positive' agonist activities.

- TR activity assessment: The specific aims of TR activity assessment are to i.) conduct a more finely tuned assessment of a "positive" TI's activity metrics, and ii.) to repeat the cytotoxicity assessment. Additional Positive and Negative Control items are included in the procedure for TR activity

assessment. No further analyses will be conducted on those TIs determined in the dose-range finding to be devoid of activity against both TRa and TRb.

2.2 SOP changes from previous version

The SOP has been modified in the following points:

- Par 9.3.2 Acceptance Criteria dose range finding TRα and TRβ assays: modification of Fold-Activation (FA) threshold values for both TRα and TRβ
- Par 9.3.5 Acceptance Criteria Cytotoxicity live Cell Multiplex Assay: elimination of Z' as criteria for LCMA cytotoxicity assay
- Par. 10.1 Preparation of the assay plates and exposure of the TR reporter cells: Step 7a_addition of an alternative dilution factor for TI treatments preparation; Step 7f_addition of preparation of RI EC_{100}.
- Par. 10.3.2 Acceptance criteria for TRα and TRβ assays: modification of Fold-Activation (FA) threshold values for both TRα and TRβ; Modification of %RA threshold of PC
- Par 10.3.5 Acceptance Criteria Cytotoxicity live Cell Multiplex Assay: elimination of Z' as criteria for LCMA cytotoxicity assay

3 Principles of the assays

3.1 INDIGO's Thyroid Hormone Receptors Assays

The TRα and TRβ assays are luminescence-based assay systems; endpoint readings are in terms of Relative Luminescence Units (RLU). Each assay utilizes Human Embryonic Kidney cells engineered to provide constitutive, high-level expression of the Thyroid Hormone Receptor alpha (NR1A1) or Thyroid Hormone Receptor beta (NR1A2). These reporter cells express hybrid thyroid hormone receptors in which their respective native N-terminal DNA Binding Domain (DBD) sequence have been replaced with that of the yeast GAL4 DBD sequence. Accordingly, the resident luciferase reporter gene is functionally linked to a tandem array of GAL4 upstream activation sequences (UAS). Thus, quantifying changes in luciferase expression in the treated *vs.* untreated reporter cells, following 24 hr exposure to a test item, provides a specific and sensitive measure of changes in TR activity without collateral induction/suppression of the target genes that are otherwise regulated by the activated TR transcription factors.

3.2 INDIGO's Live Cell Multiplex Assay (LCMA)

The LCMA is a fluorescence-based assay; endpoint readings are in terms of Relative Fluorescence Units (RFU). The LCMA quantifies relative changes in the number of live cells remaining in assay wells following 24 hr exposure to a test item (TI). The assay protocol and chemistry are optimised to be run in multiplex with any of INDIGO's other 96-well luminescence-based nuclear receptor assays. The LCMA utilises Calcein-AM, a hydrophobic, non-fluorescent molecule that readily crosses cellular membranes. Once in the intracellular environment, it is hydrolyzed by endogenous esterases in a time- and temperature-dependent manner. The resulting product, calcein, is both hydrophilic and highly fluorescent. Due to its high charge density there is no appreciable loss/efflux of calcein from the intracellular compartment during the short reaction period of the LCM Assay. Normalizing calcein fluorescence measured from the test item-treated cells against the fluorescence measured from the untreated (Solvent Control) cells provides a sensitive, quantitative measure of the relative numbers of live cells remaining in the treated assay wells.

The described *in vitro* method is based on two separate and distinct assay readouts (Cytotoxicity-based Dose-Finding and Thyroid Hormone Receptor activation) performed in a multiplexed format. Therefore, a single procedure that unifies these two assays is presented in step-by-step detail.

4 Proprietary and/or Confidentiality Issues

INDIGO Biosciences maintains as proprietary all information pertaining to expression and reporter vector sequences, the identity of genetic regulatory elements and their configurations, as well as the formulations of all media components, detection reagents, and cryo-preserved reporter cells. Assay kit components are for research purposes only. They are not for therapeutic, diagnostic, or contact use in humans or animals.

5 Health and Safety Issues

a.) General Precautions

General safety instructions have to be followed and appropriate protective safety equipment worn at all times. Unknown and coded chemicals have to be considered potential endocrine disruptors and must be handled with extreme care, as well as negative control, positive control and reference item.

b.) Hazard Statement (H) / Precautionary Statement (P):

Triiodothyronine (T3): H302
17β-Estradiol (E2): H351-H360Fd-H362 / P201-P263-P281-P308 + P313
Sobetirome: Not hazardous compound according to GHS
Staurosporine: H340-H350 / P201-P202-P280-P308 + P313-P405-P501

6 Definitions and Abbreviations

Acceptance criteria: Criteria for when results can be accepted, i.e. a set of well-defined parameters describing aspects of the *in vitro* method such as range for positive and negative controls. All acceptance criteria have to be met for an experiment to be considered valid.
Agonist: A substance that binds to a specific receptor and triggers a response in the cell. It mimics the action of an endogenous ligand that binds to the same receptor.
CV: Coefficient of variation
Cytotoxicity: Harmful effects to cell structure or function ultimately causing cell death. It can be reflected by a reduction in the number of cells present in the well at the end of the exposure period or a reduction of the capacity for a measure of cellular function when compared to the concurrent vehicle control.
DMSO: Dimethyl sulfoxide
E2: 17-beta-Estradiol; CAS# 50-28-2
EC_{50}: The half-maximal effective concentration of a test chemical
FA: Fold Activation. Test Item (TI) activity normalized to Solvent Control (SC) activity.
LCMA: Live Cell Multiplex Assay
LCMA-PC: Live Cell Multiplex Assay Positive Control Cytotoxicant Staurosporine (CAS# 62996-74-1. Staurosporine
%LC: Percent (%) Live Cells
NC: Negative control item. Separate part of a test system treated with a chemical for which it is known that the test system will not respond. In this SOP, the negative control item E2 provides evidence that the test system is not responsive under the actual conditions of the assay.
PBS w/o CaMg: Phosphate Buffered Solution without Ca2+ and Mg2+
PC: Positive control item. Separate part of the test system treated with a chemical for which it is known that the test system will respond. In this SOP the positive control item Sobetirome provides evidence that the test system is responsive under the actual conditions of the assay.
%RA: Percent (%) Activation relative to the maximal activation value of RI (*i.e.,* RI EC_{100}). RI, PC, NC and TI activities at each of their respective treatment concentration will all be normalized to RI EC_{100}.
RI: Reference item. A chemical used to provide a basis for comparison with the test item. In this SOP T3 is the reference item.
RI EC_{50}: Concentration of the reference item T3 at which 50% of its maximum response is observed.
RI EC_{100}: Concentration of the reference item T3 at which maximum response is observed

RFU: Relative Fluorescence Unit
aRFU: Average Relative Fluorescence Unit
RLU: Relative Luminescence Unit
aRLU: Average Relative Luminescence Unit
SC: Solvent control. The solvent (vehicle) that is used to dissolve TI, RI, PC and NC items. The SC treatment media contains solvent only, without dissolved chemical.
TI: Test item, means an article that is the subject of a study.
TI1-%RA$_{10}$, TI1-%RA$_{50}$ and TI1-%RA$_{80}$: The concentration of a TI that produces and activation response that is equivalent to 10%, 50% or 80% of the maximum activation by the reference item.
Treatment Media: a generic descriptor for cell culture media prepared to contain either TI, RI, PC, NC or SC, which is then applied to the TR reporter cells.
Z': Z' is the industry standard method to assess reference compound performance to determine if an assay is sufficiently robust for downstream screening applications. As described by Zhang, *et. al.* (1999), the theoretical limits of Z' scores are 0 – 1. Z' scores that fall below 0.5 identify failed assays, unsuitable for screening applications. Z' scores ranging between 0.5 – 1 are suited for screening applications, with increasingly higher scores correlating to higher overall assay robustness. Because Z' calculations incorporate standard deviation (SD) values, the score not only provides a readout on assay performance, but also of the precision in a user's lab technique and instrument performance.

7 Assay Test Kits, Reagents, Consumable Materials, Equipment and Reagents

7.1 Assay Test Kits to be acquired by the testing lab

The TR alpha (NR1A1, TRα) and TR beta (NR1A2, TRβ) 96-well format assays and LCM Assay kits are procured from INDIGO Biosciences (www.indigobiosciences.com). Each LCMA kit is configured to process one 96-well TR assay plate. The following table details the kit requirements for screening test items against each individual TRa and TRb assay, with each plate incorporating the Live Cell Multiplex Assay.

Dose-range finding: Preliminary assessments of TI activities against TRα and TRβ and Cytotoxicity thresholds.			
# Test Items to be screened	TRα Assay Kit, 96-well # IB01001_EUC	TRβ Assay Kit, 96-well # IB01101_EUC	Live Cell Multiplex Assay, 96-well LCM-01*
1 - 3	1	1	2
4 - 6	2	2	4
7 - 9	3	3	6
10 - 12	4	4	8
13 - 15	5	5	10
16 - 18	6	6	12

* LCMA may also be purchase as 5x 96-well format (LCM-05) and 10x96-well format (LCM-10)

TR activity assessment: Determine activity metrics for each TI demonstrated to be 'positive' against TRα or TRβ			
# of Positive Test Items	TRα Assay Kit, 96-well # IB01001_EUC	TRβ Assay Kit, 96-well # IB01101_EUC	Live Cell Multiplex Assay, 96-well LCM-01
1	1	1	2
2-3	2	2	4

In addition to TR Reporter Cells, each kit provides two optimized media for use during cell culture and in diluting test samples, the reference agonist Triiodothyronine (T3), Sobetirome as an additional positive

control, 17-b-Estradiol (E2) as a physiologically relevant negative control, Luciferase Detection Reagent, and a cell culture-ready 96-well assay plate. INDIGO's Human TRa and TRb Assay kits each contain sufficient materials to run one 96-well assay plate. The respective aliquots of TR Reporter Cells are provided as single-use reagents. Once thawed, they can NOT be refrozen or maintained in extended culture with any hope of retaining downstream assay performance.

7.2 Reagents and Materials Provided with the INDIGO Assay Kits

The reagents and materials listed below are provided in each of the INDIGO TRa and TRb assay kits. Note that only the TRα and TRβ reporter cells are different between the two assay kits. All other reagent and media formulations are identical and may be used interchangeably during assay setups.

- TRα or TRβ reporter cells, cryopreserved
- Cell Recovery Medium
- Compound Screening Medium
- Detection Substrate
- Detection Buffer
- 96-well collagen-coated assay plate (white, sterile, cell-culture ready)
- LCMA Buffer
- LCMA Substrate
- Reference Agonist, T3 (3,3',5-TriIodo-L-thyronine, CAS# 6893-02-3); 50 µM stock in DMSO, to use as a 500x-concentrated solution.
- Positive Control Agonist, Sobetirome (also known as GC 1, CAS# 211110-63-3); 500 µM stock in DMSO, ready to use as a 500x-concentrated solution.
- Negative Control, E2 (17-beta-Estradiol, CAS# 50-28-2); 500 µM stock in DMSO, ready to use as a 500x-concentrated solution.

The following reagents are provided in the INDIGO LCMA kit

- LCMA Buffer
- LCMA Substrate, 300x-concentrated
- Positive Control Cytotoxicant Staurosporine (CAS# 62996-74-1); 4.0 mM stock in DMSO, ready to use as a 500x-concentrated solution.

7.3 Reference and control items

The dose-range finding and TR activity assessment procedures utilize reference and control items that are supplied in the INDIGO assay kits. They are provided as ready-to-use 500x-concentrated solutions prepared in DMSO.

a.) Used for dose-range finding and TR activity assessment:

Reference (RI): T3, 50 µM
LCMA Positive Control (LCMA PC) cytotoxicant: Staurosporine 4,000 µM

b.) Used only for TR activity assessment:

Positive Control (PC): Sobetirome, 500 µM
Negative Control (NC): E2, 500 µM

7.4 INDIGO Assay Kit Storage

Assay kits are shipped on dry ice. Upon receipt, individual kit components must be stored at the temperatures indicated on their respective labels. More simply, the entire kit may be stored at -80°C until use.

7.5 Reagents to be provided by the testing lab

- Dimethylsulfoxide (DMSO, anhydrous): CAS 67-68-5; 50 ml bottle that has been opened to air for less than 3 months. Opened bottles of DMSO should be sparged with Ar or N2.
- 95 – 100% Ethanol
- PBS (without Ca^{+2} and Mg^{+2})
- 70% alcohol wipes, or squirt bottle + tissues
- Sterile ultrapure water or deionised water

7.6 Equipment Requirements of the testing lab

- Calibrated Incubator (37°C, 5% CO_2 and ≥ 70% humidified atmosphere) for mammalian cell culture
- Cell culture-rated laminar flow hood.
- Sonication 37°C water bath
- Calibrated 8-channel pipette, either an electronic repeat-dispensing or manual pipette and tips suitable for dispensing 50 µl, 100 µl, and 200 µl volumes
- Calibrated manual pipettes: 2.0, 10, 20, 100, and 1000 µl maximum dispensing volume
- Vortex mixer
- Software for non-linear regression analyses and curve-fitting of assay data.
- Plate reader capable of luminescence and fluorescence measurements, with the following minimum requirements

Requirements for Fluorescence Measure	Recommended Fluorescence filters [Ex:485nm | Em:535nm]
Requirements for Luminescence Measure	Integration time: 500 ms

7.7 Consumable Materials to be provided by the testing lab

- Container of dry ice
- Media basins, sterile
- *Plates for preparing serial dilutions of TI and RI stocks:* Clear 0.2mL PCR Strip tubes (e.g., Sigma AXYPCR0212C) <u>or</u> 96 well Polypropylene Deep Well V-Bottom (or U-Bottom) (e.g., Corning CLS 3957)
- *For preparing TI and RI treatment media:* Sterile, reusable multi-chamber media reservoirs (*e.g.,* Dual Solution Reservoir, Heathrow Scientific #HS20821A) <u>or</u> Sterilized 96 deep-well blocks (*e.g.,* Axygen Scientific, #P-2ML-SQ-C-S)
- Sample tube: Polypropylene round tube 5 mL, BD Falcon, #352054
- *For TI solubility testing:* 4 ml clear glass vial closed with a screw-top cap fitted with PTFE lined solvent-resistant seal (*e.g.,* Thermo Scientific B7800-2)

8 Preparation of test item Stock Solutions and confirming TI solubility in Assay and Treatment Media

OVERVIEW: Stock solutions of TIs are to be prepared the day before setting up the TR assays. DMSO is the first, and preferred, solvent to use in preparing TI stocks. The TI stocks will be diluted 500 times into cell culture media to prepare the treatment media used in the assays. Both TI stocks and diluted treatment media should be prepared fresh on the day of experiment.

Note: For confirmation of solubility, it is recommended to weigh sufficient amount of TI to prepare a minimum volume of 0.5 ml in a clear glass vial.

8.1 Preparing test item stocks

Prepare Test item stock solutions as follows:

i. Weigh a net amount (e.g., 10 mg to 25 mg) of each TI directly into a 1.5 ml or 2 ml clear glass vial that can be closed with a screw-top cap fitted with an O-ring or comparable solvent-resistant, liquid resistant seal.

ii. *Molarity Method*: in order to determine the volume of anhydrous DMSO (ml) required to prepare a 50 mM stock solution, knowing the molecular weight of a TI (g/mol or Dalton) and the weighted TI mass (mg), use the following formula:

$$\text{Volume solvent (ml)} = \frac{\text{Weight test item (mg)}}{\text{Molecular weight }\left(\frac{\text{g}}{\text{mol}}\text{ or Dalton}\right) * 50\text{ (mM)}} * 10^{03}$$

Dispense the determined volume of DMSO into the vial, cap it, then use a vortex mixer to gain complete dissolution of the TI. Confirm transparency of the solution. This stock will be diluted 500 times in final assay

Gravimetric Method: If the molecular weight of a TI is not available, the gravimetric method will be used, starting with 50 mg/ml in DMSO. Weigh a minimum of 25 mg and dispense the appropriate volume of DMSO into the vial, cap it, then use a vortex mixer to gain complete dissolution of the TI. Confirm transparency of the solution. This stock will be diluted 500 times in assay setup.

8.2 Test item solubility

The majority of small organic compounds can be successfully solubilised in DMSO. However, some TIs will require more effort to achieve complete solubility as a 50 mM or 50 mg/ml stock. If a TI is initially resistant to dissolution in DMSO apply the following procedures, in the order presented:

i. DMSO solutions may be warmed to 37°C for 10 minutes followed by extensive vortex mixing; this sequence may be repeat two more times. If available, a more effective method for solubilising recalcitrant TIs is to perform repeated 10 minute periods in a sonication water bath (37°C), followed by extensive vortex mixing. Check the solution for transparency.

ii. If dissolution of the TI as a 50 mM or 50 mg/ml stock was not successful, add a second equal volume of DMSO to the solution and repeat the mixing process. Check the solution for transparency. Note that this will reduce the stock concentration to 25 mM or 25 mg/ml, however it will still be used as a stock that will be diluted 500 times in the assay setup. Be sure to account for the change in starting concentration for this TI in the final data analyses.

iii. If dissolution of the TI as a 25 mM or 25 mg/ml stock was not successful, add a third equal volume of DMSO to the solution and repeat the mixing process. Check the solution for transparency. Note that this will reduce the stock concentration to 12.5 mM or 12.5 mg/ml, however it will still be used as a stock that will be diluted 500 times in the assay setup.

iv. If dissolution of the TI as a 12.5 mM or 12.5 mg/ml stock was not successful, then start over. Reweigh TI and prepare a stock solution of 5 mM or 5 mg/ml. Repeat steps i, ii and iii for a maximum of 2 times (i.e., to 1.25 mM or 1.25 mg/ml).

v. If it is not possible to achieve complete solubility of the TI as a 1.25 mM or 1.25 mg/ml stock in DMSO, then start over. Reweigh TI and use 95 – 100% Ethanol to generate a 50 mM or 50 mg/ml stock solution. Do NOT heat a solution prepared in ethanol! Check the solution for transparency. As

before, additional equal volumes of ethanol may be applied if necessary, resulting in a 25 and 12.5 mM or 25 and 12.5 mg/ml stock that will be used diluted 500 times in the assays setup.

vi. If the TI is not soluble as a 12.5 mM or 12.5 mg/ml stock in ethanol, then start over. Reweigh TI and prepare a stock solution of 5 mM or 5 mg/ml. Repeat points iv and v for a maximum of 2 times (i.e., to 1.25 mM or 1.25 mg/ml).

vii. If the TI is not soluble as a 1.25 mM or 1.25 mg/ml stock in ethanol, then start over. Reweigh the TI and use PBS(w/o CaMg) to generate a 50 mM or 50 mg/ml stock solution. This stock solution may be warmed to 37°C to aid in dissolving the TI. Check the solution for transparency. As before, an additional equal volume of PBS (w/o CaMg) may be applied if necessary, resulting in a 25 mM and 12.5 mM or 25 mg/ml and 12.5 mg/ml stock that will be diluted 500 times in the assays setup.

viii. If the TI is not soluble as a 12.5 mM or 12.5 mg/ml stock in PBS(w/o CaMg), then start over. Reweigh TI and prepare a stock solution of 5 mM or 5 mg/ml. Repeat points iv and v for a maximum of 2 times (i.e., to 1.25 mM or 1.25 mg/ml).

ix. If it is not possible to achieve 100% dissolution of a TI at a minimum stock concentration of 1.25 mM or 1.25 mg/ml in DMSO > Ethanol > PBS, then remove that chemical from the screening process.

8.3 Test item solubility in Treatment Medium (CSM)

Perform a 500-fold dilution of each prepared TI stock solution into INDIGO's Compound Screening Medium (CSM) supplied in the kit. This is accomplished by adding 2 µl of the TI stock solution into 998 µl CSM. Mix the solution using a vortex mixer. If the media appears hazy, warm the solution at 37°C for 10 minutes and vortex mix. If the media appears transparent, or only very slightly hazy without noticeable precipitate, the TI stock solution will be advanced to the dose-range finding procedure; store at +4°C. Discard the preliminary CSM test media.

8.4 Test item insolubility in CSM

If a TI is resistant to complete dissolution in CSM treatment media, producing significant haziness or precipitate in the solution, then apply the following procedures.

i. Prepare a 2-fold dilution of the TI stock using the original solvent that was used during its preparation (typically DMSO). Then repeat the process of making a 500-fold dilution into CSM, vortex as before; warm if necessary. Check the media for transparency. A slight haze is acceptable, and the TI stock solution will be advanced to the dose-range finding procedure; store at +4°C. Note that the TI stock concentration was reduced by half, however, it will still be diluted 500 times in the setup. Be sure to note the reduced concentration of concentrated stock.

ii. If the treatment media still presents a significant haze, use the original solvent to make second 2-fold dilution of the TI stock. Repeat the process of making a 500-fold dilution into CSM, vortex; warm if necessary. Check for solubility. A slight haze is acceptable, and the TI stock solution will be advanced to the dose-range finding procedure; store at +4°C. Note that the TI stock concentration was again reduced by half, however, it will be used as a stock in the assay setup. Be sure to note the reduced concentration of concentrated stock.

iii. Repeat point i for a maximum of 4 times. If the above steps fail to resolve TI insolubility in the treatment medium, remove the chemical from further screening.

9 Dose-range finding

Preliminary assessments of TI activities against TRα and TRβ and Cytotoxicity thresholds.

OVERVIEW

a.) Workflow. TIs are to be tested in the TRα and TRβ assays at the same time. This SOP integrates INDIGO's Live Cell Multiplex Assay (LCMA). Freshly prepared TI Stocks will be used for the assay. The Dose-range finding is completed over two days. **Figure 2** provides an overview of the multiplexed TR + LCM Assay workflow.

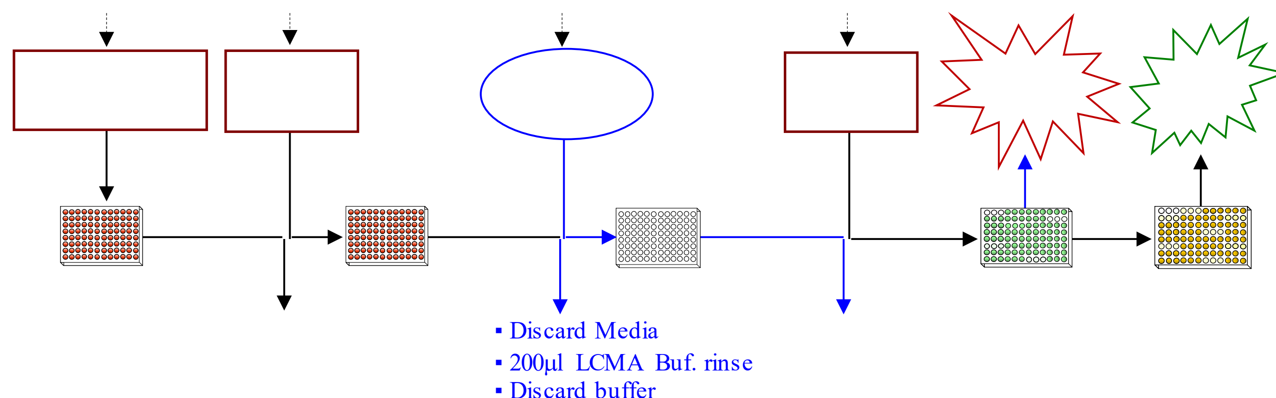


Figure 2. Overview of the **workflow for dose range finding and TR assays.** Text and arrows in blue font denote the LCM Assay.

b.) Plate layout Dose-range finding. The following procedure is scaled for the setup of one plate for *each* TR assay: TRα PLATE and TRβ PLATE. However, multiple assay plates for each TR may be setup at one time to maximise workflow efficiencies and sample throughput. PLATE setups for each TR assay are depicted in **Figure 3.**

TI treatment Conc.		1	2 (T1)	3 (T1)	4 (T1)	5 (T2)	6 (T2)	7 (T2)	8 (T3)	9 (T3)	10 (T3)	11	12
High	A												
	B		C1	C1	C1	C1	C1	C1	C1	C1	C1		
	C		C2	C2	C2	C2	C2	C2	C2	C2	C2	RI	
	D		C3	C3	C3	C3	C3	C3	C3	C3	C3	EC_{100} T3	
	E		C4	C4	C4	C4	C4	C4	C4	C4	C4	T3	
Low	F		C5	C5	C5	C5	C5	C5	C5	C5	C5		
	G		SC	SC	SC	LCMA PC (Stauro)			LCMA-BKG No cells + Solvent				
	H												
			Controls										

Figure 3. **Plate layout for the LCMA+TRα and LCMA+TRβ assays.** Each Plate setup consists of five treatment concentrations for each of three TI's, a fixed EC_{100} concentration of RI (T3), Solvent Controls (SC), a fixed concentration of the Positive Control cytotoxicant Staurosporine (LCMA PC) and wells devoid of cells but treated with SC treatment media to provide 'Background RFU' determinations in the LCM Assay (LCMA BKG). Average RFU from the SC wells will correlate to "100% Live Cells". Wells shaded grey will contain sterile ultrapure water or deionised water readouts from these wells will not be used.

9.1 Preparation of the assay plates and exposure of the TR reporter cells

(All steps must be performed using aseptic technique!)

This procedure details the processing of 2 assay plates, corresponding to "PLATE 1" for each TR, with a maximum of 3 TI's per run. However, the values highlighted in white font are 'scalable' numbers, meaning that they will be adjusted in accordance with the *actual* number of assay plates being run for each TR. *For*

example, if the number of TIs being run requires the use of 4 plates for each TR assay, then the numbers in white font will be multiplied by a factor of 4.

Step 1) Thaw Cell Recovery Medium (CRM) *and* Compound Screening Medium (CSM).
To set up 1 TRα and 1 TRβ assay plate:
a.) remove 4 tubes of CRM from freezer storage. Place the tubes in a 37°C water bath to thaw the media. Once thawed, leave CRM at 37°C until it is used in *Step 2*,
and,
b.) remove 2 tubes of CSM from freezer storage. Thaw CSM using a 37°C water bath. CSM will first be used in *Step 3*, and then again in *Step 8.*

Step 2) *Rapid* **Thaw of the Reporter Cells.**
First, retrieve the tubes of **CRM** from the 37°C water bath and sanitize their outside surfaces with a 70% ethanol swab.

Second, retrieve 1 tube of **TRα** AND 1 tube of **TRβ Reporter Cells** from -80°C storage, place them directly into a dry ice bucket and transport the cells to the laminar flow hood. When ready to begin, transfer the tubes of reporter cells into a rack and, *without delay*, perform a rapid thaw of the cells as follows:

a.) transfer 2x **9.5 ml** of 37°C **CRM** directly into the tube of frozen **TRα** cells, *and*
b.) transfer 2x **9.5 ml** of 37°C **CRM** directly into the tube of frozen **TRβ** cells.
c.) Place each tube of Reporter Cells into a 37°C water bath for 5 - 10 minutes.

The resulting volume of each individual cell suspension will be 21 **ml**.

Step 3) Setup **LCM Assay Background Control wells.** During the 5 – 10 minutes cell recovery period dispense **200 µl /well** of **CSM** into each of the three wells designated as "LCMA BKG_No Cells + solvent". Well locations are depicted in the PLATE 1 template provided in Figure 3.

Step 4) Dispense Reporter Cells.

a.) Retrieve from the water bath the tubes of the Reporter Cell Suspension. Sanitise the outside surface of each tube using a 70% alcohol swab. If more than 1 tube of cells was thawed for the TRα assays and the TRβ assays, pool the individual common tubes by combining them in a media basin; mix by gentle rocking. Use an electronic, repeat-dispensing, 8-chanel pipette to dispense **200 µl / well** of respective TR cell suspensions as follows:

PLATE 1: dispense cells into all wells *except* for those designated to be "LCMA BKG_No Cells + solvent" control wells and edge-wells (refer to Figure 3.)

PLATE 2, 3, 4 … *etc.*: dispense cells into all wells of the assay plates (refer to Figure 3.)

NOTE: Increased well-to-well variation will occur if cells are allowed to settle during the dispensing period. Take care to ensure precision in dispensing exact volumes across the assay plate.

b.) To mitigate the occurrence of 'edge-effects', remove 1 tip from the 8-chanel pipette and dispense **~ 100 µl** of sterile dH2O into all inter-well spaces within the assay plate(s).

Step 5) Pre-incubate reporter cells. Place the assay plate into a cell culture incubator for 4.5 hr ± 30 min.

During the 4.5 hr pre-incubation period, perform Steps 6 and 7:

Step 6) Prepare TI stocks and Control stocks.

a.) Prepare fresh stock solution as described in section 8

b.) Retrieve a tube of T3 from one of the TR assay kits. Retrieve a tube of Staurosporine from the LCM Assay kit. Allow the tubes to thaw and equilibrate to room temperature under low light.

Step 7) Dilute TI, T3, and DMSO to generate final concentrations of respective treatment media.
200 µl / well of each treatment media will be dispensed into the designated wells of the assay plate(s) (refer to **Figures 3)**. Follow the specific instructions, below, to prepare each treatment media.

a.) **Preparation of Test Item Treatment Media:** Follow the dilution scheme and treatment media preparation strategy presented in **Appendix 1**.
I*n brief*,
- Starting with the prepared Stocks of TIs, a total of five treatment concentrations will be prepared per TI. Use DMSO (or the alternate solvent selected on basis of solubility testing) to prepare serial 8-fold dilutions. Use 0.2 ml PCR strip-tubes, or U- or V-bottom plates, to prepare these intermediate dilutions.

- All intermediate TI stocks prepared in DMSO then undergo a 2-step dilution process (first dilution 1/20 followed by 1/25 dilution) using CSM to yield the final concentrations of treatment media for each TI. Use a sterile multi-chamber media reservoir or 96 deep-well block to prepare the final TI treatment media.

b.) **Preparation of RI Treatment Media:** Utilise the stock of **T3** (50 µM) from either of the TR assay kits. Transfer **5.0 µl** of T3 Stock into **2.495 ml** of CSM; mix. The resulting concentration is 0.10 µM, which is an EC_{100} concentration in both TR assays.

c.) **Preparation of LCMA-PC Treatment Media:** Utilise the stock of **Staurosporine** (4.0 mM) from one LCM Assay kit. Transfer **5.0 µl** of Staurosporine Stock into **2.495 ml** of CSM; mix. The resulting concentration is 8 µM, sufficient to elicit ≥ 85% cell death.

d.) **Preparation of SC Treatment Media:** The preparation of TI Treatment Media, as described above and depicted in Appendix 1, will also generate SC media. Assuming that an alternative solvent was not used during the preparation of the concentrated TI Stock solutions, the SC media will comprise **0.2% DMSO.** In *Step 9a*, SC treatment media will be dispensed into replicate wells seeded with cells, AND into wells with "no cells" and designated as LCMA BKG (no cells + solvent).

Step 8) At the end of the 4.5 hr ± 30 min pre-incubation period discard the culture media. To avoid trauma to the reporter cell, do not remove media by pipetting but perform a 'wrist-flick' to manually eject the liquid into a waste container. *Gently* tap the inverted plate onto a clean absorbent paper towel to remove residual droplets. Cells will remain tightly adhered to well bottoms.

Step 9) Dispense **Treatment Media.**
a.) Dispense **200 µl / well** of the prepared TI, RI, and SC treatment media into the designated wells of the assay plates (refer to **Figure 3**). For PLATE 1, dispense sterile ultrapure or deionised wated into wells shaded grey in **Figure 3**.

b.) As before, to mitigate the occurrence of 'edge-effects', dispense **~ 100 µl** of sterile dH2O into all inter-well spaces within the assay plate.

Step 10) Place the assay plate in a cell culture incubator for 24±1 hours.

Step 11) In preparation for next day's activities, retrieve the following reagents from freezer storage and place them in a dark refrigerator (4°C) to thaw overnight.
2 tubes of **Detection Substrate** from TR assay kits
2 tubes of **Detection Buffer** from TR assay kits

2 tubes of **LCMA Buffer** from LCM Assay kits
2 tubes of **LCMA Substrate** from LCM Assay kits

9.2 Quantification of luminescence and fluorescence

(Subsequent manipulations may be performed on a bench top)

Step 12) Equilibrate assay reagents to room temperature. Approximately 30 minutes prior to the end of the 24±1 hr incubation period, remove from the refrigerator the tubes of various assay reagents described in *Step 11*. Place them in a dark area to equilibrate to room temperature. Do NOT warm Detection Substrate above room temperature. Once at room temperature, *gently* invert each tube several times to ensure homogenous solutions.

Step 13) Prepare LCMA Detection Reagent. To perform the LCM Assay on 2 plates:

a.) Transfer **2 x 6 ml** of **LCMA Buffer** into a single tube, then

b.) Dispense into the LCMA Buffer **2 x 20 µl** of **LCMA Substrate**; mix gently. This preparation of *LCMA Detection Reagent* will be used in *Step 16*.

Step 14) Discard **treatment media from the TR Reporter cells.** At the end of the 24±1 hr incubation period discard treatment media from the assay plate. Manually eject the liquid into an appropriate waste container. *Gently* tap the inverted plate onto a clean absorbent paper towel to remove residual droplets.

Step 15) Rinse**.** Dispense **200 µl / well** of the **LCMA Buffer**. Gently rinse the wells by tilting the plate side-to-side 2-3 times, then manually eject the rinse buffer.

Step 16) Transfer preparations of **LCMA Detection Reagent** into a media basin. Dispense **50 µl / well** across the entire assay plate. Incubate the plate in a *dark location* at room temperature for **15 minutes**.

Step 17) During the 15 minute incubation period perform the following:

a.) turn on the plate reader and select *fluorescence* mode reads using the filter combination [Ex:485nm | Em:535nm]. Set the instrument to perform a single 5 second "plate shake" prior to reading the first assay well. For convenience, set the instrument to read all wells.

and,

b.) prepare **Luciferase Detection Reagent (LDR)**. For 2 assay plates, transfer the entire volumes of 2 tubes of **Detection Buffer** into 2 tubes of **Detection Substrate**, thereby generating a **2x 12 ml** volumes of **LDR**. Mix by *gently* inverting the tubes (*avoid foaming*), then pool all individual volumes of LDR by transferring them into a media basin.

Step 18) At the 15 minute incubation timepoint manually discard the LCMA Reagent from wells of the assay plate. Tamp the plate gently on a clean absorbent paper to remove residual drops. *No rinse step is required*.

Step 19) Dispense **100 µl / well** of the prepared **LDR**.

Step 20) Quantify **fluorescence (RFU).**

Step 21) Quantify **luminescence (RLU)**. Reconfigure the plate-reader to "luminescence" mode. Set read-time to 0.5 second (500 mSec) per well and perform the measure.

9.3 DATA ANALYSES Dose-range finding

9.3.1 Data analysis Dose-range finding TRα and TRβ Assays

For each treatment, the activation of TRα and TRβ agonist is calculated form **RLU** using a method represented by the following formulae (*i. – vii.)*:

i. **Calculate Average RLU (aRLU).** Calculate the aRLU of replicate treatment concentrations for TI, RI and SC, as follows: $aRLU = (RLU_1 + RLU_2 + ... + RLU_N) / N$

ii. **Calculate Standard Deviation (SD) of RLU.** Use the formula below, to calculate the SD of replicate treatment concentrations for TI, RI and SC.

$$SD = \sqrt{[(RLU_1 - aRLU) + (RLU_2 - aRLU) + ... + (RLU_N - aRLU)] / N}$$

iii. **Calculate Percent Coefficient of Variation (%CV) of RLU.** Calculate %CV of replicate treatments concentration for TI, RI, and SC, as follows: $\% CV = (SD / aRLU) \times 100$

iv. **Calculate RI Fold-Activation (FA) for RI.** Calculate FA for the RI at its EC_{100} concentration (T3 at 0.10 µM). Note: Do not use background subtracted aRLU values. FA^{RI} provides one of the minimum criteria for confirming the validity of assay performance for each of the TRs (*Step 24*):

Calculate FA of RI	
RI EC_{100} (T3; 0.10 µM):	$FA^{T3(0.10\ \mu M)} = (aRLU^{T3(0.10\ \mu M)} / aRLU^{SC})$

v. **Calculate BKG-Subtracted aRLU values.** Subtract the SC aRLU value from respective TI, RI, and SC aRLU values, as follows:

Calculation of Background-Subtracted Average RLU Values	
RI treatment:	$aRLU^{RI\text{-}BKG} = aRLU^{RI} - aRLU^{SC}$
TI treatments:	$aRLU^{TI\text{-}BKG} = aRLU^{TI} - aRLU^{SC}$
SC treatment:	$aRLU^{SC\text{-}BKG} = aRLU^{SC} - aRLU^{SC} = \mathbf{0}$

vi. **Calculate % Relative-Activation (%RA).** Use "BKG-subtracted aRLU" values to calculate %RA of RI, TI, and SC treatments relative to the RI (T3; 0.10 µM) activity value, as follows:

Calculation of % Relative Activation of TRs	
RI treatment:	$\%RA^{RI} = (aRLU^{T3(0.10\ \mu M)\text{-}BKG} / aRLU^{T3(0.10\ \mu M)\text{-}BKG}) \times 100 = \mathbf{100\%}$
TI treatments:	$\%RA^{TI} = (aRLU^{TI\text{-}BKG} / aRLU^{T3(0.10\ \mu M)\text{-}BKG}) \times 100$
SC treatment:	$\%RA^{SC} = (aRLU^{SC\text{-}BKG} / aRLU^{T3(0.10\ \mu M)\text{-}BKG}) \times 100 = \mathbf{0\%}$

vii. **Calculate Z' value.** This calculation utilises the RI aRLU and its corresponding SD, and the SC aRLU and its corresponding SD. Note: Do *not* use background subtracted aRLU values. Z' provides one of the minimum criteria for determining validity of the assay (see section 9.3.2).
$Z' = 1 - [3*(SD^{T3\ 0.10\ \mu M} + SD^{SC}) / (aRLU^{T3\ 0.10\ \mu M} - aRLU^{SC})]$

9.3.2 ACCEPTANCE CRITERIA dose-range finding TRα and TRβ Assays.

Confirm that the performance of the TRα and TRβ assays meets the following acceptance criteria for the Reference ligand, T3, at its EC_{100} concentrations. If these acceptance criteria are not met, then there was a fault in the assay components, in the setup or execution of the assays, or data reduction. Carefully review the SOP and FA and Z' calculations. If it is determined that assay performance failed, then start over. If the Z' score fell below 0.5, consider ways to improve dispensing technique to increase precision.

Assay	*RI-EC$_{100}$ Minimum Acceptance Criteria*	
	Fold-Activation (FA)	Z'
TRα	≥ 300	≥ 0.5
TRβ	≥ 500	≥ 0.5

9.3.3 ACTVITY THRESHOLD ASSESSMENT_TI activities.

i.) Apply the following minimum criteria to assign an activity status for each TI

Independent Assays	*Minimum Threshold*	*Activity Status*
TRα TRβ	TI %RA ≥ 10%	Active
	TI %RA < 10%	Not Active

ii.) Proceed to LCMA Data Analyses to determine TIs respective cytotoxicity profiles and record results. TIs that meet or exceed Relative Activation = 10% for either TR will be advanced to subsequent TR activity assessment. TIs that fail to meet the established minimum threshold for activity are excluded from TR activity assessment

9.3.4 Data analyses cytotoxicity Live Cell Miltiplex Assay.

For those few TIs that show positive activity in the TR assays, the % Live Cells (%LC) for each treatment concentration is calculated form **RFU** using a method represented by the following formulae (*i. – v.)*

***i.* Calculate Average RFU (aRFU)**. Use the formula as follows to calculate the aRFU of replicate treatment concentrations for TI, RI, SC, LCMA PC, and LCMA BKG :
aRFU = (RFU$_1$ + RFU$_2$ + …. + RFU$_N$) / N

***ii.* Calculate Standard Deviation (SD) of RFU.** Use the formula below to calculate the SD of replicate treatment concentrations for TI, RI, SC, LCMA PC, and LCMA BKG.

$$SD = \sqrt{\frac{[(\mathrm{RFU}_1 - \mathrm{aRFU}) + (\mathrm{RFU}_2 - \mathrm{aRFU}) + \ldots\ldots + (\mathrm{RFU}_N - \mathrm{aRFU})]}{N}}$$

***iii.* Calculate Percent Coefficient of Variation (%CV) of RFU**. Calculate %CV of replicate treatments concentration for TI, RI, SC, LCMA PC, and LCMA BKG, as follows:
% CV = (SD / aRFU)*100

***iv*. Calculate values of 'aRFU – BKG'.** Subtract "LCMA BKG" aRFU value from the aRFU values of TI, RI, SC, LCMA PC, and LCMA BKG treatments, as follows:

Calculation of Background-Subtracted Average RFU Values	
TI treatments:	$aRFU^{TI\text{-}BKG} = aRFU^{TI} - aRFU^{LCMA\ BKG}$
RI treatment:	$aRFU^{RI\text{-}BKG} = aRFU^{RI} - aRFU^{LCMA\ BKG}$
SC treatment:	$aRFU^{SC\text{-}BKG} = aRFU^{SC} - aRFU^{LCMA\ BKG}$
LCMA PC treatment:	$aRFU^{PC\text{-}BKG} = aRFU^{PC} - aRFU^{LCMA\ BKG}$
LCMA BKG treatment:	$aRFU^{BKG\text{-}BKG} = aRFU^{LCMA\ BKG} - aRFU^{LCMA\ BKG} = \mathbf{0}$

***v*. Calculate Percent Live Cells (%LC) relative the SC treated cells.** For TI, RI, SC, LCMA-PC, and LCMA-BKG treatments, divide 'BKG-Subtracted aRFU' by 'SC aRFU-BKG', then multiply by 100 to determine "%", as follows:

Calculation of % Live Cells	
SC treatment:	$\%LC^{SC} = (aRFU^{SC\text{-}Bkg} / aRFU^{SC\text{-}Bkg}) \times 100 = \mathbf{100\%}$
TI treatments:	$\%LC^{TI} = aRFU^{TI\text{-}Bkg} / aRFU^{SC\text{-}Bkg}) \times 100$
RI treatment:	$\%LC^{RI} = aRFU^{RI\text{-}Bkg} / aRFU^{SC\text{-}Bkg}) \times 100$
LCMA PC treatment:	$\%LC^{PC} = aRFU^{PC\text{-}Bkg} / aRFU^{SC\text{-}Bkg}) \times 100$
LCMA BKG treatment:	$\%LC^{BKG} = aRFU^{Bkg\text{-}Bkg} / aRFU^{SC\text{-}Bkg}) \times 100 = \mathbf{0\%}$

9.3.5 ACCEPTANCE CRITERIA Cytotoxicity Live Cell Multiplex Assay

Apply the following acceptance criteria to confirm the performance of the LCMA. If the acceptance criteria are not met, then there was a fault in the assay components, in the setup or execution of the LCM assays, or data reduction. Carefully review the SOP and %LC. If it is determined that assay performance failed, then start over.

Independent Assays	*PC Minimum Acceptance Criteria*	*Status of LCMA Performance*
	LCMA-PC (Staurosporine; 8 µM)	
TRα-LCMA TRβ-LCMA	%LC < 20%	Pass
	%LC ≥ 20%	Fail

9.3.6 CYTOTOXICITY THRESHOLD ASSESSMENT_TI treatment concentrations.

i.) Apply the following criteria to identify the highest concentration of each TI that is non-cytotoxic in the TR assays.

Independent Assays	*Threshold Criteria*	*TI Cytotoxicity Status for each treatment concentration*
TRα-LCMA TRβ-LCMA	%LC ≥ 80%	Non-Cytotoxic
	%LC < 80%	Cytotoxic

ii.) The highest non-cytotoxic concentration for each TI that shows activity against one, or both, of the TR's will be advanced to the TR Activity Assessment for further analysis.

10 TR Activity Assessment

Assessments of TI activity metrics against TRa and TRb, including repeated Cytotoxicity analyses.

OVERVIEW: Only those TIs that were identified in the dose-range finding to produce %RA ≥ 10% against either TRα or TRβ will be advanced to TR Activity Assessment. Testing will begin at the highest non-cytotoxic treatment concentration identified in the dose-range finding.

The overall workflow is the same as that depicted in **Figure 2**, and again integrates the Live Cell Multiplex Assay (LCMA) with the TRα and TRβ assays. The fundamental procedure for the TR activity assessment is very similar to that used in the dose-range finding, with the following significant changes: *i.)* the starting concentration of TI in the treatment media will be adjusted to correspond to the highest concentration that does not induce cytotoxicity, as determined in the dose-range finding, *ii.)* assay setup will include Positive and Negative Controls for the TR assays, *iii.)* changes in plate setups, and *iv.)* expanded data analyses.

PLATE 1 for each TR assay will be configured as depicted in **Figure 4. PLATES 2, 3 … etc.,** will be configured as depicted in **Figure 5**.

PLATE 1 — T3 Treatment Concentrations (columns 2–9: Low ← → High); Controls (columns 10–11)

		1	2	3	4	5	6	7	8	9	10	11	12
	A												
RI (T3)	B		C8	C7	C6	C5	C4	C3	C2	C1	NC (E2)	LCMA PC (Stauro.)	
	C		C8	C7	C6	C5	C4	C3	C2	C1			
	D		C8	C7	C6	C5	C4	C3	C2	C1			
TI-1	E		SC	C7	C6	C5	C4	C3	C2	C1	PC (Sobetirome)	LCMA BKG (NoCells+SC)	
	F			C7	C6	C5	C4	C3	C2	C1			
	G			C7	C6	C5	C4	C3	C2	C1			
	H												

TI Treatment Concentrations (columns 3–9: Low ← → High)

Figure 4. Template for PLATE 1: The LCMA+TRα and LCMA+TRβ assays. Each Plate 1 setup consists of seven treatment concentrations of one TI and eight of RI T3, Solvent Controls (SC), and a fixed concentration of a TR negative control (NC) item, TR Positive Control (PC) item, and LCMA PC (Staurosporine). As before, wells devoid of cells but treated with SC treatment media are included to provide 'Background RFU' in the LCM Assay (LCMA BKG). Average RFU from the SC wells will correlate to "100% Live Cells". Wells shaded grey will contain dH2O; readouts from these wells will not be used.

PLATE 2 — Controls (columns 10–11)

		1	2	3	4	5	6	7	8	9	10	11	12
	A												
TI-2	B		SC	C7	C6	C5	C4	C3	C2	C1	RI (EC_{100} T3)	LCMA PC (Stauro.)	
	C			C7	C6	C5	C4	C3	C2	C1			
	D			C7	C6	C5	C4	C3	C2	C1			
TI-3	E		SC	C7	C6	C5	C4	C3	C2	C1		LCMA BKG (NoCells+SC)	
	F			C7	C6	C5	C4	C3	C2	C1			
	G			C7	C6	C5	C4	C3	C2	C1			
	H												

TI Treatment Concentrations (columns 3–9: Low ← → High)

Figure 5. Template for PLATE 2, 3, etc. Additional plates for the LCMA+TRα and LCMA+TRβ assays will each comprise 2 TIs presented in seven treatment concentrations, and will include SC, a fixed EC_{100} concentration of (T3), a fixed concentration of LCMA-PC, and LCMA BKG control wells.

10.1 Preparation of the assay plates and exposure of the TR reporter cells

(All steps must be performed using aseptic technique!)

As before, this SOP details the processing of 2 assay plates: PLATE 1 for each TR, which will test only one TI. However, as before, the values highlighted in white font are 'scalable' numbers, and are to be adjusted in accordance with the *actual* number assay plates being run for each TR.

Step 1) Thaw Cell Recovery Medium (CRM) *and* Compound Screening Medium (CSM).
To set up 1 TRα and 1 TRβ assay plate:
a.) remove 4 tubes of CRM from freezer storage. Place the tubes in a 37°C water bath to thaw the media. Once thawed, leave CRM at 37°C until it is used in *Step 2*,
and,
b.) remove 2 tubes of CSM from freezer storage. Thaw CSM using a 37°C water bath. CSM will first be used in *Step 3*, and then again in *Step 8.*

Step 2) *Rapid* **Thaw of the Reporter Cells.**
First, retrieve the tubes of **CRM** from the 37°C water bath and sanitize their outside surfaces with a 70% ethanol swab.

Second, retrieve 1 tube of **TRα** AND 1 tube of **TRβ Reporter Cells** from -80°C storage, place them directly into a dry ice bucket and transport the cells to the laminar flow hood. When ready to begin, transfer the tubes of reporter cells into a rack and, *without delay*, perform a rapid thaw of the cells as follows:

a.) transfer 2x **9.5 ml** of 37°C **CRM** directly into the tube of frozen **TRα** cells, *and*
b.) transfer 2x **9.5 ml** of 37°C **CRM** directly into the tube of frozen **TRβ** cells.
c.) Place each tube of Reporter Cells into a 37°C water bath for 5 - 10 minutes.

The resulting volume of each individual cell suspension will be **21 ml**.

Step 3) Setup **LCM Assay Background Control wells.** During the 5 – 10 minute cell recovery period dispense **200 µl /well** of **CSM** into each of the three wells designated as "LCMA BKG_No Cells + SC". Well locations are depicted in the PLATE 1 template provided in Figure 5.

Step 4) Dispense Reporter Cells.

a.) Retrieve from the water bath the tubes of the Reporter Cell Suspension. Sanitize the outside surface of each tube using a 70% alcohol swab. If more than 1 tube of cells was thawed for the TRα assays and the TRβ assays, pool the individual common tubes by combining them in a media basin; mix by gentle rocking. Use an electronic, repeat-dispensing, 8-chanel pipette to dispense **200 µl / well** of respective TR cell suspensions. For **PLATE 1** and *(if applicable)* **PLATE 2** for each TR assay, dispense cells into all wells *except* for those designated to be "LCMA BKG_No Cells + SC" control wells and edge-wells (refer to **Figures 4** and **5**.)

NOTE: Increased well-to-well variation will occur if cells are allowed to settle during the dispensing period. Take care to ensure precision in dispensing exact volumes across the assay plate.

b.) To mitigate the occurrence of 'edge-effects', remove 1 tip from the 8-chanel pipette and dispense **100 µl** of sterile water into all inter-well spaces within the assay plate(s).

Step 5) Pre-incubate reporter cells. Place the assay plate into a cell culture incubator for 4 .5 hr ± 30 min.

During the 4.5 hr pre-incubation period, perform Steps 6 and 7:

Step 6) Prepare stocks of TR 'activity-positive' TIs, and Control stocks.

a.) Prepare stock solution as described in section 8.

b.) From one of the TR assay kits retrieve one tube each of T3, Sobetirome, and E2. From the LCM Assay kit Retrieve a tube of Staurosporine. Allow the tubes to thaw and equilibrate to room temperature under low light.

Step 7) Dilute TI, T3, Sobetirome, E2, and DMSO to generate final concentrations of respective treatment media. 200 µl / well of each treatment media will be dispensed into the designated wells of the assay plate(s) (refer to **Figures 4** and **5**). Follow the specific instructions, below, to prepare each treatment media.

***a.)* Prepare Seven concentrations of TI Treatment Media:** Follow the dilution scheme and treatment media preparation strategy presented in **Appendix 2 (A and B)**.

I*n brief*,

- Use the TI and DMSO (or the alternative solvent selected during solubility testing) to prepare a TI stock corresponding to 500x the highest treatment concentration of the TI that did not induce cytotoxicity in the dose-range finding.

- As depicted in **Appendix 2A**, prepare six subsequent serial **3-fold** dilutions using DMSO. If the application of 3-fold dilution factor leads to the generation of an incomplete dose-response sigmoidal curve the dilution factor can be increased to **4-fold** in subsequent runs as depicted in Appendix 2B

- All of these various intermediate stocks will then undergo a 2-step dilution process using CSM, to yield the final concentrations of treatment media for each TI.

***b.)* Prepare Eight concentrations of RI (T3) Treatment Media:** Start with the 500x-concentrated stock of **T3** (50 µM) from either of the TR assay kits. As depicted in **Appendix 3**, prepare seven subsequent serial **3-fold** dilutions using DMSO. All of these various intermediate 500x-concentrated stocks will then undergo a 2-step dilution process using CSM, to yield the final concentrations of treatment media. The final T3 concentrations in the assay are: 100, 33, 11, 3.7, 1.2, 0.41, 0.14 and 0.046 nM.

***c.)* Preparation of PC (Sobetirome) Treatment Media:** Utilize the 500x-concentrated stock of **Sobetirome** (500 µM) from either of the TR assay kits. Transfer **5.0 µl** of Sobetirome Stock into **2.495 ml** of CSM; mix. The resulting concentration is 1.0 µM, which is an EC_{100} concentration for this agonist in both TR assays.

***d.)* Preparation of NC (SC) Treatment Media:** Utilize the 500x-concentrated stock of **E2** (500 µM) from either of the TR assay kits. Transfer **5.0 µl** of E2 Stock into **2.495 ml** of CSM; mix. The resulting concentration is 1.0 µM, which should display no activity in either TR assay.

***e.)* Preparation of LCMA-PC Treatment Media:** Utilize the 500x-concentrated (4.0 mM) stock of **Staurosporine** from one LCM Assay kit. Transfer **5.0 µl** of Staurosporine Stock into **2.495 ml** of CSM; mix. The resulting concentration of 8 µM is sufficient to elicit ≥ 85% cell death.

***f.)* Preparation of RI EC_{100} (T3) Treatment Media:** Utilize the 500x-concentrated stock of **T3** (50 µM) resulting concentration is 0.1 µM, which is an EC_{100} concentration for the reference item.

***g.)* Preparation of SC Treatment Media:** The preparation of TI Treatment Media, as described above and depicted in Appendix 2 (A and B), will also generate SC media comprising **0.2% DMSO.** In *Step 9a*, SC treatment media will be present into replicate wells seeded with cells, AND into wells with "no cells" designated as LCMA BKG.

Step 8) At the end of the 4.5 hr ± 30 min pre-incubation period discard the culture media. The use of a 'tip based' method is discouraged. For speed and efficiency in processing a small number of plates, and to avoid trauma to the reporter cell, the preferred method is to perform a 'wrist-flick' to manually eject the liquid into a waste container. *Gently* tap the inverted plate onto a clean absorbent paper towel to remove residual droplets. Cells will remain tightly adhered to well bottoms.

Step 9) Dispense **Treatment Media.**

a.) Dispense **200 µl / well** of the prepared TI, PC, NC, and SC treatment media into the designated wells of the assay plates. Dispense extra CSM or sterile dH2O into wells shaded grey.
(refer to **Figures 5** and **6**).

b.) As before, to mitigate the occurrence of 'edge-effects', dispense **~ 100 µl** of dH2O into all inter-well spaces within the assay plate.

Step 10) Place the assay plate in a cell culture incubator for 24±1 hours.

Step 11) In preparation for next day's activities, retrieve the following reagents from freezer storage and place them in a dark refrigerator (4°C) to thaw overnight.

2 tubes of **Detection Substrate** from TR assay kits
2 tubes of **Detection Buffer** from TR assay kits
2 tubes of **LCMA Buffer** from LCM Assay kits
2 tubes of **LCMA Substrate** from LCM Assay kits

10.2 Quantification of luminescence and fluorescence

(Subsequent manipulations may be performed on a bench top)

Step 12) Equilibrate assay reagents to room temperature. Approximately 30 minutes prior to the end of the 24±1 hr incubation period, remove from the refrigerator the tubes of various assay reagents described in *Step 11*. Place them in a dark area to equilibrate to room temperature. Do NOT warm Detection Substrate above room temperature. Once at room temperature, *gently* invert each tube several times to ensure homogenous solutions.

Step 13) Prepare LCMA Detection Reagent. To perform the LCM Assay on 2 plates:

a.) Transfer 2 **x 6 ml** of **LCMA Buffer** into a single tube, then

b.) Dispense into the LCMA Buffer 2 **x 20 µl** of **LCMA Substrate**; mix gently. This preparation of *LCMA Detection Reagent* will be used in *Step 16*.

Step 14) Discard **treatment media from the TR Reporter cells.** At the end of the 24±1 hr incubation period discard treatment media from the assay plate. Manually eject the liquid into an appropriate waste container. *Gently* tap the inverted plate onto a clean absorbent paper towel to remove residual droplets.

Step 15) Rinse**.** Dispense **200 µl / well** of the **LCMA Buffer**. Gently rinse the wells by tilting the plate side-to-side 2-3 times, then manually eject the rinse buffer.

Step 16) Transfer preparations of **LCMA Detection Reagent** into a media basin. Dispense **50 µl / well** across the entire assay plate. Incubate the plate in a *dark location* at room temperature for **15 minutes**.

Step 17) During the 15 minute incubation period perform the following:

a.) turn on the plate reader and select *fluorescence* mode reads using the filter combination [Ex:485nm | Em:535nm]. Set the instrument to perform a single 5 second "plate shake" prior to reading the first assay well. For convenience, set the instrument to read all wells;

and,

b.) prepare **Luciferase Detection Reagent (LDR)**. For 2 assay plates, transfer the entire volumes of 2 tubes of **Detection Buffer** into 2 tubes of **Detection Substrate**, thereby generating a 2x **12 ml** volumes of **LDR**. Mix by *gently* inverting the tubes (*avoid foaming*), then pool all individual volumes of LDR by transferring them into a media basin.

Step 18) At the 15 minute incubation timepoint manually discard the LCMA Reagent from wells of the assay plate. Tamp the plate gently on a clean absorbent paper to remove residual drops. *No rinse step is required*.

Step 19) Dispense **100 µl / well** of the prepared **LDR**.

Step 20) Quantify fluorescence (RFU).

Step 21) Quantify luminescence (RLU). Reconfigure the plate-reader to "luminescence" mode. Set read-time per well to 0.5 second (500 mSec).

10.3 DATA ANALYSES TR activity assessment

10.3.1 10.3.1 Data analysis for TRa and TRb assays

For each treatment, the activation of TRα and TRβ agonist is calculated form **RLU** using a method represented by the following formulas (*i. – vii.)*

***i.* Calculate Average RLU (aRLU)**. Use the formula below to calculate the aRLU of replicate treatment concentrations for TI, RI, PC, NC, and SC.
$aRLU = (RLU_1 + RLU_2 + ... + RLU_N) / N$.

***ii.* Calculate Standard Deviation (SD) of RLU.** Use the formula below to calculate the SD of replicate treatment concentrations for TI, RI, PC, NC, and SC.

$$SD = \sqrt{\frac{[(\mathrm{RFU}_1 - \mathrm{aRFU}) + (\mathrm{RFU}_2 - \mathrm{aRFU}) + \ldots\ldots.. + (\mathrm{RFU}_N - \mathrm{aRFU})]}{N}}$$

***iii.* Calculate Percent Coefficient of Variation (%CV) of RLU**. Calculate %CV of replicate treatments concentration for TI, RI, PC, NC, and SC, as follows: % CV = (SD / aRLU) x 100

***iv.* Calculate RI Fold-Activation (FA)**. Calculate FA for the RI item at its EC_{100} concentration (T3 at 0.10 µM). Note: Do not use background subtracted aRLU values. FA calculations are not required for TIs or other Control items. FA^{RI} provides one of the minimum criteria for confirming the validity of assay performance for each of the TRs (See section 10.3.2):

Calculate FA of RI	
RI EC_{100} treatment (0.10 µM T3):	$FA^{T3(100\mu M)} = (aRLU^{T3(100\mu M)} / aRLU^{SC})$

***v.* Calculate BKG-Subtracted aRLU values.** Subtract the SC aRLU value from respective TI, RI, PC, NC, and SC aRLU values, as follows:

Calculation of Background-Subtracted Average RLU Values	
RI treatment:	$aRLU^{RI\text{-}BKG} = aRLU^{RI} – aRLU^{SC}$
TI treatments:	$aRLU^{TI\text{-}BKG} = aRLU^{TI} – aRLU^{SC}$
PC treatments:	$aRLU^{PC\text{-}BKG} = aRLU^{PC} – aRLU^{SC}$
NC treatments:	$aRLU^{NC\text{-}BKG} = aRLU^{NC} – aRLU^{SC}$
SC treatment:	$aRLU^{SC\text{-}BKG} = aRLU^{SC} – aRLU^{SC} =$ **0**

***vi*. Calculate % Relative-Activation (%RA).** Use "BKG-subtracted aRLU" values to calculate %RA of TI, RI, PC, NC, and SC treatments relative to the RI- EC_{100} (T3 at 0.10 µM) activity value, as follows:

Calculation of % Relative Activation of TRs	
RI treatment:	$\%RA^{RI} = (aRLU^{RI\text{-}BKG} / aRLU^{T3(0.10\mu M)\text{-}BKG}) \times 100$
TI treatments:	$\%RA^{TI} = (aRLU^{TI\text{-}BKG} / aRLU^{T3(0.10\mu M)\text{-}BKG}) \times 100$
PC treatments:	$\%RA^{PC} = (aRLU^{PC\text{-}BKG} / aRLU^{T3(0.10\mu M)\text{-}BKG}) \times 100$
NC treatments:	$\%RA^{NC} = (aRLU^{NC\text{-}BKG} / aRLU^{T3(0.10\mu M)\text{-}BKG}) \times 100$
SC treatment:	$\%RA^{SC} = (aRLU^{SC\text{-}BKG} / aRLU^{T3(0.10\mu M)\text{-}BKG}) \times 100 =$ **0%**

***vii.* Calculate Z' value.** This calculation utilizes the RI-EC_{100} aRLU and its corresponding SD, and the SC aRLU and its corresponding SD. Note: Do not use background subtracted aRLU values. Z' provides one of the criteria for determining validity of the assay (See section 10.3.2).

$Z' = 1 - [3*(SD^{T3\ 0.10\mu M} + SD^{SC}) / (aRLU^{T3\ 0.10\mu M} - aRLU^{SC})]$

viii. Plot RI and TI dose-response data. Use appropriate software to perform non-linear regression curve-fitting (variable slope, 4 parameters, least squares fit) of TIs and RI treatments for both TR assays. Plot '%RA +/- %CV *vs.* Log_{10}[Molar conc.]'. Constrain the lower plateaus to "0".

a.) RI-EC_{50}. Values of EC_{50}, log(EC_{50}) and Standard Deviation of log(EC_{50}) will be automatically calculated by the software used.

b.) Calculate %CV of RI-LogEC_{50}. From the T3 dose-response data plotted against Molar treatment concentrations:

$RI\ \%CV\ \log(EC_{50}) = [SD\ Log(EC_{50}) / |Log(EC_{50})|] \times 100$

10.3.2 ACCEPTANCE CRITERIA for TRα and TRβ Assays

Refer to the table below to confirm that the performance of the TRα and TRβ assays meet the acceptance criteria for RI (T3) dose-response data, PC-EC_{100}, and NC. If these acceptance criteria are not met, then there was a fault in the assay components, in the setup or execution of the assays, or data reduction. Carefully review the SOP and all calculations. If it is determined that assay performance failed, then start over.

Assay Metric	**Acceptance Criteria**	
	TRα Assay	**TRβ Assay**

1	Sigmoidal dose-response of RI	YES	
2	FA of RI-EC_{100} (T3; 0.10 µM)	≥ 300 FA	≥ 500 FA
3	RI-EC_{50}	≤ 0.01 µM ≤ 1.0E-08 M	≤ 0.04 µM ≤ 4.0E-08 M
4	%CV log (EC_{50}) for RI	< 3%	
5	PC %RA (Sobetirome at EC_{100}; 1 µM)	≥ 50% RA	
6	NC %RA (17-b-Estradiol; 1 µM)	< 10% RA	
7	Z' for RI-EC_{100} (T3; 0.10µM)	≥ 0.5	

10.3.3 Determine TR assay metrics for TI treatments.

From their respective dose-response plots, determine the following for each TI:

i.) **TI-%RA_{10} and TI-%RA_{50} values.** Determine the concentrations of each TI that produced a %RA value equivalent to the RI-EC_{10} and RI-EC_{50} values.

ii.) **TI-EC_{10} and TI-EC_{50}, *if possible*.** The curve-fitting software will attempt to calculate EC values for TIs. However, dose-response data for the majority of 'positive' TIs will not generate a complete sigmoidal curve. Specifically, if the upper plateau of the dose-response plot is not established, then neither TI-EC_{10} or TI-EC_{50} values can be determined accurately.

10.3.4 LCMA Data analyses.

From **RFU** Calculate the % Live Cells (%LC) for each treatment concentration, as described in the formulas below (*i.-v.*).

***i.* Calculate Average RFU (aRFU)**. Use the formula below to calculate the aRFU of replicate treatment concentrations for TI, RI, SC, LCMA PC, and LCMA BKG.

$aRFU = (RFU_1 + RFU_2 + ... + RFU_N) / N$

***ii.* Calculate Standard Deviation (SD) of RFU.** Use the formula below to calculate the SD of replicate treatment concentrations for TI, RI, SC, LCMA PC, and LCMA BKG.

$$SD = \sqrt{\frac{[(\mathrm{RFU}_1 - \mathrm{aRFU}) + (\mathrm{RFU}_2 - \mathrm{aRFU}) + \dots\dots.. + (\mathrm{RFU}_N - \mathrm{aRFU})]}{N}}$$

***iii.* Calculate Percent Coefficient of Variation (%CV) of RFU**. Calculate %CV of replicate treatments concentration for TI, RI, SC, LCMA PC, and LCMA BKG, as follows:
% CV = (SD / aRFU)*100

***iv*. Calculate values of 'aRFU – BKG'.** Subtract "LCMA BKG" aRFU value from the aRFU values of TI, RI, SC, LCMA PC, and LCMA BKG treatments, as follows:

Calculation of Background-Subtracted Average RFU Values	
TI treatments:	$aRFU^{TI\text{-}BKG} = aRFU^{TI} – aRFU^{LCMA\ BKG}$

RI treatment:	$aRFU^{RI\text{-}BKG} = aRFU^{RI} – aRFU^{LCMA\ BKG}$
SC treatment:	$aRFU^{SC\text{-}BKG} = aRFU^{SC} – aRFU^{LCMA\ BKG}$
LCMA PC treatment:	$aRFU^{PC\text{-}BKG} = aRFU^{PC} – aRFU^{LCMA\ BKG}$
LCMA BKG treatment:	$aRFU^{BKG\text{-}BKG} = aRFU^{LCMA\ BKG} – aRFU^{LCMA\ BKG} = \mathbf{0}$

***v.* Calculate Percent Live Cells (%LC) relative the SC treated cells.** Divide all values of 'BKG-Subtracted aRFU' for TI, RI, SC, LCMA-PC, and LCMA-BKG treatments by 'SC aRFU-BKG', then multiply by 100 to determine "%", as follows:

Calculation of % Live Cells	
SC treatment:	$\%LC^{SC} = (aRFU^{SC\text{-}Bkg} / aRFU^{SC\text{-}Bkg}) \times 100 = \mathbf{100\%}$
TI treatments:	$\%LC^{TI} = aRFU^{TI\text{-}Bkg} / aRFU^{SC\text{-}Bkg}) \times 100$
RI treatment:	$\%LC^{RI} = aRFU^{RI\text{-}Bkg} / aRFU^{SC\text{-}Bkg}) \times 100$
LCMA PC treatment:	$\%LC^{PC} = aRFU^{PC\text{-}Bkg} / aRFU^{SC\text{-}Bkg}) \times 100$
LCMA BKG treatment:	$\%LC^{BKG} = aRFU^{Bkg\text{-}Bkg} / aRFU^{SC\text{-}Bkg}) \times 100 = \mathbf{0\%}$

10.3.5 ACCEPTANCE CRITERIA_LCM Assays

Apply the following acceptance criteria to confirm the performance of the LCMA. If the acceptance criteria are not met, then there was a fault in the assay components, in the setup or execution of the LCM assays, or data reduction. Carefully review the SOP and %LC. If it is determined that assay performance failed, then start over.

Independent Assays	*PC Minimum Acceptance Criteria*	*Status of LCMA Performance*
	LCMA-PC (Staurosporine; 8 µM)	
TRα-LCMA TRβ-LCMA	%LC < 20%	Pass
	%LC ≥ 20%	Fail

10.3.6 ACCEPTANCE CRITERIA for TR data based on CYTOTOXICITY ASSESMENT

Apply the following criteria to confirm, based on cytotoxicity, the validity of individual treatment concentration of TIs, RI, PC and NC in the TR assays.

Independent Assays	*Items*	*Cytotoxicity Threshold Criteria*	*Status of Individual Treatments*
TRα-LCMA TRβ-LCMA	SC	%LC = 100%	CONTROLS for normalizing %LC calculations
	LCMA-BKG	%LC = 0%	
	TIs RI PC NC	%LC ≥ 80%	TR assay data **Valid**
	Tis* RI PC NC	%LC < 80%	TR assay Data **Invalid** due to cytotoxicity

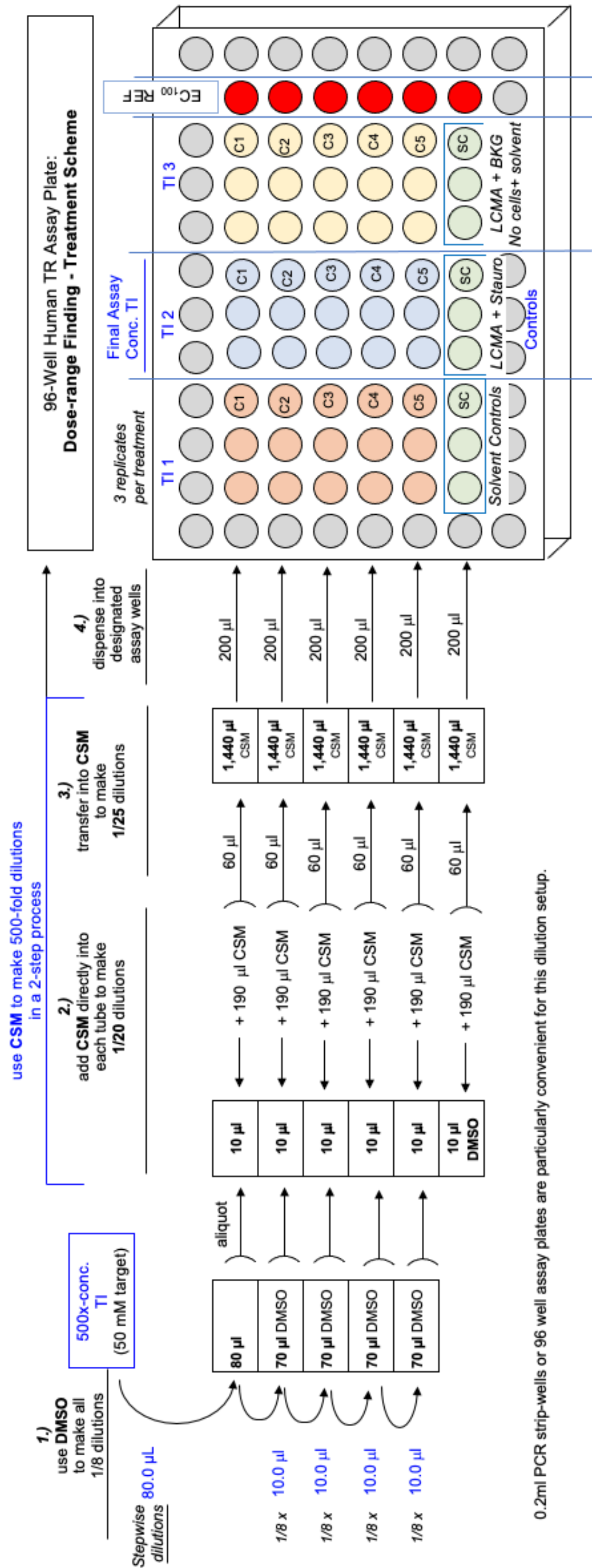
* Cytotoxicity of one TI concentration is acceptable. The TR assay metrics from this cytotoxic concentration should not be excluded from the final results.

11 Bibliography

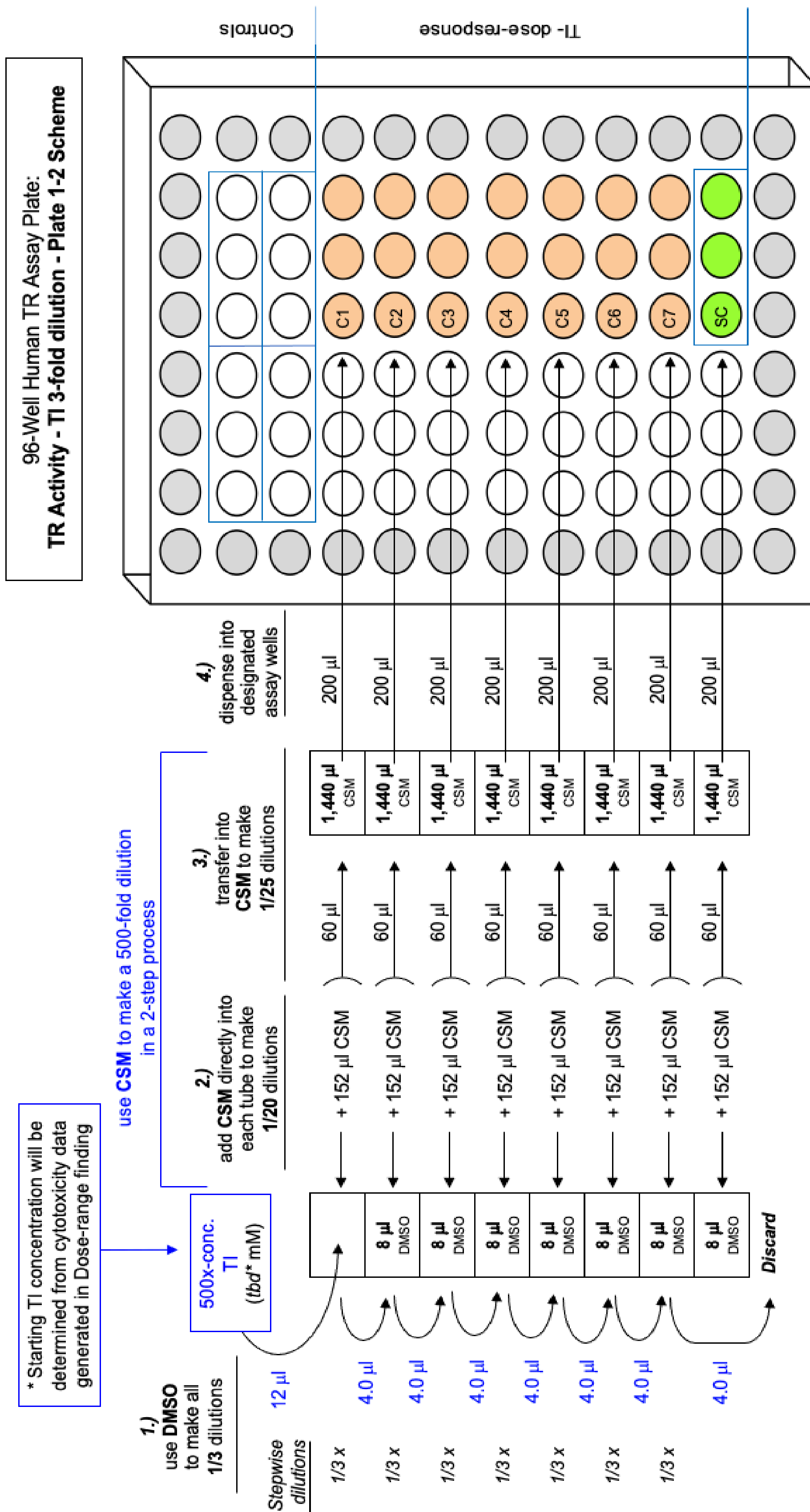
- Zhang JH, Chung TD, Oldenburg KR. (1999) A Simple Statistical Parameter for Use in Evaluation and Validation of High Throughput Screening Assays. J Biomol Screen.:4(2), 67-73.
- Human Thyroid Hormone Receptor TRα (NR1A1) TRβ (NR1A2) - Technical Manual
- Technical Manual LCMA kit (TM_LCMA)

Appendix 1: Preparing TI Treatment Media for Dose range finding

Depicted are TI assay concentrations when starting with 50 mM stocks. 500x-concentrated TI Stocks will be 50 mM if, during the solubility testing procedure, no additional volumes of solvent were required to achieve 100% solubility.

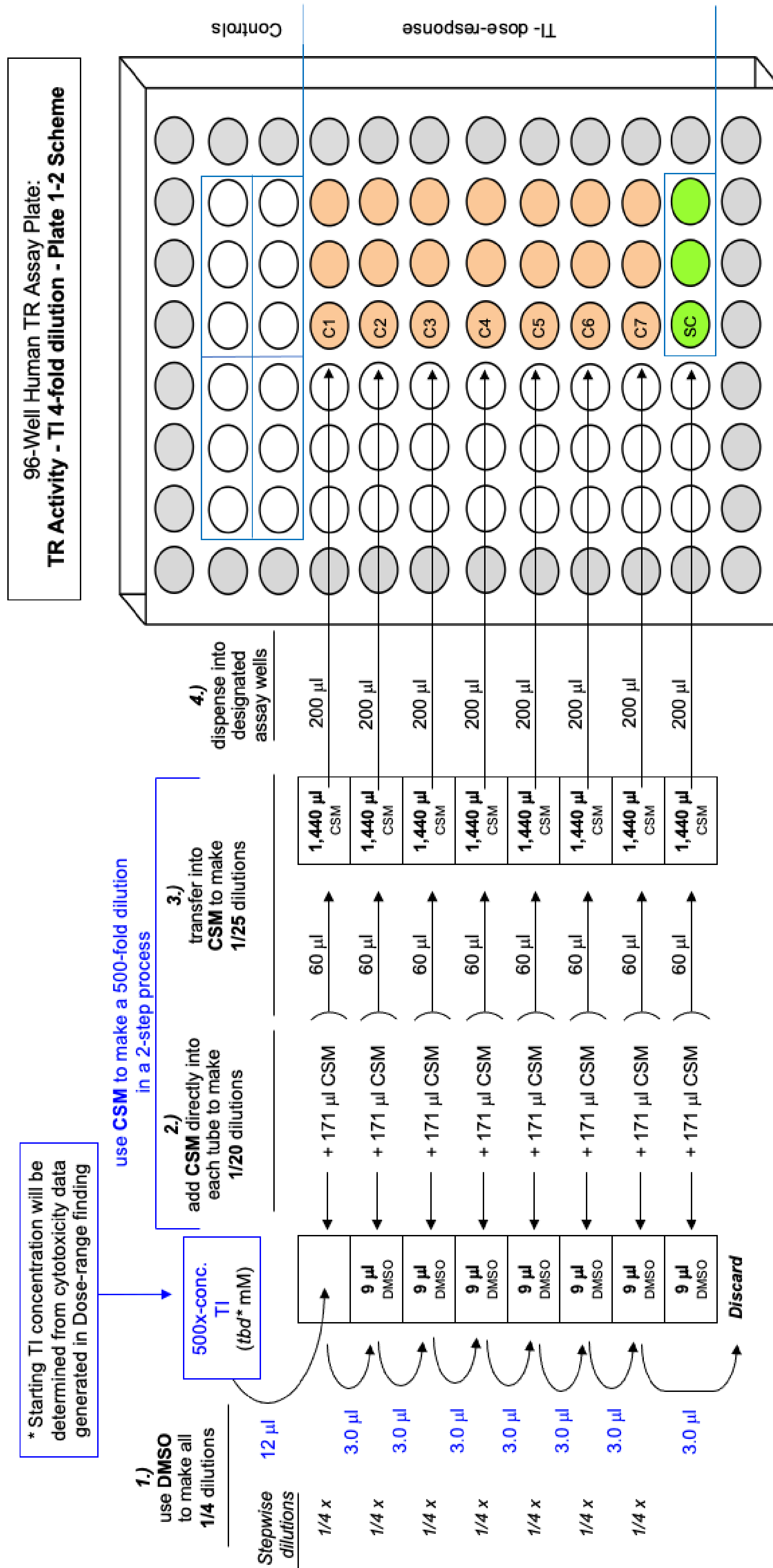


Appendix 2A: Preparing 3-Fold dilution TI Treatment Media for TR activity assessment
500x-concentrated TI Stocks will be 50 mM if, during the solubility testing procedure, no additional volumes of solvent were required to achieve 100% solubility.



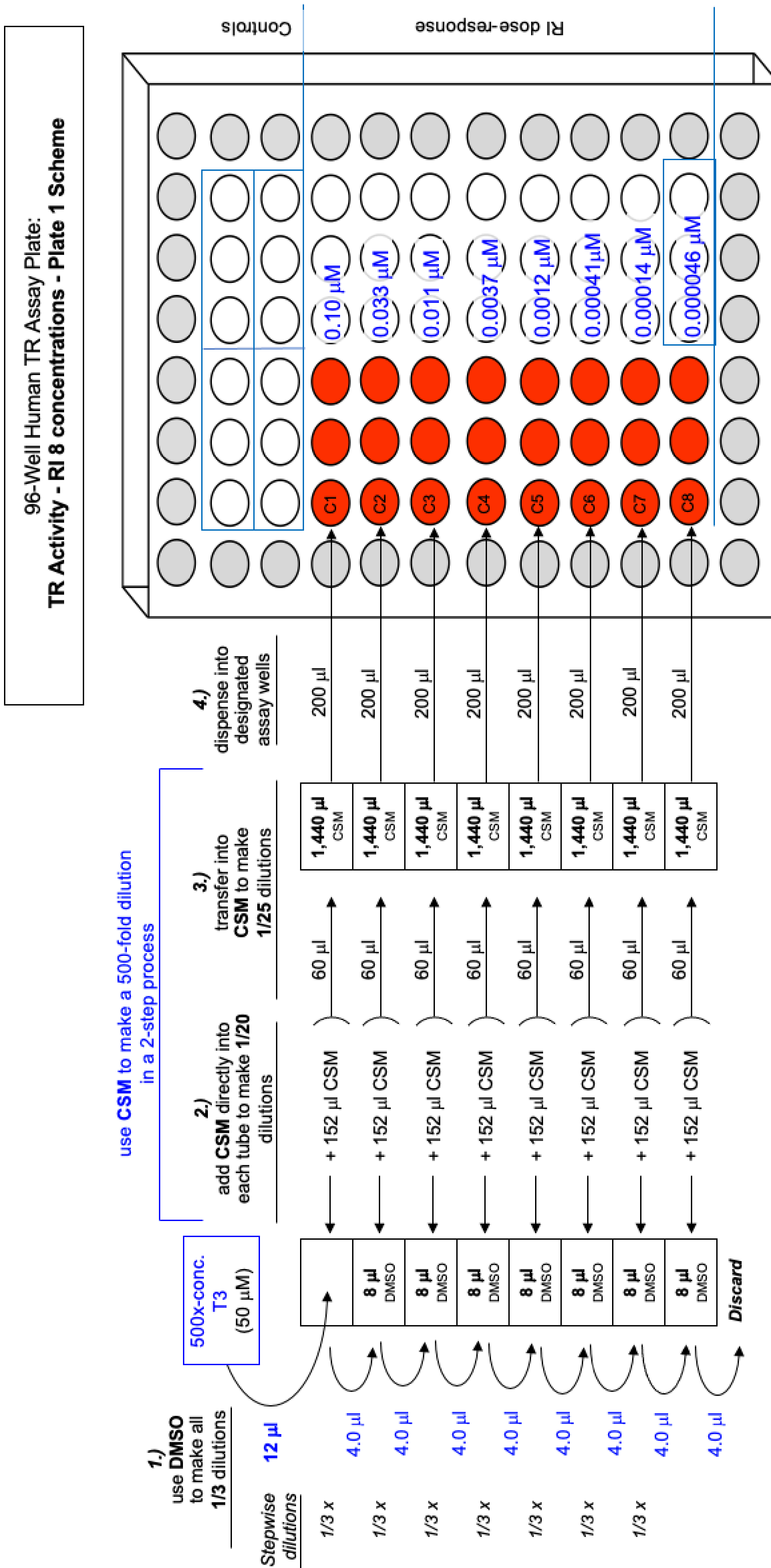
Appendix 2B: Preparing 4-Fold dilution TI Treatment Media for TR activity assessment

500x-concentrated TIs Stocks will be 50 mM if, during the solubility testing procedure, no additional volumes of solvent were required to achieve 100% solubility.



Appendix 3: Preparing RI Treatment Media for TR activity assessment

Serial dilution of 500x-concentrated RI Stock (T3; 50 µM in DMSO) using DMSO, followed by dilution with CSM to generate treatment media.



GETTING IN TOUCH WITH THE EU

In person

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

On the phone or in writing

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

— by freephone: 00 800 6 7 8 9 10 11 (certain operators may charge for these calls),

— at the following standard number: +32 22999696,

— via the following form: european-union.europa.eu/contact-eu/write-us_en.

FINDING INFORMATION ABOUT THE EU

Online

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

EU publications

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

EU law and related documents

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

Open data from the EU

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

The European Commission's science and knowledge service

Joint Research Centre

JRC Mission

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

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

@EU_ScienceHub

EU Science Hub - Joint Research Centre

EU Science, Research and Innovation

EU Science Hub

EU Science